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NADES-Based Extracts of Selected Medicinal Herbs as Promising Formulations for Cosmetic Usage

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Abstract: As a functional extraction medium, natural deep eutectic solvents (NADESs) can dissolve various plant metabolites. Those solvents provide untapped potential for creating novel green extracts with distinctive phytochemical signatures and unique biological activities. This is particularly relevant given the rising need for eco-friendly and sustainable skin care products. The main aim of this work was to optimize the most efficient natural deep eutectic solvents (NADESs) for extracting bioactives from 18 medicinal herbs applicable to the cosmetic industry. Selection of the most potent herbs involved assessing their conventional extracts for tyrosinase inhibition, antioxidant activity, and keratinocyte cytotoxicity. Moreover, we analyzed the phenolic profile using ultra-high-pressure liquid chromatography/mass spectrometry (UHPLC/MS) and spectrophotometric assays such as total phenolic (TPC) and flavonoid content (TFC). Using the COSMO-RS method, we modeled the solubility of 12 phenolics in 64 virtual NADESs and selected the 7 most promising ones for further experimental validation. NADESs, including betaine-urea, betaine-proline, and betaine-lysine, were computationally chosen and demonstrated the highest levels of TPC and antioxidative capacity, as confirmed by in vitro assays. The proposed combination of NADES herbal extracts represents a promising natural constituent for the cosmetic industry.

Keywords: medicinal herbs from Serbia; tyrosinase inhibition; radical scavenging activity; keratinocytes; UHPLC-MS; COSMO-RS; natural deep eutectic solvents; skin anti-aging

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1. Introduction

The growing global consciousness regarding the use of natural, renewable, and sustainable ingredients in the cosmetics industry has reached remarkable levels. Conventional organic solvents, once commonplace, pose substantial challenges due to their toxicity, flammability, and adverse environmental impact. In response, the industry is shifting toward more sustainable and eco-friendly alternatives. Green cosmetics products not only prioritize natural raw materials and ingredients but also emphasize the sustainability of the entire production process. By doing so, they contribute to a reduced carbon footprint, aligning with the urgent need for responsible practices in the beauty sector [1,2].

Natural deep eutectic solvents (NADESs) are a new generation of green solvents that meet all principles of green chemistry [3]. NADESs are two or multi-component mixtures designed from different plant secondary metabolites acting as hydrogen bond

donors (HBDs) and acceptors (HBAs). The mixture's melting point decreases by forming intermolecular interactions, resulting in a liquid homogeneous phase of different viscosities—the eutectic mixture. By tailoring the components of NADESs, such as organic acids, amino acids, and sugars, as well as the molar ratios of NADES components, solvents of different physical and chemical properties can be obtained, thus leading to high selectivity in extraction [4–6]. The main advantages of NADESs are their low cost, non-toxicity, biodegradability, maximum atom economy, and ease of preparation. NADESs function as solubilizing agents, serving as carriers of active compounds and improving the skin permeability of polar compounds [7]. In addition, it has been observed that NADESs can often enhance the biological activity of the extracts compared to conventional solvents [8]. These facts suggest that NADESs should be used more in the cosmetic industry and could eventually replace traditional organic solvents completely.

The toxicity and cytotoxicity of deep eutectic solvents (DESs) have undergone extensive investigation through numerous in vitro and in vivo assays [9,10]. It is noteworthy that the toxicity profile is influenced by the DES's inherent characteristics, the molar ratio of its components, and the dosage administered [9]. Notably, certain components of DESs, such as choline chloride, are prohibited in European cosmetics due to their toxic properties [9,11]. Moreover, organic acids like tartaric, oxalic, and malonic acids are not preferred due to their propensity to significantly increase the cytotoxicity in specific DES formulations [9]. Given that sugars present in NADES are entirely natural and safe, and amino acids are commonly used in cosmetics as safe ingredients, the selection of NADES components holds promise for safe cosmetic use [12]. Additionally, betaine has emerged as a suitable and entirely natural substitute for choline chloride, being utilized as a food additive and found to be non-toxic at all studied concentrations [13].

Computational models play a crucial role in understanding and enhancing natural product extraction processes, providing valuable insights into the fundamental mechanisms involved [14,15]. The application of computational models to predict the performance of NADES extraction ensures efficiency, economic viability, and environmental sustainability. One notable model for this purpose is COSMO-RS (Conductor-like Screening MOdel for Real Solvents), which utilizes quantum mechanics and statistical thermodynamics to simulate molecular behavior in a solution. COSMO-RS predicts various thermodynamic and transport properties, including solubility, partitioning, and viscosity [16].

Skin aging follows various transformations in skin physiology, and these changes can significantly influence self-perception and self-confidence. Enzymes within the extracellular matrix, including collagenase, elastase, and tyrosinase, become more active over time. These enzymes are involved in the degradation of crucial skin molecules responsible for maintaining elasticity, hydration, and structural integrity. The effects of their increased activity include thinner skin, sagging, wrinkles, dryness, reduced elasticity, and hyperpigmentation. The overactivity of tyrosinase enzyme contributes to accelerated skin pigmentation during aging. While tyrosinase normally produces a protective skin pigment known as melanin, excessive activation leads to issues like melasma, hyperpigmentation age spots, and freckles, which some may find aesthetically undesirable [17–21].

Free radicals are reactive molecules generated in organisms during normal physiological processes but also due to a variety of external factors, including UV radiation, exposure to xenobiotics, pollution, smoking, etc. Further, these molecules play a significant role in various biological processes but can also lead to oxidative stress and damage when their levels become excessive [22]. Oxidative stress occurs when free radicals interact with biomacromolecules such as DNA, proteins, and lipids in cells, leading to their damage. This process contributes to premature aging [23]. Furthermore, free radicals directly influence the activation of the mentioned enzymes, further inducing a loss of skin elasticity and the appearance of wrinkles [24].

Herbs consist of diverse specialized compounds, such as phenolic compounds and terpenoids, renowned for their abilities to combat melanin production, act as antioxidants, and provide valuable means in the strategy against aging [25–28]. They are useful in controlling

many diseases, including the prevention and treatment of skin disorders, particularly skin aging processes. Given the significance of adopting green chemistry principles in the cosmetic sector, we recognized the need for developing an effective eco-friendly extraction method enriched with phytochemicals with potential anti-aging effects on the skin.

In this regard, 18 herbal extracts were chosen and tested using five in vitro spectrophotometric assays: antioxidative (AO) capacity (DPPH and ABTS), total flavonoid (TFC), total phenolic content (TPC), and tyrosinase inhibition. Green tea (GT) was used as a reference sample in these experiments. Characterization of the extracts included the quantification of 26 phenolic compounds present in the plants using UHPLC-MS/MS. Given the critical importance of dosage in cosmetic product formulation, we characterized plant extracts by assessing their cytotoxicity on human keratinocytes (HaCaT cells).

Therefore, we aimed to select the six most potent herbs and design the NADESs for their mixture using the COSMO-RS approach. The AO capacity and TPC were conducted to compare the designed NADES solvents with a conventional and three NADESs already employed in cosmetics. The most efficient NADESs were identified as valuable ingredients for cosmetics products.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and reagents used in this study were provided by commercial suppliers. Ethanol, methanol, dimethylsulfoxide, glycerol, and dichloromethane were purchased from ZORKA Pharma (Šabac, Serbia). Free radical 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was purchased from Fluka (Buchs, Switzerland). Phosphate buffer (disodium phosphate and monosodium phosphate), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), tyrosinase enzyme from mushrooms, 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic (Trolox, T), protocatechuic acid (PCA), quercetin 3-*O*-glucoside (Q-3-*O*-G), 3,4-dihydroxy-L-phenylalanine (L-DOPA), caffeic acid (CAFA), aesculin, syringic acid (SYA), chlorogenic acid (CHA), rutin (RU), *p*-hydroxybenzoic acid (*p*-hydroxyBA), aesculetin, isorhamnetin (ISORH), isoorientin (ISOOR), kaempferol (K), vitexin (VIT), rosmarinic acid (RA), p-coumaric acid (*p*-COUM), kaempferol-3-*O*-glucoside (K-3-*O*-G), taxifolin (TAX), naringenin, ellagic acid (ELLA), quercitrin, aromadendrin (AD), luteolin, eriodictyol (ED), hispidulin (HP), quercetin, naringin gallic acid (GA), betaine, urea, L-proline, L-lysine, and kojic acid were bought from Sigma-Aldrich (Darmstadt, Germany). D-glycose, D-fructose, xylitol, and D-sorbitol were purchased from Thermo Scientific (Waltham, MA, USA). Ascorbic acid was provided by Betahem (Belgrade, Serbia). Folin–Ciocalteu (FC) reagent, potassium persulfate, and sodium carbonate were obtained from Merck (Darmstadt, Germany). Sucrose was bought from a local grocery store (Maxi Supermarket, Belgrade, Serbia).

2.2. Herbal Material

2.2.1. Conventional Extractions

Medicinal herbs were selected and classified into six different families (*Lamiaceae*: wild thyme (1), basil (2), peppermint (3), oregano (4), rosemary (5), winter savoury (6), mountain germander (17), and wall germander (19); *Rosaceae*: midland hawthorn (7), agrimony (9), red raspberry (13), blackberry (14), and dog rose (16); *Plantaginaceae*: broadleaf plantain (10); *Hypericaceae*: St John's wort (11); *Moraceae*: white mulberry (12); and *Asteraceae*: chamomile (15) and yarrow (18)). The medicinal herbs investigated in the current study were obtained by the Institute of Medicinal Plant Research "Dr. Josif Pancic", Belgrade, Serbia, while mulberry was cultivated in Ogladjenovac (Valjevo), Serbia (Table S1). The green tea plant, a well-known anti-aging plant (*Theaceae*, 8), obtained by "Dr. Josif Pancic", was used as the reference sample.

The raw herbal starting materials were milled into powder for 5 min using a home miller (Gorenje, Belgrade, Serbia), then 1 g of herbs material was mixed with 10 mL of extraction solvent, methanol:dichloromethane (1:1, *v*/*v*), and ultrasound-assisted extraction

was applied as an eco-friendly method to extract plant metabolites. The extracts were collected and centrifuged, and the solvents were evaporated to dry rest using a distilling rotary (IKA-Werke, Breisgau, Germany). The plant-dried residues were dissolved in methanol and stored in the fridge until further use.

2.2.2. NADESs Preparation and Green Extractions of Plant Mixture

The components forming the eutectic mixture were initially mixed and heated using a mechanical stirrer at 80 °C until the formation of a transparent phase was observed. Subsequently, a specific water content was added, and stirring continued for an additional 30 min. After forming the extraction solvent, 10 mL of the NADES was transferred to a flask, and precisely 1 g of the plant mixture was added. The selection of plants for the mixture was based on the results of spectrophotometric assays and cytotoxicity assessments. Phenolics from the plant material were then extracted for 30 min at a temperature of 50 °C. Following extraction, the plant material was separated by centrifugation at 12,326$\times$ g for 10 min, while the NADES extract was purified using solid-phase extraction (SPE) for characterization. Methanol and water were used as the reference conventional solvents.

2.2.3. Solid-Phase Extraction of Plant Mixture NADES Extracts

In brief, the sorbent (silica RP-C18 gel, Agilent Technologies–Bond Elut C18) underwent conditioning with water (5 mL) followed by methanol (5 mL), and the resulting supernatant (4 mL) was loaded. For the removal of non-adsorbed compounds and NADES components, water (10 mL) was used. The elution of the phenolics was carried out using methanol (6 mL), and the resultant methanolic extracts were stored in opaque glass vials at −20 °C until analysis.

2.3. LC-MS

Quantification of the phenolic compounds in herbal extracts was performed using the Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) configured with a diode array detector (DAD) and a triple quadrupole mass spectrometer (TSQ Quantum Access Max, Thermo Fisher Scientific). A Hypersil gold C18 column (50 × 2.1 mm, 1.9 µm particle size) was used for the separation of the phenolic compounds. The mobile phase consisted of acetic acid (0.2%) in water (v/v) (A) and acetonitrile (B). The injection volume was 10 µL, while the flow rate of the mobile phase was 0.4 mL/min.

A triple quadrupole mass spectrometer (qqqMS) equipped with a heated electrospray ionization (HESI) source was set as follows: sheet gas (N_2) pressure of 50 AU, spray voltage of 4000 V, vaporizer temperature of 450 °C, auxiliary gas pressure of 20 AU, and ion sweep gas pressure of 0 AU. The skimmer offset was 0 V, and the capillary temperature was set to 320 °C. For the phenolic compounds, a negative ion mode was applied, while the collision energy was 30 eV.

Working standard solutions were prepared by dissolving the stock solution of a pure compound to obtain a concentration of 100 mg/mL, and the other calibration levels were obtained by diluting the solutions with methanol. The dilution ranged from 0.025 to 1.000 mg/L. Calibration curves were constructed by plotting the peak areas of the standards against their concentrations ($R^2 > 0.9998$), and quantification of the phenolic compounds was performed using the external standard quantification procedure [14].

2.4. Spectrophotometric Assays

For the 96-well microplate spectrophotometric assays, herbal extracts were standardized to a concentration of 100 µg DW per gram of plant material (100 µg DW/g) to determine the AO capacity (DPPH and ABTS). A tyrosinase inhibition assessment was performed on the herbal extracts at a concentration of 100 µg/mL of extract. To obtain the most potent enriched extract (NADES), the efficiency of green extractions of the herbal mixture was determined by measuring the AO capacity (DPPH) and the quantification of the TP content.

2.4.1. Total Phenolic Content (TPC)

The Folin–Ciocalteu method was used for the determination of the total phenolic content (TPC) of the herbal (NADES) extracts [29]. A 0.5 mL aliquot of diluted NADES extract was mixed with 0.5 mL distilled water and 2.5 mL of 10% FC reagent (v/v). The mixture was incubated for 5 min at room temperature, followed by the addition of 2 mL of 7.5% Na_2CO_3 (w/v) to the reaction mixture. After shaking and 2 h of incubation at room temperature, the absorbance was measured at 765 nm using a GBC UV–Visible Cintra 6 spectrophotometer. The standard curve for the total phenols was constructed using gallic acid standard solutions (20–120 mg/L). The results were expressed as milligrams of gallic acid equivalents per gram of dried weight (mg GAE/g).

2.4.2. Total Flavonoid Content (TFC)

The total flavonoid content of the analyzed samples was determined using the Pekal and Pyrzynska method with some modifications [30]. Then, 0.3 mL of a diluted herbal extract was mixed with 3.4 mL of 30% methanol (v/v). Further, 0.15 mL of $NaNO_2$ (0.5 mol/L) and 0.15 mL of $AlCl_3$ (0.15 mol/L) were added to the reaction solution. After 5 min of incubation, a NaOH solution (1 mL of 1 mol/L) was added, and each tube was thoroughly shaken. The absorbance was measured at 506 nm (GBC UV–Visible Cintra 6 spectrophotometer). A calibration curve based on standard rutin solutions was used to estimate the TFC, expressed in milligrams of rutin equivalents per gram of dried weight (mg RUE/g).

2.4.3. DPPH Radical Scavenging Assay

The DPPH• scavenging capacity of the plant NADES extracts was determined by the standardized in vitro protocol [31]. In a 1.5 mL vial, 280 µL of ethanolic DPPH• solution (0.24 mg/mL, initial absorbance of 1.09) was combined with 10 µL of diluted herbal (NADES) extract and 10 µL of absolute ethanol. Five replicates were prepared for each sample. Following a 24 h incubation period, 250 µL from each replicate was transferred into microtiter plates. Subsequently, the decrease in absorbance at 517 nm was measured using a BioTek 800 TS spectrophotometer (Agilent Technologies, Inc., Headquarters, Santa Clara, CA, USA). Standard solutions of Trolox were utilized for the calibration curve construction. The results were expressed as a percentage of DPPH radical inhibition (%) and micromoles of Trolox equivalents per gram of dried weight (mmol(mM)TE/g).

2.4.4. ABTS•+ Radical Scavenging Assay

The reduction power of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•) radical cation was applied as another method for assessing the radical scavenging capacity. The ABTS•+ solution was prepared by mixing 7 mmol/L of ABTS solution in water and 245 mmol/L of potassium persulfate in proportions that yielded a final concentration of 2.45 mmol/L of potassium persulfate and stored in the dark at room temperature for 16 h [32]. After dilution, the reaction solution was adjusted to an absorbance of 0.7028 at 734 nm, then 10 µL of diluted herbal extracts were mixed with 190 µL of ABTS•+ solution, followed by an immediate absorbance measurement at 734 nm using a BioTek 800 TS spectrophotometer. To construct a calibration curve, standard solutions of Trolox (ranging from 10 to 500 µmol/L) were employed. The results were reported as both a percentage of ABTS radical inhibition and micromoles of Trolox equivalents per gram of dried weight (mmol(mM)TE/g).

2.4.5. Mushroom Tyrosinase Inhibition Assay

The inhibitory activity of the tyrosinase enzyme was determined using a 96-well microtiter plate spectrophotometric protocol, with slight modifications [33]. L-DOPA served as the substrate for the assay. A 140 µL phosphate buffer solution (pH 6.8; 20 mmol/L) was mixed with 20 µL of herbal extract (diluted in phosphate buffer) and 20 µL of enzyme solution in buffer (480 IU/mL). Further, the mixture was incubated for 10 min at room temperature. Additionally, the reaction mixture was mixed with 20 µL of 5 mmol/L

L-DOPA, which resulted in the formation of a brown color in the wells. We measured the decreasing absorbance at 475 nm after 20 min using a BioTek 800 TS spectrophotometer (Agilent Technologies, Inc., Headquarters, USA). The obtained results were expressed as a percentage of the enzyme inhibition (%):

$$I(\%) = 100 \cdot [(A - B) - (C - D)]/[A - B],$$

where A: negative control of the sample, B: blank, C: positive control of the sample, and D: negative control of an enzyme. Kojic acid was used as a positive control for this experiment.

2.5. Cytotoxicity Assay

2.5.1. Cell Culture

Human keratinocyte (HaCaT; AddexBio, No. T0020001) cells were cultured as monolayers in Dulbecco's Modified Eagle's Medium (DMEM). The DMEM was supplemented with 1% non-essential amino acid (NEAA), 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 10% fetal bovine serum (FBS). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.5.2. Determination of Target Cell Survival

The HaCaT cells were plated at a density of 10,000 cells per well in 96-well microtiter plates. After allowing the cells to adhere for 24 h, polyphenolic extracts were added into the wells at a final concentration of 100 µg/mL. Control wells received only a nutrient medium. All experiments were conducted in triplicate. Cell viability was assessed using the MTT assay after 24 h of treatment, following a slightly modified version of Mosmann's procedure [34]. In brief, 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline, PBS) was added to each well and incubated for 2 h at 37 °C in a humidified atmosphere containing 5% CO_2. Subsequently, the medium was carefully aspirated, and 200 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan complexes. Absorbance was measured at 530 nm for the test wavelength and 670 nm for the reference wavelength using a BioTek 800 TS spectrophotometer (Agilent Technologies, Inc., Headquarters, USA). The data were presented as a percentage of viability, with untreated cells considered as 100%.

2.6. COSMO-RS Modeling

The solubility of 12 compounds, namely quercetin, luteolin, hispidulin, rutin, dihydrokaempferol, eriodyctiol, naringenin, caffeic acid, 4-hydroxybenzoic acid, syringic acid, rosmarinic acid, and chlorogenic acid, was predicted in 64 virtual NADESs. These NADESs comprised 1:1 mixtures of betaine, carnitine, creatine, and ornithine (serving only as hydrogen bond acceptors—HBAs) and glycerol, dextrose, sorbitol, xylitol, sucrose, urea, ascorbic acid, L-proline, lysine, tyrosine, and fructose (acting as both hydrogen bond donors—HBDs and HBAs). Additionally, for comparative analysis, the solubility of these polyphenolics in water and methanol was predicted.

The σ-profiles for 4-hydroxybenzoic acid, betaine, carnitine, ornithine, creatine, tyrosine, and proline were retrieved from the ADF sigma profile database. For all other compounds' screening charges, their densities were computed by first optimizing their geometry at the semiempirical PM7 level [35] with the COSMO water model. Subsequently, single-point calculations were performed employing a small core TZP basis set, the Becke-Perdew (GGA:BP86) functional, and the relativistic scalar ZORA method for gas phase calculations [14]. These charges were then converted into activity coefficients (γ) using the COSMO-RS method implemented in the ADF2022.101 program [36] (https://www.scm.com/product/cosmo-rs/ (accessed on 14 March 2023)). The solubility parameter (β), directly proportional to the mutual solvent–solute affinity, was calculated as $\beta = 1/\gamma$ [37].

3. Results and Discussion

3.1. Total Phenolics and Flavonoid Contents (TPCs and TFCs)

The phenolic acids, flavonoids, and glycosides contents in the herbal extracts were evaluated by measuring the total phenolic (TPC) and total flavonoid content (TFC) (Table 1). The TPC varied between 0.21 ± 0.19 and 7.65 ± 1.23 mg GAE/g, with the GT extract showing the highest TPC at 12.37 ± 1.38 mg GAE/g, serving as the reference. Red raspberry extract had the lowest TPC, while rosemary extract exhibited the highest TPC. Notably high TPCs, surpassing half of GT's TPC, was observed in oregano (6.67 ± 0.77 mg GAE/g), agrimony (6.75 ± 0.50 mg GAE/g), and wall germander (7.59 ± 0.92 mg GAE/g) extracts.

Table 1. The bioactive content of the 18 herbal extracts and GT: TPC expressed as mg of gallic acid equivalents per g of plant material and TFC expressed as mg of rutin equivalents per g of plant material.

Number Designation	Common Name	TPC mg GAE/g	TFC mg RUE/g
1	Wild thyme	2.95 ± 0.41	13.89 ± 0.42
2	Basil	5.76 ± 0.45	2.64 ± 0.24
3	Peppermint	2.07 ± 0.41	4.72 ± 0.39
4	Oregano	6.67 ± 0.77	19.30 ± 0.80
5	Rosemary	7.65 ± 1.23	13.53 ± 1.23
6	Winter savoury	0.56 ± 0.25	1.99 ± 0.22
7	Midland hawthorn	4.48 ± 1.54	9.31 ± 0.85
8	Green tea	12.37 ± 1.38	78.09 ± 4.34
9	Agrimony	6.75 ± 0.50	21.33 ± 1.07
10	Broadleaf plantain	2.88 ± 0.26	17.97 ± 0.82
11	St John's wort	3.11 ± 0.41	8.82 ± 0.42
12	White mulberry	0.88 ± 0.11	1.50 ± 0.12
13	Red raspberry	0.21 ± 0.19	1.24 ± 0.41
14	Blackberry	1.03 ± 0.06	1.32 ± 0.14
15	Chamomile	1.85 ± 0.47	11.58 ± 0.43
16	Dog rose	1.53 ± 0.84	5.36 ± 0.77
17	Mountain germander	0.82 ± 0.35	4.71 ± 0.31
18	Yarrow	0.93 ± 0.02	2.57 ± 0.26
19	Wall germander	7.59 ± 0.92	28.61 ± 1.00

In terms of TFC, the range in the herbal extracts varied from 1.24 ± 0.41 to 28.61 ± 1.00 mg RUE/g, with GT extract displaying the highest TFC of 78.09 ± 4.34 mg RUE/g. Similar to TPC, red raspberry extract had the lowest TFC, while wall germander extract had the highest. Notably high TFCs, around more than a quarter of the reference extract's values, were found in broadleaf plantain (17.97 ± 0.82 mg RUE/g), oregano (19.30 ± 0.80 mg RUE/g), and agrimony (21.33 ± 1.07 mg RUE/g) extracts. Oregano, agrimony, and wall germander extracts demonstrated notable levels of both TP and TF content.

3.2. Antioxidative (AO) Capacity Assays

The herbal extracts' AO capacity was determined using DPPH and ABTS spectrophotometric assays measuring the radical scavenging activity (Table 2). The results from the DPPH and ABTS assays indicated that all extracts possessed an AO capacity, albeit with varying degrees of radical inhibition potential. Both assays showed a generally similar trend in activity, supported by a high correlation coefficient (R^2 = 0.8305). Among the herbal extracts, midland hawthorn extract exhibited the lowest radical inhibition (0.27 ± 0.03%, DPPH; 12 ± 1%, ABTS). Conversely, wall germander (72 ± 3%, DPPH; 60 ± 2%, ABTS), winter savoury (47 ± 2%, DPPH; 45 ± 3%, ABTS), mountain germander (45 ± 2%, DPPH; 28 ± 2%, ABTS), peppermint (66 ± 2%, DPPH; 40 ± 2%, ABTS), agrimony (36 ± 2%, DPPH; 26 ± 1%, ABTS), and yarrow (36 ± 5, DPPH; 26 ± 2, ABTS) were identified as the most potent radical scavengers. Notably, wall germander, winter savoury, and peppermint

demonstrated a higher AO capacity compared to GT (42 ± 5%, DPPH; 41 ± 3%, ABTS), while agrimony exhibited significant inhibition with a value comparable to GT.

Table 2. The AO capacity of the 18 herbal extracts and GT (100 µg of DW/g of plant material): expressed as a percent of DPPH and ABTS radical inhibition and µM of Trolox equivalents per g of plant material.

Number Designation	Common Name	DPPH RSA (%)	ABTS RSA (%)	DPPH mM TE/g	ABTS mM TE/g
1	Wild thyme	25 ± 2	26 ± 3	0.18 ± 0.01	0.18 ± 0.02
2	Basil	10 ± 4	13 ± 1	0.06 ± 0.01	0.11 ± 0.01
3	Peppermint	66 ± 2	40 ± 2	0.46 ± 0.01	0.27 ± 0.01
4	Oregano	6.8 ± 3	18 ± 3	0.06 ± 0.01	0.14 ± 0.01
5	Rosemary	4.8 ± 1.9	13 ± 2	0.04 ± 0.01	0.14 ± 0.06
6	Winter savoury	47 ± 2	45 ± 3	0.33 ± 0.01	0.32 ± 0.01
7	Midland hawthorn	0.27 ± 0.03	12 ± 1	0.01 ± 0.01	0.10 ± 0.01
8	Green tea	42 ± 5	41 ± 3	0.29 ± 0.03	0.29 ± 0.02
9	Agrimony	36 ± 2	26 ± 1	0.24 ± 0.01	0.19 ± 0.01
10	Broadleaf plantain	10 ± 1	11 ± 1	0.07 ± 0.0	0.09 ± 0.01
11	St John's wort	24 ± 3	24 ± 2	0.17 ± 0.02	0.18 ± 0.01
12	White mulberry	12 ± 1	19 ± 2	0.08 ± 0.01	0.15 ± 0.01
13	Red raspberry	18 ± 3	24 ± 2	0.13 ± 0.02	0.18 ± 0.01
14	Blackberry	24 ± 4	24 ± 1	0.15 ± 0.01	0.18 ± 0.01
15	Chamomile	25 ± 7	21 ± 1	0.19 ± 0.02	0.16 ± 0.01
16	Dog rose	14 ± 4	13 ± 1	0.08 ± 0.01	0.11 ± 0.01
17	Mountain germander	45 ± 2	28 ± 2	0.32 ± 0.01	0.20 ± 0.01
18	Yarrow	36 ± 5	26 ± 2	0.24 ± 0.01	0.19 ± 0.01
19	Wall germander	72 ± 3	60 ± 2	0.51 ± 0.02	0.42 ± 0.01

It is widely acknowledged that the reference sample of green tea possesses a strong AO capacity due to compounds known as catechins [38]. However, agrimony demonstrated potent AO activity comparable to the reference sample [39]. The high AO capability observed in wall germander and agrimony can be attributed to their heightened levels of TP and TF content, probably due to the presence of active phenols and flavonoids within these extracts (as outlined in Table 1). The highest quantities of quercetin (50 ± 4 mg/kg) and chlorogenic acid (43 ± 3 mg/kg) were measured in wall germander, consistent with values reported in the literature (Table 3) [40]. These compounds have been recognized as strong inhibitors of radicals, potentially accounting for the elevated antioxidant capacity of the wall germander extract [41,42]. The AO effect of winter savoury is influenced by other classes of compounds besides phenolics. Furthermore, free radicals have been implicated in skin hyperpigmentation resulting from oxidative stress induced by ultraviolet and visible light exposure. Therefore, the potential importance of herbal extracts lies in their capacity to scavenge these radicals, which could be pivotal in hindering such skin concerns.

Table 3. List of quantified phenols determined in the herbal extracts and GT (mg/kg ± SD). Measurements were performed in triplicate. NF—not found.

	Aesculin	***p*-HydroxyBA**	**Aesculetin**	**ISOOR**	**Q-3-*O*-G**	**TAX**	**PCA**	**SYA**	**CHA**	**CAFA**	**RU**	**VIT**	***p*-COUM**
1	3.62 ± 0.18	84 ± 5	30.7 ± 1.1	7 ± 1	24 ± 1	32 ± 2	17.2 ± 0.9	18.53 ± 0.24	23 ± 3	38 ± 2	NF	2.4 ± 0.2	3.03 ± 0.12
2	5.50 ± 0.28	70 ± 3	41 ± 2	0.49 ± 0.03	28 ± 2	NF	7.91 ± 0.37	9.45 ± 0.18	8.9 ± 0.3	53 ± 1	61 ± 3	NF	7 ± 1
3	0.67 ± 0.03	42.4 ± 2.4	12.99 ± 1.29	NF	1.85 ± 0.03	0.97 ± 0.03	2.99 ± 0.2	2.99 ± 0.06	12.9 ± 0.8	17.2 ± 0.7	33 ± 2	NF	3.0 ± 0.5
4	5.80 ± 0.28	64 ± 4	36 ± 2	6.63 ± 0.39	3.91 ± 0.18	100 ± 5	12.1 ± 1.0	13.9 ± 0.3	25.7 ± 1.9	48 ± 3	41 ± 6	9.7 ± 0.4	7 ± 1
5	1.05 ± 0.06	20.1 ± 1.0	13.03 ± 0.51	NF	0.39 ± 0.03	NF	3.21 ± 0.34	2.90 ± 0.14	0.49 ± 0.02	11.7 ± 0.8	0.89 ± 0.04	NF	NF
6	0.56 ± 0.03	10.7 ± 0.7	2.28 ± 0.02	NF	2.61 ± 0.09	8.8 ± 0.5	1.99 ± 0.11	1.76 ± 0.03	0.58 ± 0.05	3.17 ± 0.07	0.82 ± 0.04	NF	1.34 ± 0.04
7	3.40 ± 0.17	10.1 ± 0.4	0.98 ± 0.05	0.80 ± 0.04	NF	201 ± 9	1.34 ± 0.07	5.18 ± 0.10	6.3 ± 0.1	101 ± 5	0.99 ± 0.03	99 ± 5	NF
8	10.80 ± 0.54	22.5 ± 0.6	1.00 ± 0.05	0.80 ± 0.04	NF	29 ± 2	0.6 ± 0.1	6.77 ± 0.17	NF	5.26 ± 0.47	3.8 ± 0.2	137 ± 7	12.7 ± 0.5
9	14 ± 1	2.47 ± 0.14	5 ± 1	3.37 ± 0.20	8.68 ± 0.34	167 ± 9	0.29 ± 0.02	12 ± 1	12.9 ± 0.52	4.66 ± 0.48	0.01 ± 0.01	150 ± 8	11.5 ± 0.4
10	8.15 ± 0.29	4.61 ± 0.23	7.0 ± 0.5	6 ± 1	1.51 ± 0.06	0.78 ± 0.03	0.71 ± 0.11	4.64 ± 0.11	24 ± 2	23 ± 3	NF	0.83 ± 0.03	NF
11	36 ± 4	2.12 ± 0.10	2.88 ± 0.05	1.91 ± 0.11	0.69 ± 0.03	179 ± 10	0.38 ± 0.07	39 ± 1	8.8 ± 1.1	14 ± 1	1.31 ± 0.06	552 ± 9	NF
12	0.39 ± 0.02	3.80 ± 0.16	0.57 ± 0.01	0.67 ± 0.02	NF	52 ± 3	0.28 ± 0.03	0.28 ± 0.02	15.4 ± 2.0	53 ± 4	NF	81 ± 5	0.02 ± 0.01
13	18 ± 1	5.32 ± 0.27	35 ± 3	23 ± 2	11.51 ± 0.58	3.39 ± 0.17	0.19 ± 0.01	18.6 ± 0.4	13.68 ± 1.14	19 ± 1	NF	NF	NF
14	2.97 ± 0.08	2.84 ± 0.08	3.33 ± 0.09	2.26 ± 0.24	1.29 ± 0.06	3.28 ± 0.17	0.13 ± 0.02	3.21 ± 0.11	4.66 ± 0.21	3.00 ± 0.02	0.01 ± 0.01	9 ± 1	NF
15	7.98 ± 0.19	26.4 ± 0.9	15 ± 2	11 ± 1	1.29 ± 0.07	19.70 ± 0.99	0.39 ± 0.01	8.50 ± 0.32	14.8 ± 0.4	77 ± 4	NF	4.40 ± 0.15	NF
16	36 ± 2	4.71 ± 0.10	0.58 ± 0.05	0.60 ± 0.05	8.92 ± 0.55	22 ± 2	0.69 ± 0.03	36 ± 1	10 ± 1	2.38 ± 0.06	NF	4.40 ± 0.23	0.01 ± 0.01
17	11 ± 1	1.02 ± 0.05	1.37 ± 0.06	1.12 ± 0.06	NF	2.38 ± 0.08	0.29 ± 0.02	0.98 ± 0.02	15.9 ± 0.7	3.80 ± 0.18	NF	4.08 ± 0.13	NF
18	1.52 ± 0.06	1.22 ± 0.08	6.97 ± 0.48	5.02 ± 0.31	1.25 ± 0.06	1.89 ± 0.09	0.24 ± 0.02	1.22 ± 0.06	16.8 ± 0.5	33 ± 4	0.02 ± 0.01	8.15 ± 0.39	0.02 ± 0.01
19	28 ± 2	16.5 ± 0.4	10.5 ± 0.9	7.99 ± 0.43	3.82 ± 0.07	6.01 ± 0.29	0.48 ± 0.03	9.6 ± 0.5	43 ± 3	26.9 ± 1.8	0.02 ± 0.01	7 ± 1	NF
	K	**HP**	**ISORH**	**RA**	**AD**	**ED**	**LUT**	**Q**	**Naringenin**	**ELLA**	**Naringin**	**K-3-*O*-G**	**Quercitrin**
1	12 ± 1	1.45 ± 0.08	0.9 ± 0.1	470 ± 9	12 ± 1	56 ± 3	49 ± 4	30 ± 3	32.8 ± 2.8	40 ± 2	42 ± 3	14.92 ± 1.15	154 ± 6
2	3.1 ± 0.4	3 ± 1	1.6 ± 0.2	446 ± 6	0.02 ± 0.01	4.76 ± 0.05	12.7 ± 1.2	0.7 ± 0.1	4.09 ± 1.04	1.49 ± 0.07	NF	12.2 ± 0.89	2.88 ± 0.08
3	2.44 ± 0.18	0.94 ± 0.03	NF	345 ± 7	0.02 ± 0.01	8.93 ± 0.76	10.6 ± 0.6	1.31 ± 0.04	12.16 ± 1.04	NF	310 ± 9	2.65 ± 0.08	0.09 ± 0.02
4	22 ± 3	0.58 ± 0.03	NF	542 ± 13	142.5 ± 6.65	254 ± 9	183 ± 6	14 ± 1	269 ± 5	69 ± 5	9.91 ± 0.50	2.36 ± 0.08	0.53 ± 0.04
5	6.06 ± 0.38	25 ± 3	NF	134 ± 11	NF	6.51 ± 0.96	9 ± 1	0.8 ± 0.1	1.49 ± 0.11	NF	NF	1.70 ± 0.06	0.29 ± 0.03
6	1.68 ± 0.17	0.12 ± 0.02	0.11 ± 0.01	95.1 ± 434	22.8 ± 1.9	15.96 ± 2.19	12 ± 1	0.75 ± 0.05	43.8 ± 2.4	6.6 ± 0.4	0.41 ± 0.01	1.17 ± 0.05	0.30 ± 0.01
7	1.99 ± 0.02	8 ± 1	NF	3.41 ± 0.12	3.99 ± 0.38	NF	7 ± 1	2.63 ± 0.20	10.45 ± 0.95	4.48 ± 0.14	NF	NF	42 ± 4
8	0.86 ± 0.03	8 ± 2	0.05 ± 0.01	NF	2.98 ± 0.04	1.08 ± 0.76	0.5 ± 0.1	NF	33.4 ± 3.1	5.7 ± 0.4	3.33 ± 0.17	2.02 ± 0.08	26 ± 1
9	1.31 ± 0.03	5.70 ± 0.95	0.03 ± 0.01	640 ± 9	3.88 ± 0.07	NF	1.0 ± 0.2	0.50 ± 0.02	23 ± 2	6. ± 1	29 ± 2	NF	48 ± 4
10	0.32 ± 0.03	NF	2 ± 1	1.65 ± 0.08	15.81 ± 0.85	NF	1.05 ± 0.08	24 ± 2	0.44 ± 0.02	NF	NF	NF	1.90 ± 0.10
11	1.03 ± 0.03	7.12 ± 0.38	0.13 ± 0.02	150 ± 4	5.32 ± 0.66	0.81 ± 0.04	0.99 ± 0.12	0.91 ± 0.02	430 ± 9	4.11 ± 0.18	1.06 ± 0.07	0.53 ± 0.03	12 ± 2
12	0.04 ± 0.01	3.0 ± 0.3	NF	0.32 ± 0.01	1.08 ± 0.04	0.39 ± 0.02	0.3 ± 0.1	0.68 ± 0.04	1.93 ± 0.03	6.0 ± 0.5	2.93 ± 0.09	0.18 ± 0.02	60 ± 4
13	0.2 ± 0.1	2.91 ± 0.17	0.04 ± 0.01	2.0 ± 0.5	1.31 ± 0.02	NF	0.47 ± 0.09	NF	5.41 ± 0.54	2.85 ± 0.09	55 ± 3	NF	7 ± 1
14	1.00 ± 0.03	2.17 ± 0.19	0.04 ± 0.01	0.30 ± 0.02	5.93 ± 0.36	NF	0.60 ± 0.05	0.24 ± 0.04	2.56 ± 0.22	1.01 ± 0.04	49 ± 3	NF	10 ± 1
15	2.0 ± 0.1	8.3 ± 0.67	1.55 ± 0.02	NF	2.0 ± 0.5	0.76 ± 0.02	5 ± 1	7 ± 1	89 ± 3	NF	NF	0.3 ± 0.1	14 ± 1
16	1.02 ± 0.03	6 ± 1	0.10 ± 0.01	78 ± 5	3.59 ± 0.12	0.71 ± 0.01	2.5 ± 0.5	NF	32 ± 3	8.0 ± 0.5	179 ± 10	NF	18.5 ± 0.5
17	4.0 ± 0.5	NF	0.30 ± 0.02	9 ± 1	2.27 ± 0.02	NF	1.0 ± 0.1	23 ± 4	1.08 ± 0.06	0.61 ± 0.03	NF	0.11 ± 0.08	11.6 ± 0.9
18	0.15 ± 0.02	2.63 ± 0.14	1.69 ± 0.08	0.20 ± 0.03	0.69 ± 0.04	NF	0.29 ± 0.03	29 ± 3	6.89 ± 0.40	NF	1.49 ± 0.07	0.65 ± 0.07	10.45 ± 0.95
19	30 ± 3	6.12 ± 0.23	0.47 ± 0.02	NF	6.71 ± 0.08	NF	1.59 ± 0.04	50 ± 4	1.24 ± 0.03	0.76 ± 0.03	0.85 ± 0.03	NF	5.35 ± 0.31

3.3. Cytotoxicity and Tyrosinase Inhibition Assays

HaCaT cells serve as a valuable in vitro model for evaluating cytotoxicity, especially in toxicology, pharmaceuticals, and cosmetics, since there is a good correlation with the results obtained using in vivo experimental models [43]. The cytotoxicity results (Figure 1A) revealed different effects on the HaCaT cell viability of the examined extracts in the applied concentration of 100 µg/mL. Peppermint, midland hawthorn, and yarrow induced a notable but not statistically significant reduction in cell viability (viability: 72.5 ± 10.9%, 80.0 ± 4.7%, and 76.4 ± 7.9%, respectively) compared to the untreated cells. Importantly, rosemary, St John's wort, and white mulberry displayed significant cytotoxicity (viability: 31.4 ± 8.5%, 51.4 ± 13.4%, and 67.0 ± 1%, respectively). Interestingly, agrimony, broadleaf plantain, dog rose, and wall germander showed significant proliferative effects on HaCaT cells (viability: 130.8 ± 13.9%, 132.4 ± 14.5%, 148.7 ± 4.7%, and 160.0 ± 11.1%, respectively). The plant extracts with no notable effects on HaCaT cell viability were the wild thyme, basil, oregano, winter savoury, red raspberry, blackberry, chamomile, mountain germander, and GT reference extracts.

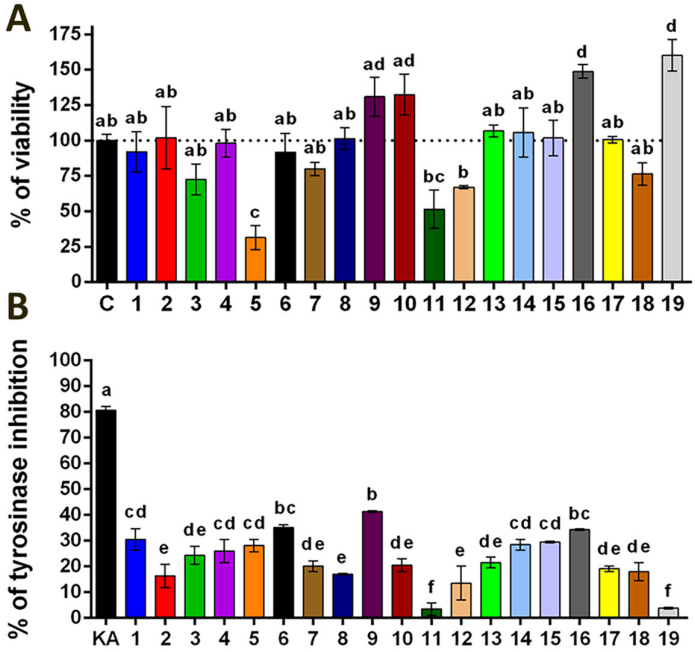

Figure 1. (**A**) The viability percentage of HaCaT cells following exposure to phenolic extracts at a concentration of 100 µg/mL (1–19) and a control (C). (**B**) Tyrosinase inhibition of 18 investigated herbs (1–19), GT (8), and a standard of kojic acid (KA). Different letters above the columns denote significant differences at $p < 0.05$ according to Tukey's multiple comparisons test.

Milutinovic and colleagues studied wall germander extract's impact on colon carcinoma and HaCaT cells, finding no cytotoxic effects on HaCaT cells and even a slight increase in proliferation, which aligns with our findings [44].

The tyrosinase inhibition of the examined extracts at a 100 µg/mL concentration and KA (81 ± 6%) as a known tyrosinase inhibitor was tested (Figure 1B). Among the extracts, agrimony displayed the highest tyrosinase inhibition (42 ± 1%), followed by winter savoury (35 ± 1%), dog rose (34 ± 1%), and wild thyme (31 ± 6%). All other tested extracts exhibited approximately the same potential for tyrosinase inhibition, whereas St John's wort and wall germander extracts demonstrated negligible potential for tyrosinase inhibition (3 ± 2% and 4 ± 2%, respectively).

This study aimed to identify new natural resources that could be utilized for cosmetic purposes in skin lightening. It is known that flavonoids with a hydroxyl group at C-3′ of ring B, a hydroxyl group at C-3, and a carbonyl group at C-4 of ring C enhance tyrosinase inhibitory effects [45,46]. Our study quantified taxifolin, vitexin, and quercitrin as the

primary metabolites in agrimony. These phenolics meet one or more conditions from the statement above for potent tyrosinase inhibition, likely causing high inhibition in this plant extract. Indeed, the data indicate a high inhibition of these compounds against tyrosinase [47–50]. Additionally, in the aforementioned sample, a high amount of caffeic acid, a confirmed tyrosinase inhibitor, was quantified [51]. To the best of our knowledge, there is limited available data regarding the tyrosinase inhibition potential of agrimony. The existing information suggests that it exhibits low potency in tyrosinase inhibition, while, in our study, this sample, even at low concentration, acted as the most potent inhibitor among these plants (42 ± 1%) [52]. Wild thyme also represents a relatively underexplored plant, with only one result in the literature indicating moderate inhibition. Our research confirms this finding (31 ± 6%) [52].

According to the available literature, there has been only one study that explored the impact of winter savory on tyrosinase inhibitory activity [53]. In our research, this herb demonstrated moderate tyrosinase inhibition, showing the second-best inhibitory activity (35 ± 1%) and exhibiting characteristics of a potent in vitro anti-pigmentation agent. Limited information exists on the tyrosinase inhibitory activity of midland hawthorn. Our results demonstrated moderate inhibitory activity at low concentrations (21 ± 3%). Other samples, such as peppermint, broadleaf plantain, and chamomile, exhibited almost identical inhibition as found in the literature [53]. Surprisingly, red raspberry (22 ± 3%) and dog rose (35 ± 1%) showed moderate levels of tyrosinase inhibition in our study, contrary to descriptions in the literature where they are reported to have weak or no inhibitory properties [53].

A correlation was observed between high tyrosinase inhibition activity and elevated concentrations of specific phenolics in the most effective extracts. Wild thyme and dog rose contained high levels of naringenin and naringin (Table 3). Wild thyme showed a noteworthy amount of eriodictyol, ellagic acid, and luteolin. Previous studies have demonstrated that luteolin effectively inhibits tyrosinase-catalyzed oxidation in both cell-free extracts and living cells [54]. Additionally, ellagic acid has been described in the literature as an effective inhibitor of this enzyme both in vivo and in vitro [55]. Notably, Fan and colleagues investigated flavonoids from food sources and found that all the mentioned flavonoids exhibited satisfactory binding energies to tyrosinase. Among them, luteolin demonstrated the highest binding affinity, which aligns with our findings [46]. In the agrimony extract, the highest level of rosmarinic acid (640 ± 9 mg/kg) was found. The content of the same significant phenols in this plant has been confirmed in the literature [56]. Rosmarinic acid, found in high concentrations in the samples of wild thyme, agrimony, and dog rose, may be associated with their significant tyrosinase inhibition, considering that this compound is known to act as a potent tyrosinase inhibitor [57]. Similarly, quercitrin, found in the highest concentration in wild thyme and agrimony, has demonstrated potent anti-melanogenic properties [50].

3.4. UHPLC-DAD-MS/MS

Using UHPLC-MS analysis, 26 phenolic compounds were quantified in our extracts (Table 3). The major phenolic acids found in the herbal extracts were syringic acid, *p*-hydroxybenzoic acid, protocatechuic acid, caffeic acid, chlorogenic acid, and rosmarinic acid. The main flavonoid glycosides, isoquercitrin, quercitrin, and kaempferol-3-O-glucoside, were found in the herbal extracts.

Among the extracts, agrimony and dog rose exhibited significant inhibitory effects on tyrosinase and proliferative effects on the HaCaT cells. Agrimony extract contained the highest levels of rosmarinic acid (640 ± 9 mg/kg) and notable amounts of *p*-coumaric acid (11.5 ± 0.4 mg/kg), vitexin (150 ± 8 mg/kg), taxifolin (167 ± 9 mg/kg), and quercitrin (48 ± 4 mg/kg). Dog rose extract showed the highest aesculin content (36 ± 2 mg/kg) and substantial levels of syringic acid (36 ± 1 mg/kg), rosmarinic acid (78 ± 5 mg/kg), and naringin (179 ± 10 mg/kg). Wild thyme and winter savoury also displayed considerable potency in tyrosinase inhibition. Wild thyme was rich in rosmarinic acid (470 ± 9 mg/kg),

protocatechuic acid (17.2 ± 0.9 mg/kg), *p*-hydroxybenzoic acid (84 ± 5 mg/kg), kaempferol-3-O-glucoside (14.92 ± 1.15 mg/kg), and quercitrin (154 ± 6 mg/kg). Winter savoury, although not notable for any specific phenolic compound, exhibited significant tyrosinase inhibition. Broadleaf plantain extract, containing notable amounts of chlorogenic acid (24 ± 2 mg/kg) and isorhamnetin (6 ± 1 mg/kg), demonstrated significant proliferative effects on HaCaT cells. However, wall germander extract showed the highest potency for HaCaT cell proliferation despite poor tyrosinase inhibition and contained substantial levels of quercetin (50 ± 4 mg/kg) and kaempferol (30 ± 3 mg/kg). These results suggest that the observed effects arise from the harmonized complex combination of phenolic compounds obtained through specific established procedures and solvents utilized during plant processing and extraction rather than the efficacy of any individual compound present in significant quantities.

3.5. Selection of the Most Potent Plants for Cosmetics Formulations

As a result of previous experiments, it was concluded that herbal extracts of wild thyme, agrimony, and wall germander proved to be effective radical scavengers. Additionally, herbal extracts of wild thyme, winter savoury, agrimony, and dog rose demonstrated exceptional depigmentation properties, while agrimony, broadleaf plantain, dog rose, and wall germander showed effectiveness as skin cell protectors. Furthermore, a significant proliferative effect on keratinocyte cells was a criterion for selecting herbs for incorporation into the herbal mixture, given that keratinocytes are important for skin re-epithelialization and recovery. Bearing this in mind, plants exhibiting the highest degree of proliferation were chosen for the herbal blend: agrimony, broadleaf plantain, dog rose, and wall germander. Individual extracts exhibiting multiple biological activities and proliferative or at least no cytotoxic effects on HaCaT cells were selected for the plant mixture. Finally, the plant mixture contained wild thyme, winter savoury, agrimony, broadleaf plantain, dog rose, and wall germander, all mixed in equal proportions.

3.6. NADESs Designs for Phenolic Extraction from the Herbal Mixture

Addressing the complexities of diverse mixtures of natural phenolic compounds derived from plant origins is highly in demand, particularly for the initial pre-screening and predicting their extractability to obtain mixtures with desired biological activity using natural deep eutectic solvents (NADESs) instead of conventional solvents. For this purpose, the *in silico* COSMO-RS method was employed.

3.6.1. COSMO-RS Screening of NADESs

The screening charge densities of hispidulin and syringic acid (Figure 2a), representing flavones and carboxylic acids found in the studied plant materials, illustrate the distribution of the negative electrostatic potential (ESP) around carbonyl and hydroxyl oxygens, with a positive ESP near hydroxyl group hydrogens. These screening charge densities were then converted into σ-profiles. Subsequently, the activity coefficients and their reciprocal values (solubility parameter β) were calculated for each of the 12 phenolics across 64 theoretical NADESs. Detailed data are available in the Electronic Supplementary Information, Table S2. Figure 2b,c reveal that carnitine-based and betaine-based NADESs exhibit a higher affinity for hispidulin and syringic acid compared to other mixtures. Additionally, these high-affinity NADESs show higher β values for all phenolics when compared to conventional solvents like water and methanol. This observation can be attributed to the complementarity of hydrogen-bonding regions for solvents and solutes, along with the significant non-polar area in σ-profiles (Figure 2d). These findings suggest the significant role of hydrogen bonding and van der Waals interactions in solubilizing these compounds in NADESs. After analyzing the results of the COSMO-RS screening, we further focused on investigating only betaine-based NADESs due to their usage in cosmetics [58].

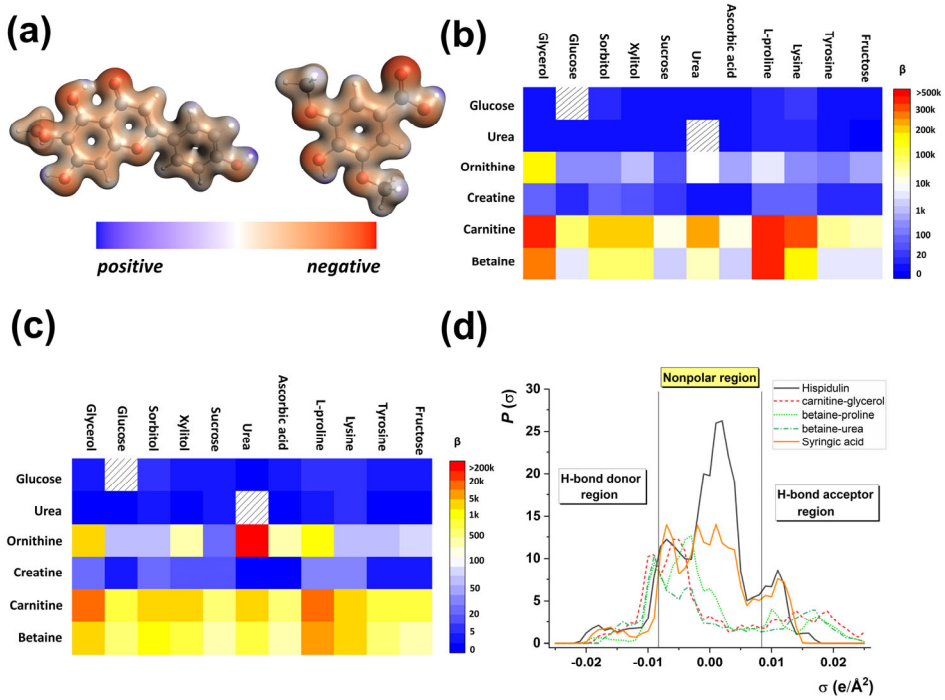

Figure 2. (**a**) Screening charge densities for hispidulin (**left**) and syringic acid (**right**); Heatmaps representing the solubility of (**b**) hispidulin and (**c**) syringic acid in 1:1 mixtures of corresponding NADES constituents. (**d**) Sigma profiles of two phenolics and three NADESs with the highest affinities for these compounds. For additional data, including the solubility parameters for 10 other studied compounds, refer to Table S2 in the Electronic Supplementary Information (ESI).

3.6.2. NADES Selection, Preparation, and the Extraction of Phenolic Compounds

Considering that ionic liquids are often incompatible for use in cosmetics, we have opted for NADESs as a green alternative to conventional organic solvents for cosmetic applications [59]. Bearing in mind the results of the COSMO-RS screening and previously reported NADESs for cosmetic applications, we synthesized ten betaine-based NADESs (Table 4).

Table 4. The abbreviations and components of the investigated NADESs.

Green Solvents		
Abbreviation	**NADES Composition**	**Molar Ratio (Water, *w*/*w*)**
N1	Betaine/Glycerol	1/1 (30%)
N2	Betaine/Xylitol	1/1 (30%)
N3	Betaine/Sucrose	1/1 (30%)
N4	Betaine/Urea	1/1 (30%)
N5	Betaine/Ascorbic acid	1/1 (30%)
N6	Betaine/L-proline	1/1 (30%)
N7	Betaine/Lysine	1/1 (30%)
GF1	Betaine/Glucose	1/1 (23%)
GF2	Betaine/Sorbitol	1/1 (23%)
GF3	Betaine/Fructose	1/1 (23%)
Conventional solvents		
M	Methanol	
W	Water	

We prepared seven proposed (N1–N7) and three reference NADESs (GF1–GF3) [11] constructed from plant metabolites such as glycerol; ascorbic acid; urea; sugars (glucose, fructose, sucrose, xylitol, and sorbitol); and amino acids (lysine and proline) that acted like

hydrogen bond donors (HBDs). Also, raw materials compatible with the cosmetic industry, such as betaine (a greener substitute for choline chloride), were used as bio-based and cost-effective materials [11].

The main drawback of NADESs is their high viscosity. The viscosity can be reduced by adding water to the NADESs or by increasing the temperature. To avoid disrupting the hydrogen bonds responsible for NADES formation or extraction, it is permissible to add a maximum of 50% (w/w) water [56]. The addition of a specific amount of water plays a crucial role in certain extractions [60]. Therefore, we added 23–30% water for easier handling of the NADESs.

Through the experimental validation of COSMO-RS, the proposed NADESs proved to be moderate to excellent green extraction media for biologically active compounds from the herbal material. The NADES extracts demonstrated a superior ability to extract phenolics as compared to both conventional solvents and reference NADESs, making them a more efficient and eco-friendlier alternative for cosmetics solvents (see Table 5).

Table 5. DPPH and TPC values of the herbal mixture NADES extracts (N1–N7), reference NADES extracts used in cosmetics (GF1–GF3), and conventional extracts (M and W). The results are presented as the mean ± standard deviation.

Extracts Designation	TPC mg GAE/g	DPPH mM TE/g
N1	16.0 ± 0.2	5.28 ± 0.46
N2	16.3 ± 0.5	5.09 ± 0.26
N3	10.1 ± 0.2	3.80 ± 0.34
N4	33 ± 1	15.06 ± 0.39
N5	11.8 ± 0.6	0.25 ± 0.15
N6	36.6 ± 0.6	18.14 ± 0.26
N7	82 ± 4	19.58 ± 0.09
GF1	0.58 ± 0.03	0.25 ± 0.01
GF2	9.5 ± 0.6	2.67 ± 0.01
GF3	0.62 ± 0.03	0.28 ± 0.02
M	1.85 ± 0.08	0.47 ± 0.05
W	1.87 ± 0.01	0.45 ± 0.03

It was concluded that the nature of the HBD played a significant role in the extraction efficiency. NADESs containing a component with basic nitrogen as the HBD component in their structure extracted the highest amounts of phenolics (N4, N6, and N7), with N7, featuring lysine as the second component, showing the most promising performance. NADES N7 extracted more than twice the amount of phenols compared to N4 and N6. Additionally, N7 extracted between 8.6 times and 141 times more phenols compared to the reference NADESs, while extracting 44 times more phenols compared to the water and methanol extracts, representing a new promising alternative to organic solvents used in cosmetics. These findings are consistent with the predicted activity coefficients obtained after COSMO-RS screening. Additionally, mixtures GF1 (glucose), GF3 (fructose), N3 (sucrose), and N5 (ascorbic acid) proved to be the poorest extractors of phenolic compounds, which aligns with the COSMO-RS predictions. An exception was GF2 (sorbitol), which extracted slightly more phenolics, as also predicted by COSMO-RS.

Betaine, as a component of NADES, would be an effective addition to cosmetic products, because it acts as a confirmed hydrating agent due to the formation of strong hydrogen bonds with water, making it an ideal addition for skin hydration. Additionally, betaine has been confirmed to reduce skin irritation, further enhancing its appeal for cosmetic use [61].

3.7. Quality Assessment of 18 Investigated NADESs from the Plant Mixture

TP Content and AO Capacity of the Investigated NADESs

The TP content and AO capacity for seven designed NADESs (N1–N7) were compared with three extracts obtained using patented cosmetical NADESs (GF1–GF3) and conventional methanolic (M) and water extracts (W) (Table 5).

The good correlation (R^2 = 0.8139) between the results of the TPC and DPPH tests indicates that the AO capacity is strongly influenced by the phenolic compounds present in the samples. In the examined NADES extracts, phenolic compounds were detected in a range of 10.1 ± 0.2 mg GAE/g to 82 ± 4 mg GAE/g. NADES N7 quantified the highest amounts of phenols, while N3 quantified the least. It was observed that NADES N1 (16.0 ± 0.2 mg GAE/g) and N2 (16.3 ± 0.5 mg GAE/g), followed by N3, N5 (11.8 ± 0.6 mg GAE/g), and GF2 (9.5 ± 0.6 mg GAE/g), as well as N4 (33 ± 1 mg GAE/g) and N6 (36.6 ± 0.6 mg GAE/g), quantified similar amounts of phenolics from the plant mixture. The reference NADES extracts proved to be inferior extractors of phenols, ranging between 0.58 ± 0.03 mg GAE/g and 9.5 ± 0.6 mg GAE/g, with GF1 quantifying the lowest amount of phenols and GF2 quantifying the highest. Methanol (1.85 ± 0.08 mg GAE/g) and water (1.87 ± 0.01 mg GAE/g) extracted more phenolics compared to the GF1 and GF3 mixtures, albeit significantly less than any of the other investigated NADES extracts.

A similar trend was observed in the DPPH test, where NADES N7 (19.58 ± 0.09 mM TE/g) emerged as the most potent radical inhibitor, while N5 (0.25 ± 0.15 mM TE/g) exhibited the lowest radical inhibition. N7 demonstrated a significantly higher AO capacity, displaying between 7 to 78 times greater radical inhibitory ability compared to the reference NADESs, 43 times higher AO capacity than water, and 42 times higher capacity than methanol. NADES N4 (15.06 ± 0.39 mM TE/g) and N6 (18.14 ± 0.26 mM TE/g) also showed a high AO capacity consistent with their quantified TP. The AO capacity of GF1–GF3 ranged from 0.25 ± 0.01 to 2.67 ± 0.01 mM TE/g, with GF2 exhibiting the highest and GF1 the lowest AO capacity. All proposed NADES mixtures demonstrated superior radical inhibition compared to those currently used in cosmetics, except for the proposed N5 (ascorbic acid, HBD). The conventional solvents exhibited almost identical AO capacities (0.47 ± 0.05 mM TE/g, M; 0.45 ± 0.03 mM TE/g, W), which were higher than GF1, GF3, and N5 but significantly lower than all the other proposed NADESs.

Proposed NADESs N4, N6, and N7 contained the highest TP contents, aligning with their highest values of AO capacity.

4. Conclusions

This study highlights the potential of natural deep eutectic solvents (NADESs) as a valuable extraction medium for specialized plant metabolites. By dissolving bioactives from 18 medicinal herbs, NADESs offer a green and sustainable approach to creating novel cosmetic extracts. Experimental validation through the green extraction of an herb mixture confirmed NADESs N4 (betaine-urea), N6 (betaine-proline), and N7 (betaine-lysine) as the most effective solvents, surpassing the reference NADESs (GF1–GF3) and conventional extracts in the AO assays and TP content. These promising natural constituents hold significant promise for the cosmetics industry, addressing the growing demand for eco-friendly and effective skincare products. Future research should explore their practical applications and potential benefits in formulations aimed at combating skin aging and promoting overall skin health. Moreover, carnitine-based and ornithine-based NADESs identified through COSMO-RS models represent promising solvents for experimental validation. In summary, NADESs offer a green pathway to harnessing the power of medicinal herbs. Their potential impact on skincare formulations could revolutionize the industry, providing consumers with sustainable bioactive cosmetic products.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/pr12050992/s1: Table S1: List of plants with data on their common and Latin names, plant family names, and plant parts used for extraction. Table S2: Activity coefficients and solubility parameters for 12 phenolic compounds in the designed NADESs.

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