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Natural Deep Eutectic Solvent-Based Extraction of *Malva sylvestris* L.: Phytochemical Content, Antioxidant and Antimicrobial Potential

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Abstract

Malva sylvestris L. is a herbaceous plant, distributed worldwide, rich in biological active compounds, and known for its health benefits. In this study, extracts from different parts (leaves, flowers, and roots) of this plant were prepared using green classic (70% ethanol) and natural deep eutectic solvents (NADESs) based on choline chloride and acetic acid (NADES1) or glycerol (NADES2). Their antioxidant, antibacterial (against *B. cereus*, *S. aureus*, *E. coli*, and *P. aeruginosa*), and antifungal activity (against *P. chrysogenum*, *F. oxysporum*, *A. parasiticus*, *A. flavus*, *A. niger*, *A. carbonarius*, and *A. ochraceus*) were compared. Ethanolic extracts were characterized with the highest total contents of phenols, flavonoids, and condensed tannins. Ethanolic and NADES flower extracts were the richest in the antioxidants tested. Alkaloids were extracted in low quantities. The experimentally determined antioxidant potential of the extracts proved the highest DPPH scavenging activity of ethanolic extracts and the lowest of NADES1 extracts. The ABTS scavenging capacity of NADES1 and ethanolic extracts displayed comparable results, while NADES2 extracts were characterized as having the highest FRAP activity. NADES1 extracts manifested pronounced antibacterial activity, partially due to the low pH of the pure solvent, as well as inconsistent antifungal activity—from moderate to a complete lack of activity. A strong positive correlation was reported between the DPPH radical scavenging capacity and phenolic compound content. Future detailed investigations on the mechanism of the antimicrobial activity of NADES1 extracts are necessary to clarify the observed phenomenon of the decreased antifungal potential of NADES1 extracts compared to the pure solvent NADES1.

Keywords: *Malva sylvestris*; ethanol; NADESs; antibacterial activity; antioxidants; antifungal potential



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1. Introduction

Nowadays, in the “post-antibiotic era”, the increasing number of cases of drug-resistant bacterial strains and the side effects of synthetic drugs are leading to a vital need for new efficient antimicrobial agents [1,2]. Moreover, many of the commonly used synthetic antibiotics (amoxicillin clavulanate, azithromycin, ciprofloxacin, amoxicillin,

and cephalixin) and antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tetra-butylhydroquinone (TBHQ), are found to be potentially damaging to the liver [3,4]. Butylated derivatives are potentially carcinogenic as well [3]. These findings have provoked increased interest in the search for and investigation of natural compounds as efficient alternative biologically active agents. In this respect, plants have been considered as one of the basic sources of natural substances with therapeutic potential [5].

Malva sylvestris L. is a herbaceous plant of the genus *Malva* that comprises more than 100 species [6], and is a weed distributed worldwide, including in Europe, where it is known as “common mallow” [7]. Based on its known health potential, *M. sylvestris* has recently become an object of scientific research. Traditionally, the plant has been used to treat various diseases, like coughs, colds, diarrhea, dysentery, hypertension, and skin irritation [8]. Its aerial parts can be consumed [9] and it displays a satisfactory ability to clean the colon, thus having a healing effect on gastrointestinal lesions [10]. Scientific research reports that extracts of *M. sylvestris* possess anti-inflammatory, antioxidant, anti-diabetes, wound-healing [9,11–13], and immunomodulatory properties [14], as well as promising antiviral effects against HIV [15] and COVID-19 [6] infections. These healing properties are a function of the biologically active substances it contains. Polysaccharides, carotenoids, polyphenols, fatty acids, ascorbic acid, and tocopherol have been extracted from its edible parts in various concentrations [14,16]. The plant extracts contain tannins, polysaccharides, flavonols, essential oil, anthocyanidins, flavones, anthocyanins, mucilagen, leucoanthocyanidines, coumarins, and terpenoids such as diterpenes, sesquiterpenes, and monoterpenes [16–19].

The new production practices strive to be environmentally friendly, and this includes the use of green (eco-friendly) solvents. When these solvents are also selective and effective, they can lead to production cost savings [20]. Such promising solvents are mixtures of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) of naturally occurring compounds called natural deep eutectic solvents (NADESs). They have a low melting point and are nontoxic, renewable, cheaper, and easy to prepare [21]. However, they have high viscosity. This is their main drawback, because it causes issues with mass transfer and hinders the extraction process. To address this, water is often added in amounts of up to 30%, but this reduces their effectiveness in extracting non-polar compounds [20]. Choline chloride-based NADESs are often preferred because they are widely available, easy to use, inexpensive, non-toxic, and stable in moist conditions, meeting several green chemistry principles. Their main advantage lies in the phase separation process, as it allows for the easy isolation of the solvent via simple decantation [22], and a big extraction capacity against polyphenolic compounds, known as strong antioxidants [23]. Compared to conventional solvents, NADESs often show higher extractability against polyphenols, which can be explained by the formation of a strong intramolecular structure with the compounds dissolved [24]. In addition, the NADES-obtained extracts often display more pronounced antioxidant potential [21].

There are no data shared in the world’s scientific literature about the use of NADESs for the extraction of biologically active compounds from *M. sylvestris*. In the search for eco-friendly solvents, in this study, our research team aimed to compare the potential of NADESs with “green” classic ethanol in the preparation of *M. sylvestris* extracts through an assessment of their phytochemical content and antioxidant, antibacterial, and antifungal activity.

2. Materials and Methods

2.1. Materials

2.1.1. Biological Material

The objects of this research are leaves (L), flowers (F), and roots (R) from *M. sylvestris* L. (Figure 1). The plant material was collected from the village of Sulitsa, Stara Zagora region, Mt. Sredna Gora, Bulgaria (42°433264 N, 25°477991 E). Aerial plant parts were harvested in July 2024, and the roots at the end of the vegetative period in October 2024. The voucher specimens from the studied population are kept in the herbarium of the Agricultural University in Plovdiv under number SOA 063591. The plant material was air dried in the shade at room temperature and ground in a mechanical grinder (final powder size less than 400 µm). The samples were stored in dark and cool rooms at 16–18 °C prior to the analysis.

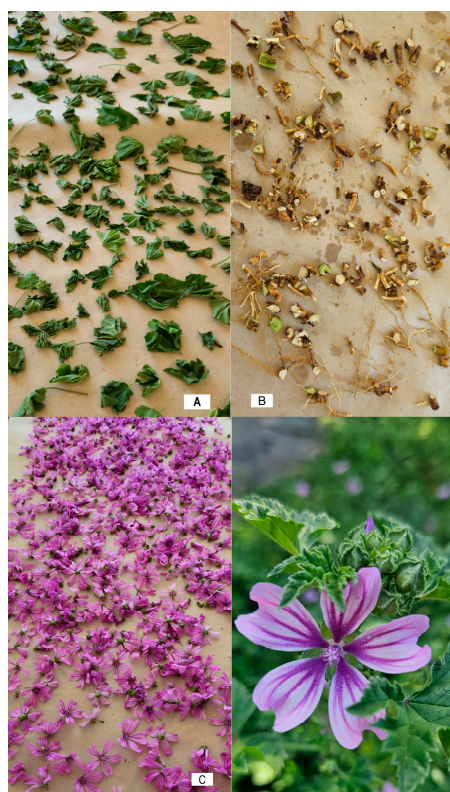


Figure 1. Biological material (leaves (A), roots (B) and flowers (C)) from *M. sylvestris* L.

2.1.2. Chemicals

The chemicals and solvents applied in the present study were purchased from Sigma-Aldrich (Saint Louis, MO, USA): DPPH (2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl, $C_{18}H_{12}N_5O_6$, CAS No.: 1898-66-4), ABTS (ABTSTM chromophore, diammonium salt, $C_{18}H_{18}N_4O_6S_4 \cdot (NH_3)_2$, CAS No.: 30931-67-0), ethanol (EtOH, C_2H_5OH , p.a. $\geq 99.8\%$), NaOH (p.a., HPLC), HNO_3 (p.a., HPLC), HCl (ACS reagent, 37%), Folin–Ciocalteu’s phenol reagent, Na_2CO_3 (powder, $\geq 99.5\%$, ACS reagent), glycerol ($C_3H_8O_3$, CAS No.: 56-81-5), acetic acid (CH_3COOH , CAS No.: 64-19-7, glacial, ACS reagent, $\geq 99.7\%$), citric acid ($(HO(COOH)(CH_2COOH)_2$, CAS No.: 77-92-9, ACS reagent, $\geq 99.5\%$), choline chloride ($((CH_3)_3N(Cl)CH_2CH_2OH$, CAS No.: 67-48-1, $\geq 99\%$), Trolox ($C_{14}H_{18}O_4$, CAS No.: 53188-07-1, 97%), ferric 2,4,6-tripyridyl-s-triazine (TPTZ, $C_{18}H_{12}N_6$, CAS No.: 3682-35-7, for spectrophotometric det. (of Fe), $\geq 98\%$), $FeCl_3$ (CAS No.: 7705-08-0, reagent grade, 97%), $FeSO_4 \cdot 7H_2O$ (CAS No.: 7782-63-0, ACS reagent, $\geq 99.0\%$), gallic acid ($(HO)_3C_6H_2CO_2H$, CAS No.: 149-91-7, ACS reagent, $\geq 98.0\%$), $NaNO_3$ (CAS No.: 7631-

99-4, ACS reagent, $\geq 99.0\%$), AlCl_3 (CAS No.: 7446-70-0, reagent grade, 98%), ($\pm$)-catechin hydrate ($\text{C}_{15}\text{H}_{14}\text{O}_6 \cdot x\text{H}_2\text{O}$, CAS No.: 7295-85-4), vanillin (4-(HO) C_6H_3 -3-(OCH₃)CHO, CAS No.: 121-33-5, $\geq 97\%$), KCl (CAS No.: 7447-40-7, ACS reagent, 99.0–100.5%), brom-cresol green ($\text{C}_{21}\text{H}_{14}\text{Br}_4\text{O}_5\text{S}$, CAS No.: 76-60-8, ACS reagent, dye content 95%), atropine ($\text{C}_{17}\text{H}_{23}\text{NO}_3$, CAS No.: 51-55-8, certified reference material, pharmaceutical secondary standard), and chloroform (CHCl_3 , CAS No.: 67-66-3, for analysis EMSURE[®] ACS, ISO, Reag. Ph Eur).

2.1.3. Microorganisms Studied

In this study, the reference bacterial strains *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 and reference fungal strains *Penicillium chrysogenum* NBIMCC 129, *Fusarium oxysporum* NBIMCC 125, *Aspergillus parasiticus* NBIMCC 2001, *Aspergillus flavus* NBIMCC 916, *Aspergillus niger* NBIMCC 3252, *Aspergillus carbonarius* NBIMCC 3391, and *Aspergillus ochraceus* NBIMCC 2002 were included. The fungal strains were purchased from the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC), Bulgaria. All strains were stored at 0–4 °C.

2.2. Sampling and Extract Preparation

2.2.1. Using NADESs

The NADESs were prepared using the heat stirring method. The NADES mixtures were prepared by mixing a hydrogen bond acceptor (HBA) with a hydrogen bond donor (HBD) at a 1:1 molar ratio in round-bottom flasks equipped with a magnetic stirrer. Ultrapure water was added in the ratio detailed in Table 1, to keep its viscosity low enough at room temperature. The eutectic solvents were stirred for 8 h at 80 °C. When a homogenous, without any precipitates, transparent liquid was formed, the NADESs were transferred to glass hermetically closed vessels and stored at room temperature in the dark.

Table 1. Extracts prepared from different plant organs of *M. sylvestris* L.

ID	Plant Organ	Solvent
L1 F1 R1	Leaf Flower Root	NADES1 Choline chloride + Citric acid + Water (1:1 mol/mol) + 30% w/w Water
		Choline chloride + Citric acid + Water (1:1 mol/mol) + 30% w/w Water
		Choline chloride + Citric acid + Water (1:1 mol/mol) + 30% w/w Water
L2 F2 R2	Leaf Flower Root	NADES2 Choline chloride + Glycerol (1:1 mol/mol) + 30% w/w Water
		Choline chloride + Glycerol (1:1 mol/mol) + 30% w/w Water
		Choline chloride + Glycerol (1:1 mol/mol) + 30% w/w Water
L3 F3 R3	Leaf Flower Root	70% v/v Ethanol in water 70% v/v Ethanol in water 70% v/v Ethanol in water

Then, 3 g of plant sample was weighed on an analytical balance and suspended in 40 mL of solvent (1.5:20, w/v). The extraction process was carried out by mixing the dried samples and the NADESs in a water bath for 60 min at 50 °C, followed by centrifugation for

35 min, at room temperature and $5300\times g$ on a Heraeus Labofuge 200 centrifuge (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.2. Using Classical Solvent, 70% *v/v* Ethanol in Water

A definite amount of each ground sample was weighed on an analytical balance and suspended in a solvent at a ratio of 1.5:20 *w/v*. The extraction was carried out via ultra-sonication for 30 min at 40 °C and 80 W/m³. The extraction technique of ultra-sonication was selected due to the quantity of the extraction of the target compounds [25]. To estimate the quantification of the total content of alkaloids, the extracts prepared were lyophilized at −40 °C on a Biobase freeze dryer (Biobase Bioindustry Ltd., Jinan, China).

2.3. Determination of pH

The pH values of the crude extracts were determined using the pH meter Consort 931 (Consort bvba, Turnhout, Belgium).

2.4. Spectrophotometric Determination of the Antioxidant Activity

To determine the antioxidant activity of the investigated extracts, three methods with different mechanisms of action were applied. The protocols followed were described by Yaneva et al. [26].

2.4.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Method

In brief, 100 µL of extract was added to 3.9 mL of 100 M solution of DPPH in methanol. Absorption at 517 nm was measured on a UV-Vis spectrophotometer (Thermo Electron Scientific Instruments LLC, Madison, WI, USA) 30 min later. The results for the radical scavenging capacity were compared with Trolox and calculated via regression analysis from the linear dependence between the concentration of Trolox and absorption at 517 nm ($R^2 = 0.9984$ for the linearity of the concentration range from 5 to 50 µmol/L). The results were expressed as µmol Trolox equivalent (TE) in 1 L extract.

2.4.2. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) Assay

A solution of ABTS^{•+} cation radicals was generated via the reaction between 7 mM ABTS solution in distilled water with 2.4 mM K₂S₂O₈ in the dark for 24 h at 20 °C at a ratio of 1:1 *v/v*. ABTS was then diluted with absolute EtOH to reach an absorbance of 0.700 measured at 734 nm on a UV-Vis spectrophotometer. To determine the ABTS radical scavenging ability, 200 µL of the sample was added to 3.6 mL of ABTS solution and the absorbance was measured spectrophotometrically at 734 nm. The activity was reported as ABTS radical inhibition (%). The radical scavenging capacity was calculated using the following Equation (1):

$$ABTS (\%) = \left(1 - \frac{I_x}{I_o}\right) \times 100, \quad (1)$$

where I_x is the absorbance of the control and I_o is the absorbance of the sample.

2.4.3. Ferric-Reducing Antioxidant Power (FRAP) Assay

The FRAP reagent was added to aliquots of 0.2 mL of extracts. This reagent was previously prepared by mixing 100 mL of 300 mM sodium acetate buffer solution at pH 3.6, 10 mL of 10 mM TPZT, and 10 mL of 20 mM FeCl₃. The resulting mixture was incubated for 30 min at 37 °C. The absorbance was measured at 593 nm. The results were expressed in mgeqv FeSO₄ in 1 L of extract. The calibration curve of FeSO₄ was established within the concentration range 0.1–1.0 mM FeSO₄ ($R^2 = 0.9962$).

2.5. Determination of Total Phenolic Content (TPC)

The experimental procedure described by Yaneva et al. was applied for the determination of the TPC [26]. In brief, 1 mL of the plant extract was mixed with 5.0 mL of Folin–Ciocalteu’s reagent (1:10 dilution). Then, 4 mL of 7.5% Na₂CO₃ was added and the tubes were left at room temperature for 60 min. The absorbance at 765 nm was measured against blank on a Thermo Scientific Evolution 300 spectrophotometer (Thermo Electron Scientific Instruments LLC, Madison, WI, USA). Gallic acid (Sigma-Aldrich, St. Louis, MO, USA) solutions in ethanol ranging from 10 to 150 µg/mL were used for the calibration curve ($R^2 = 0.9998$). The TPC of each sample was expressed as mg gallic acid equivalent (GAE) in 1 L of the plant extract.

2.6. Determination of Total Flavonoid Content (TFC)

The TFC was determined via the aluminum trichloride method, using catechin as a reference material [27]. In brief, 1 mL of extract, 0.3 mL of 5% NaNO₃, and 4 mL of deionized water were mixed. Then, 0.3 mL of 10% AlCl₃ (after 5 min) and 2 mL of 1 M NaOH (after 6 min) were added in this order. The solution was homogenized and the absorbance was measured against blank at 510 nm on a UV-Vis spectrophotometer. Standard solutions of catechin hydrate in the concentration range from 10 to 150 mg/L were used to plot the calibration curve ($R^2 = 0.9999$). The TFC was expressed as mg catechin equivalent (CE) in 1 L of extract.

2.7. Determination of Total Condensed Tannin Content (TCT)

The TCT was obtained using vanillin as a reagent and catechin as a standard. The experimental procedure described by Rebaya et al. was followed [28]. In brief, 0.4 mL of extract was added to 3 mL of 4% methanolic solution of vanillin and 1.5 mL of conc. HCl. The solution was homogenized, and after 15 min, the absorbance was measured against blank at 500 nm on a UV-Vis spectrophotometer. Standard solutions of catechin hydrate (Sigma Aldrich, St. Louis, MO, USA) in the concentration range from 10 to 150 mg/L were used to plot the calibration curve ($R^2 = 0.9998$). The TCT was expressed as mg catechin equivalent (CE) in 1 L of extract.

2.8. Determination of Total Anthocyanin Content (TAntC)

The total anthocyanin content (TAntC) was measured via the pH differential method applied by Lee et al. [29]. The samples were mixed thoroughly with 20 mL of buffer pH 1.0 (0.025 M potassium chloride) and pH 4.5 (0.4 M sodium acetate buffer) and then incubated for 20 min at room temperature and centrifuged at 4 °C and 12,000 rpm for 15 min. The supernatant was then removed, and the absorbance was read at 520 and 700 nm. The anthocyanin concentration was calculated using the following formulas:

$$TAntC, \frac{mg}{L} = \frac{A * D_f * 1000}{\epsilon * L} = \frac{A * 449.2 * D_f}{26.9} \quad (2)$$

$$A = (A_{520} - A_{700})_{pH=1} - (A_{520} - A_{700})_{pH=4.5} \quad (3)$$

where A_{520} and A_{700} are the absorbance values at 520 nm and 700 nm, respectively; M_R is the molecular weight of cyanidin-3-glucoside (449.2 g/mol), which is used as a reference compound; D_f is the dilution factor; ϵ is the molar absorptivity of cyanidin-3-glucoside (26,900 L/mol/cm); and L is the path length of the cuvette (1 cm). TAntC was expressed as mg cyanidin-3-glucoside equivalent (CGE) in 1 L of extract.

2.9. Determination of Total Alkaloid Content (TAlkC)

The TAlkC was obtained via the spectrophotometric method using bromocresol green (BCG) as a reagent and atropine as a standard [30]. In brief, an aliquot amount of each extract was dissolved in 2N HCl, filtered, and the filtrate washed three times using 10 mL of chloroform. The pH of the non-organic layer was adjusted to 7 using 0.1N NaOH. Then, 5 mL of BCG solution (69.8 mg + 3 mL 2N NaOH + 5 mL distilled water warmed up, and then adjusted to 1000 mL with distilled water) and 5 mL of pH 4.7 phosphate buffer (2 M sodium sulfate adjusted to pH 4.7 with 0.2 M citric acid) were added to the neutralized solution. After homogenization, the colored complex built was extracted with 1, 2, 3, and 4 mL of chloroform. The chloroform extracts were collected, and the volume was adjusted to 10 mL. The absorbance was measured against blank at 417 nm on a UV-Vis spectrophotometer. Standard solutions of catechin hydrate in the concentration range from 40 to 120 mg/L were used to plot the calibration curve ($R^2 = 0.9993$). The TAlkC was expressed as μg atropine equivalent (AE) in 1 L of extract.

2.10. Determination of Antimicrobial Activity

To measure the antibacterial activity, an agar well diffusion method was applied, as previously described by Velichkova et al. [31]. In brief, 18–20 h bacterial cultures grown on trypticase soy agar (TSA, Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 5% defibrinated sheep blood were used to prepare inoculums in saline, corresponding to 0.5 of the McFarland turbidity standard (1.5×10^8 CFU/mL), determined on a Densilometer II (Erba Lachema, Brno, Czechia). Cation-adjusted Mueller Hinton agar (Himedia, Maharashtra, India) was poured into each Petri dish to achieve an approximately 4 mm layer height. The agar surface was streaked three times with a sterile cotton swab preliminary dipped in inoculum by swirling the dish three times to ensure the even distribution of bacteria. Then, wells with a 6 mm diameter were made using a sterile cork borer and filled with 100 μL of the extracts. A positive control with gentamicin (Himedia, Maharashtra, India) at a concentration of 10 $\mu\text{g}/\text{mL}$ and a negative control with solvent were carried out. The dishes were incubated at 37 °C for 24 h aerobically.

The antifungal activity of extracts has been evaluated via the agar well diffusion method described by Velichkova et al. [31]. Briefly, 72 h fungal cultures were grown on potato dextrose agar (PDA, Himedia, Maharashtra, India). Then, 20 mL of PDA was poured into each Petri plate. After solidification, the agar surface was streaked three times with a sterile cotton swab preliminary dipped into the fungal inoculum ($1\text{--}2 \times 10^4$ CFU/mL) by rotating the dish three times to ensure the even distribution of fungi. The wells were made with a sterile cork borer of 6.0 mm in size and were filled with 100 μL of the extract. A positive control with amphotericin B (Sigma-Aldrich, Taufkirchen, Germany) at a concentration of 25 $\mu\text{g}/\text{mL}$ and a negative control with solvent were performed. An incubation period of 3–5 days at 26 °C–28 °C was maintained.

The antimicrobial activity was assessed by measuring the inhibition zones (IZs) of microbial growth around the extracts in the wells. IZs were measured in millimeters and the wells' diameter (6 mm) was included in the values presented. Antimicrobial activity was assumed in the presence of IZs ≥ 8.0 mm. The tests were performed in triplicate to determine the reproducibility of the results. The complete experiment was conducted under strict aseptic conditions.

2.11. Statistical Analysis

All analytical assays were carried out in triplicate and expressed as mean values $\pm$ standard deviation (mean $\pm$ SD). The Pearson correlation test and linear regression analysis were also applied to determine the relationships between the biological active compound

contents and the antioxidant activities. The statistical analysis (one-way ANOVA and Fisher’s Least Significant Difference) of the data from the antimicrobial tests was performed using Statistica 10 (Statistica for Windows, StatSoft. Inc., Tulsa, OK, USA, 2010).

3. Results and Discussion

3.1. Content of Biologically Active Compounds and Antioxidant Potential

M. sylvestris L. is an edible plant best known for its water-soluble mucilage rich in nutrients like saccharides, and their acid derivates with cough-suppressing properties [32]. The aerial parts of this plant—the leaves, flowers, fruits, and seeds—have been used since 3000 BC in traditional medicine by ancient peoples in the Near East against various ailments. Common mallow contains natural byproducts with well-known antioxidant properties, e.g., fatty acids, phenols, flavonoids, and alkaloids [7]. Based on the solvent chosen, the extraction technique applied, and the plant part selected, one group of these biologically active compounds can be selectively extracted. For example, Areesanan et al. obtained a mucilage-rich extract using water and a phenol-rich extract using methanol [33]. Munir et al. extracted water mucilage from mallow leaves [34]. The authors proved that it contains carbohydrates, proteins, and amino acids and is free of toxic minerals. The scientists also reported the high antioxidant potential of this mixture.

Various classical solvents have been used to obtain an extract of *M. sylvestris* rich in antioxidants—methanol [6,33,35,36], ethanol [37–39], acetone [35], dichloromethane [6], ethyl acetate [35], and hexane [35]. In the search for eco-friendly solvents, our research team selected two NADESs based on choline chloride (ChCl) as the HBA and citric acid (CA) or glycerol (Gly) as the HBD. This selection was made because of their proven ability to extract polyphenols and because the resulting extracts often have stronger antioxidant potential [20,40,41]. In the present study, their ability to extract antioxidants from different plant parts of common mallow was compared to the “green” classic ethanol.

The results of the pH and all measured groups of biologically active compounds extracted using the selected solvents (Table 1) are summarized in Table 2. The content values are given as equivalents in 1 L of the prepared extract. In this case, the results for all extracts obtained in our study can be more easily compared. Moreover, according to scientific data, the NADES extracts can be used directly in the food industry [21].

Table 2. pH values and total content of biologically active compounds in the crude extracts from *M. sylvestris* L.

ID	pH	TPC mgGAE/L	TFC mgCE/L	TCT mgCE/L	TAntC mgCGE/L	TAlkC µgAE/L
L1	1.14 ± 0.02	165 ± 9	52 ± 4	nd *	nd *	6.5 ± 0.3
F1	0.98 ± 0.02	579 ± 16	110 ± 9	11 ± 1	0.15 ± 0.02	4.0 ± 0.2
R1	0.52 ± 0.02	39 ± 4	nd *	nd *	nd *	4.6 ± 0.2
NADES 1	−0.17 ± 0.02	-	-	-	-	-
L2	5.26 ± 0.02	132 ± 6	81 ± 5	nd *	nd *	nd *
F2	5.00 ± 0.02	168 ± 10	104 ± 8	95 ± 5	0.25 ± 0.03	nd *
R2	5.12 ± 0.02	42 ± 3	nd *	nd *	nd *	nd *
NADES 2	2.63 ± 0.02	-	-	-	-	-
L3	6.14 ± 0.01	174 ± 7	136 ± 6	84 ± 5	nd	13.9 ± 0.2
F3	5.60 ± 0.01	276 ± 9	217 ± 8	89 ± 5	19 ± 2	nd *
R3	6.57 ± 0.01	20 ± 2	nd *	17 ± 1	nd *	nd *
70% EtOH	7.91 ± 0.01	-	-	-	-	-

* nd—not detected.

The extracts with NADES1 (choline chloride + citric acid, 1:1 mol/mol + 30% *w/w* water) had a very low pH—from 0.52 ± 0.02 of R1 (root extracts) to 1.14 ± 0.02 of L1 (leaf extract). The pH of the pure NADES1 was -0.17 ± 0.02 , which explains the very high acidity of the extracts in this group.

NADES2 (choline chloride + glycerol, 1:1 mol/mol + 30% *w/w* water) showed a higher pH— 2.63 ± 0.02 —and the extracts obtained using it had a pH = 5.00 ± 0.02 , 5.12 ± 0.02 , and 5.26 ± 0.02 for flowers, leaves, and roots, respectively.

The highest pH values were measured for extracts prepared using 70% ethanol: pH = 5.60 ± 0.01 (flowers), 6.14 ± 0.01 (leaves), and 6.57 ± 0.01 (roots), which corresponded to the highest pH of the pure solvent (7.91 ± 0.01).

Expectedly, different results for the TPC, TFC, TCT, and TAntC were obtained for the different plant parts and different solvents (Table 2). But unexpectedly, the highest values were measured in the ethanolic extracts: TPC from 20 ± 2 mgGAE/L (R3) to 276 ± 9 mgGAE/L (F3); TFC from 136 ± 6 mgCE/L (L3) to 217 ± 8 mgCE/L (F3); and TCT from 17 ± 1 mgCE/L (R3) to 89 ± 5 mgCE/L (F3). Anthocyanins were extracted using 70% ethanol only from the flowers in the amount 19 ± 2 mgCGE/L. The largest amount of these antioxidants (phenolics, flavonoids, condensed tannins, and anthocyanins) was measured in flower extracts, and the lowest in the roots. Petkova et al. extracted flowers and leaves from *M. sylvestris* harvested in Bulgaria (population near the city of Plovdiv) using 70% ethanol and ultra-sonication [39]. The authors measured the TPC and TFC and found that the flowers had more of these antioxidants than the leaves, which corresponds to our results. On the contrary, Cutillo et al. measured a higher TPC in the leaves than in the flowers of this species [16]. Batiha et al. reported a flavonoid content ranging from 210.8 to 46.6 mg/g, and that anthocyanins can be found only in the flowers, which was confirmed by our research team as well [7]. The major anthocyanins of common mallow are malvidin and delphinidin. Malvidin was first isolated from petals of *Malva sylvestris* and identified by Willstätter and Mieg in 1915, hence the name [42]. According to Azab et al., this anthocyanin and its glycoside malvidin can also be found in the leaves, the most consumed plant parts [43]. They have high antioxidant potential and proven anti-inflammatory [44], anti-allergic [45], and anticancer activity [43]. Delphinidin is also an anthocyanin with antioxidant and anti-inflammatory activity [46].

The most investigated plant organ of common mallow is the leaves. A lot of research teams used only leaves for extraction [35,37,38,47–49], and the most preferred measurement unit in their works were mg equivalents in 1 g fresh weight (FW). In the present study, this unit was inappropriate, because the extracts with deep eutectic solvents cannot be processed any further, and the best option was mg equivalents per liter. Therefore, future comparisons to results obtained by other researchers could not be accurate.

A similar trend is observed for NADES extracts as for the ethanolic extracts (Table 2): the flowers were the richest in phenols and flavonoids. In the present study, only the flowers contained anthocyanins: 0.15 ± 0.02 mgCGE/L (F1) and 0.25 ± 0.03 mgCGE/L (F2), just like the ethanolic extracts. On the contrary, NADES1 and NADES2 only extracted tannins from the flowers (11 ± 1 mgCE/L and 95 ± 5 mgCE/L, respectively). NADES2 showed better extractability according to condensed tannins and anthocyanins. Compared to the classic 70% ethanol, NADES1 was a stronger extracting agent of phenols, but 70% ethanol was superior regarding flavonoid and anthocyanin extraction. NADES2 and 70% ethanol displayed a comparable ability as extracting agents of condensed tannins— 95 ± 5 mgCE/L (F2) and 89 ± 5 mgCE/L (F3), respectively. The ability of NADESs to extract plant metabolites depends on their polarity, viscosity, and pH. NADESs with high viscosity (such as ChCl/CA and ChCl/Gly) have a lower extraction ability towards phenolic compounds compared to the less viscous deep eutectic solvents [50,51]. Lowering the viscosity leads

to the intensification of cavitation phenomena, provoking the formation of stronger H-bonding between the eutectic solvent and solute, which in turn improves the extraction capacity and yield [52].

Alkaloids were extracted in low quantities, which is expected, because *M. sylvestris* is an edible plant (Table 2). Using 70% ethanol, only $13.9 \pm 0.2 \mu\text{gAE/L}$ were extracted from the leaves. Using NADES1, $6.5 \pm 0.3 \mu\text{gAE/L}$ (L1), $4.0 \pm 0.2 \mu\text{gAE/L}$ (F1), and $4.6 \pm 0.2 \mu\text{gAE/L}$ (R1) were extracted. In the extracts prepared using NADES2, no alkaloids were detected. The capacity of NADES1 to extract alkaloids from all plant parts can be explained by its stronger acidity, which stimulates milieu alkaloid solubility.

The antioxidant potential of *M. sylvestris* extracts was measured via three methods to investigate the different mechanisms of antioxidant protection: DPPH radical quenching, ferric reducing ability (via the FRAP method), and ability to reduce ABTS radical scavenging ability (Table 3 and Figures 2–4).

Table 3. Antioxidant activity of the crude extracts from *M. sylvestris* L.

ID	DPPH $\mu\text{molTE/L}$	ABTS %	FRAP mgE/L
L1	53 ± 2	97 ± 3	0.22 ± 0.04
F1	64 ± 2	99 ± 2	0.19 ± 0.02
R1	18 ± 1	65 ± 3	0.02 ± 0.01
L2	40 ± 1	83 ± 2	5.54 ± 0.16
F2	75 ± 2	96 ± 3	4.14 ± 0.08
R2	13 ± 1	72 ± 2	0.78 ± 0.07
L3	69 ± 1	63 ± 3	3.89 ± 0.12
F3	107 ± 1	99 ± 3	1.25 ± 0.06
R3	24 ± 1	61 ± 2	0.46 ± 0.05

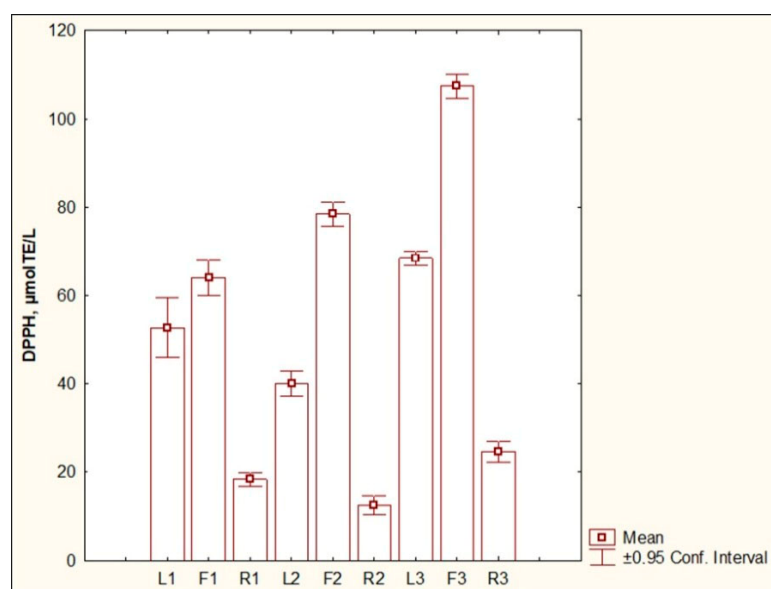


Figure 2. DPPH radical scavenging capacity of extracts from *M. sylvestris* L.

In the same solvent group, the flowers had the strongest DPPH (Figure 2) and ABTS (Figure 3) scavenging capacity: for F1— $64 \pm 2 \mu\text{molTE/L}$ and $99 \pm 2\%$; for F2— $75 \pm 2 \mu\text{molTE/L}$ and $93 \pm 3\%$; and for F3— $107 \pm 1 \mu\text{molTE/L}$ and $99 \pm 3\%$. The results obtained via the FRAP assay were reversed. The leaf extracts showed a better Fe-chelating ability: $0.22 \pm 0.04 \text{ mg Fe(II)/L}$ (L1), $5.54 \pm 0.16 \text{ mg Fe(II)/L}$ (L2), and

3.89 ± 0.12 mg Fe(II)/L (L3). The lowest DPPH, ABTS, and FRAP values were measured in the roots.

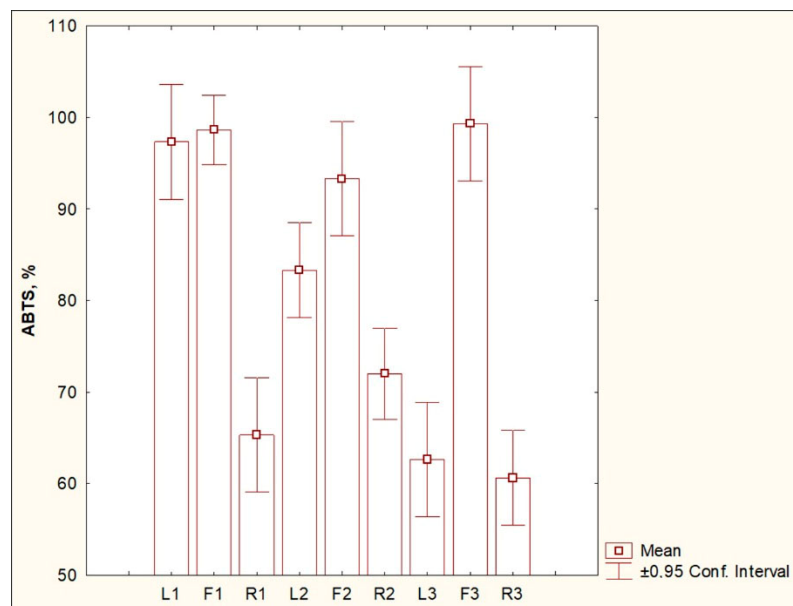


Figure 3. ABTS assay of extracts from *M. sylvestris* L.

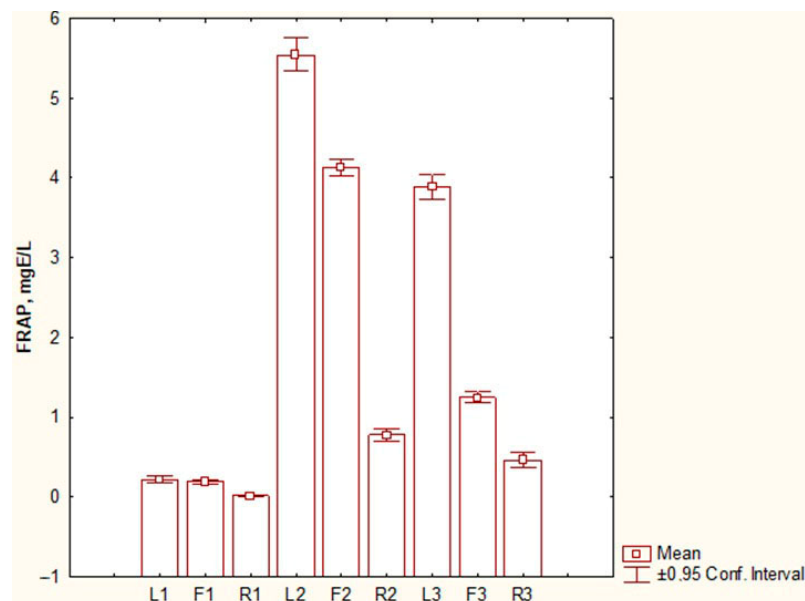


Figure 4. FRAP assay of extracts from *M. sylvestris* L.

The comparative analyses of the antioxidant potential of the extracts according to the solvent applied established the highest DPPH scavenging activity for the 70% ethanolic extracts (Figure 2): from 107 ± 1 $\mu\text{molTE/L}$ (F3) to 24 ± 1 $\mu\text{molTE/L}$ (R3), and the lowest for NADES1: from 64 ± 2 $\mu\text{molTE/L}$ (F1) to 18 ± 1 $\mu\text{molTE/L}$ (R1). The ABTS reducing capacity of NADES1 and ethanolic extracts displayed comparable results (Figure 3): from $99 \pm 2\%$ (F1) to $65 \pm 3\%$ (R1), and from $99 \pm 3\%$ (F3) to $61 \pm 2\%$ (R3), respectively. NADES2 extracts (Figure 4) were characterized as having the highest ferric-chelating ability: from 5.54 ± 0.16 mg Fe(II)/L (L2) to 0.46 ± 0.07 mg Fe(II)/L (R2). NADES1 extracts showed the lowest ferric-reducing potential: 0.22 ± 0.04 mg Fe(II)/L (L1) to 0.02 ± 0.01 mg Fe(II)/L (R1). Inverse relationships between the radical scavenging capacity and chelating ability of common mallow extracts have also been reported by other authors [48].

In order to better clarify the influence of individual groups of substances on the antioxidant activity of the obtained extracts, a correlation analysis was performed (Table 4).

Table 4. Parameter correlation matrix of extracts from *M. sylvestris* L.

	DPPH	ABTS	FRAP	TPC	TFC	TCT	TAntC	TAlkC
DPPH	1.0000	0.6588 *	0.4467	0.5887	0.9611	0.7891	0.6831	0.1298
ABTS		1.0000	0.3419	0.6854	0.5750	0.1818	0.4144	−0.2455
FRAP			1.0000	−0.0062	0.5200	0.4328	0.3714	−0.4195
TPC				1.0000	0.5933	0.1597	0.2231	0.1296
TFC					1.0000	0.7458	0.7143	0.1494
TCT						1.0000	0.4982	0.1612
TAntC							1.0000	−0.2582
TAlkC								1.0000

* Values in bold are with $r^2 > 0.5500$.

Interesting correlations between the measured parameters were established. Positive correlations with high regression coefficient values were calculated between the DPPH radical scavenging capacity (RSC) and phenolic compound contents (TPC, TFC, TCT, and TAntC). A number of researchers reported a positive correlation between the RSC measured via the DPPH method and the TPC of mallow extracts [6,33,35,47]. The strongest positive correlation established was between the RCS and TFC, with $R^2 = 0.9611$ (Table 4), which corresponded to the largest contribution of flavonoids to the radical scavenging ability. This is in accordance with the results obtained using the DPPH method for flower extracts, where the highest TFC was measured. Irfan et al. established a positive correlation between the RSC and TFC in *M. sylvestris* extracts obtained using methanol and dichloromethane [6].

The impact of the TPC and TFC on the ABTS assay was not greatly different: the correlation coefficients were 0.6854 and 0.5750, respectively. The TFC exhibited the strongest impact on the ferric-chelating ability (with a correlation coefficient $r^2 = 0.5200$), while the weakest influence on the FRAP among the phenols was registered for the anthocyanins ($r^2 = 0.3714$). The correlation coefficient between the RSC and ABTS was positive and higher ($r^2 = 0.6588$) than those between the RSC and FRAP ($r^2 = 0.4467$) and ABTS and FRAP ($r^2 = 0.3419$), which confirmed again the weak relationship between the radical scavenging capacity and chelating ability of *M. sylvestris* extracts. In addition, a negative regression between the FRAP and TPC ($r^2 = -0.0062$) was reported.

Low and even negative correlations were established for the TAlkC and the other measured parameters (Table 4). Obviously, alkaloids have no contribution to the antioxidant potential. Furthermore, the correlation between the TAlkC and anthocyanins was negative ($r^2 = -0.2582$).

Figure 5 presents the graphical results of the antioxidant capacity of NADES-based and conventional *Malva sylvestris* L. extracts obtained by other research teams [6,49,53–55]. The comparative analyses outlined that NADES-based extracts often possess higher antioxidant activity. The observed variations could be due to the fact that NADESs can dissolve a wide range of bioactive compounds, including both polar and non-polar antioxidants, better than conventional solvents like methanol, ethanol, or water. The latter leads to the more efficient extraction of antioxidant compounds such as polyphenols, flavonoids, and alkaloids. In addition, NADESs can stabilize sensitive antioxidant compounds by forming hydrogen bonds or other intermolecular interactions, which could preserve their structure and activity, preventing oxidation or degradation during extraction and storage. Moreover, the components of NADESs (e.g., carbohydrates, organic acids, amino acids, polyvalent alcohols) can sometimes have mild antioxidant properties themselves, or synergistically enhance the activity of extracted antioxidants.

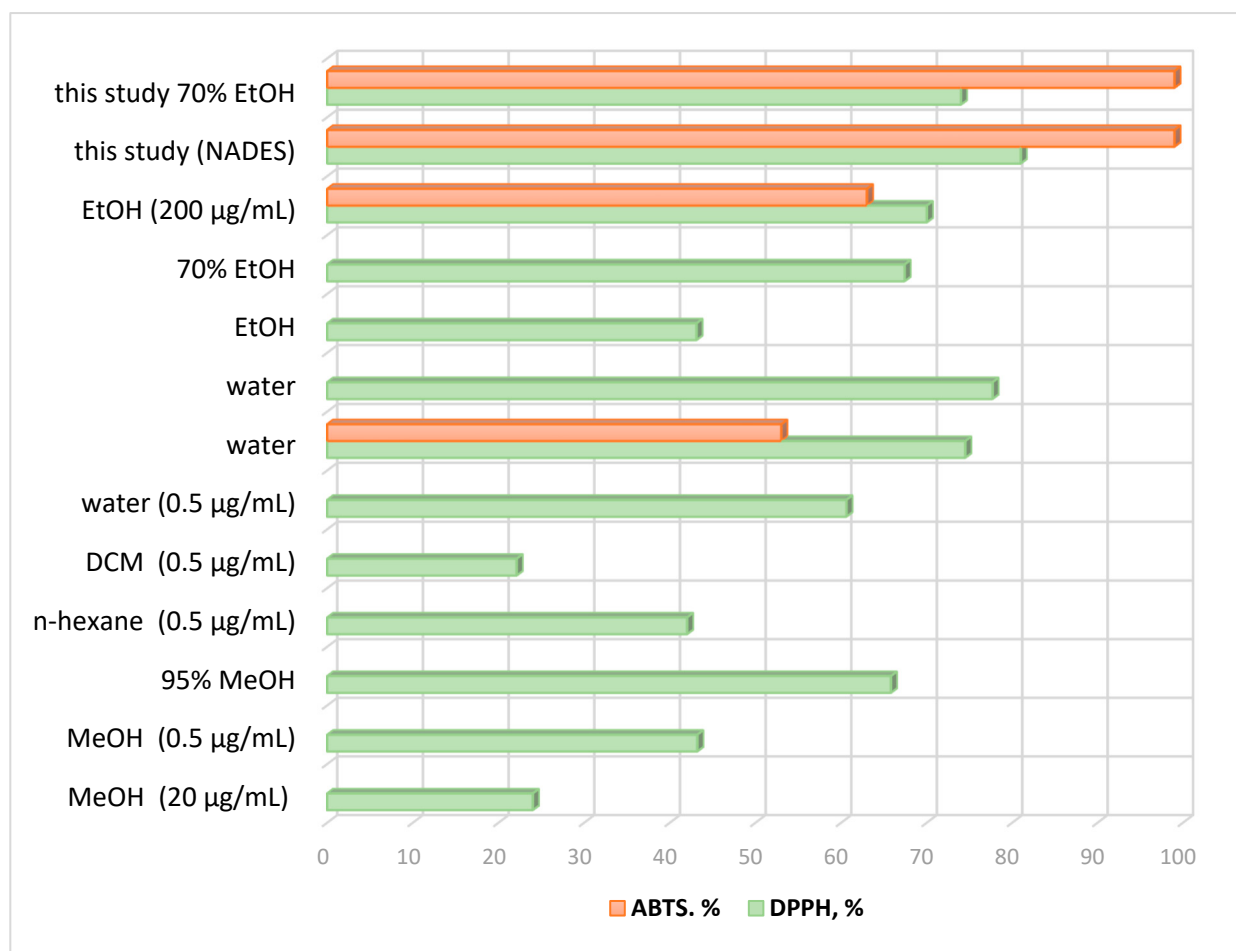


Figure 5. Comparative analyses of the DPPH and ABTS scavenging activities of *Malva sylvestris* L. extracts prepared using conventional solvents and NADESs.

3.2. Antimicrobial Activity

The antimicrobial potential of plants is among their most important physiological properties, as it provides protection of the plants against pathogenic microorganisms. This activity is dependent on the different compounds produced and exuded by plants—essential oils, phenols, flavonoids, tannins, alkaloids, glycosides, terpenes, and saponins, etc. [56,57]. Various studies have shown that the synergistic effect of phytochemicals plays a significant role in the application of plant extracts as antimicrobial agents in agriculture, biomedicine, the food industry, and cosmetics [58]. Plants are rich in flavonoids, which exhibit antibacterial activity through multiple mechanisms, including the following: (i) membrane disruption, damaging bacterial cell membranes; (ii) the inhibition of nucleic acid synthesis; (iii) the inhibition of bacterial virulence through toxin production and/or quorum sensing, which suppresses biofilm formation; (iv) the inhibition of fatty acid synthase, hindering cell envelope synthesis; (v) efflux pump inhibition; and (vi) the inhibition of ATP synthase, disrupting bacterial energy production [59–61]. The phytochemical analysis of *Malva sylvestris* showed that the most commonly used parts of the plant—the leaves and flowers—contain various bioactive substances with antimicrobial activity [7]. To the best of our knowledge, there is a lack of data in the available literature regarding the antibacterial and antifungal activity of NADESs, consisting of choline chloride + citric acid + water, and on NADES extracts against the bacteria and fungi examined in this study.

According to the data in Table 5, NADES1 (choline chloride + citric acid, 1:1 mol/mol + 30% w/w water) exhibited very high antibacterial activity against the four bacterial strains

tested. This activity was not only much higher than in the other solvents (NADES2 and 70% ethanol exhibited no or traces of activity), but also compared to the positive control gentamicin. The main reason for this extraordinarily high activity is probably the stronger acidity of NADES1 ($\text{pH} = -0.17 \pm 0.02$) compared to NADES2 ($\text{pH} = 2.63 \pm 0.02$) and ethanol ($\text{pH} = 7.91 \pm 0.01$) (Table 2). In fact, it is well known that organic acid-based NADESs exhibit strong antimicrobial activity. This is largely due to their low pH (below 3), which is much more acidic than the optimal growth pH of bacteria ($\text{pH} = 6.5\text{--}7.5$) and fungi ($\text{pH} = 5.0\text{--}9.0$). The acidic environment denatures proteins on microbial cell walls, impairing cell function [62,63]. The high viscosity of carbohydrate-based NADESs increases damage against microbial cells, and their elevated osmotic pressure further stresses microbial systems. These properties cause cell dehydration and lysis by rapidly drawing water out of the cells. Because of the delocalized cation (cholinium) of the hydrogen bond acceptor (HBA), choline chloride is believed to contribute to microbial damage by interacting with microbial cell membranes. These interactions involve the electrostatic attraction between the positively charged cholinium ions and the negatively charged cell membrane surfaces, which can disrupt the cell wall [64,65]. According to Trenzado et al., the interaction with the phospholipid bilayer and the ability to penetrate the plasma membrane of hydrophobic NADESs should lead to a disruptive effect on the membrane properties (Scheme 1) [66].

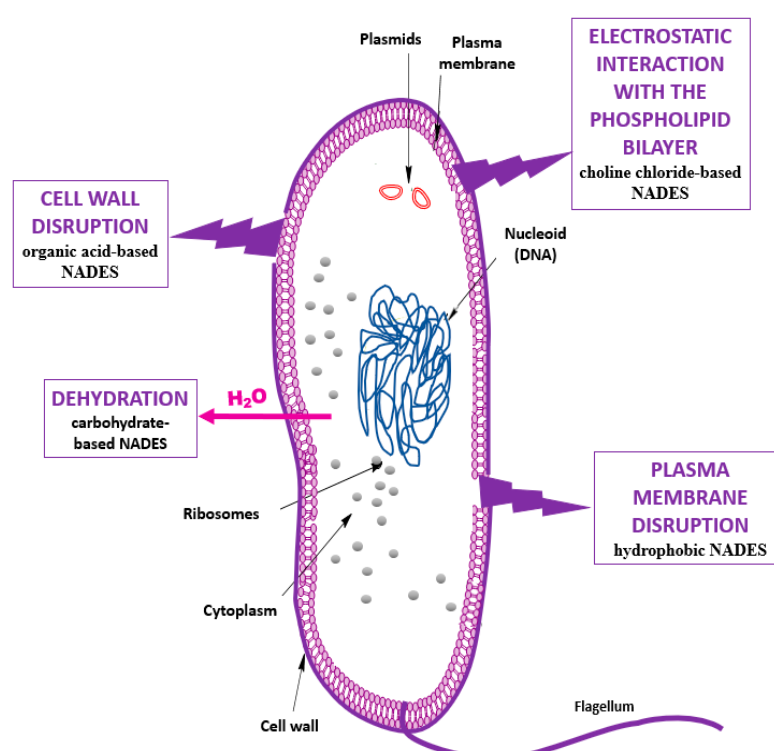
Table 5. Antibacterial activity of crude extracts of *M. sylvestris* L. determined by measuring the diameter of inhibition zones (IZs) in mm (mean $\pm$ SD) *.

ID	Diameter of Inhibition Zones (mm)			
	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>B. cereus</i>
L1	33.3 $\pm$ 0.6 ^{ac}	27.3 $\pm$ 2.3 ^{ab}	31.3 $\pm$ 2.3 ^{ac}	32.0 $\pm$ 0.0 ^{ac}
F1	33.3 $\pm$ 1.2 ^{ac}	30.0 $\pm$ 3.5 ^{ac}	32.0 $\pm$ 1.7 ^{ac}	31.3 $\pm$ 0.6 ^{ac}
R1	32.7 $\pm$ 2.3 ^{ac}	29.3 $\pm$ 0.6 ^{ac}	33.3 $\pm$ 0.6 ^{ac}	32.0 $\pm$ 0.0 ^{ac}
NADES1	30.0 $\pm$ 0.0 ^a	27.0 $\pm$ 0.0 ^a	28.3 $\pm$ 0.6 ^a	30.0 $\pm$ 0.0 ^a
L2	– **	– **	– **	– **
F2	8.0 $\pm$ 0.0 ^{ac}	– **	– **	– **
R2	– **	– **	– **	– **
NADES2	6.0 $\pm$ 0 ^a	6 $\pm$ 0 ^a	6.0 $\pm$ 0 ^a	6.0 $\pm$ 0 ^a
L3	10.7 $\pm$ 0.6 ^{ac}	6.7 $\pm$ 0.6 ^{ab}	9.3 $\pm$ 1.2 ^{ac}	8.3 $\pm$ 1.5 ^{ac}
F3	10.3 $\pm$ 0.6 ^{ac}	6.7 $\pm$ 0.6 ^{ab}	7.3 $\pm$ 0.6 ^{ab}	9.3 $\pm$ 1.2 ^{ac}
R3	9.7 $\pm$ 0.6 ^{ac}	6.7 $\pm$ 0.6 ^{ab}	9.0 $\pm$ 1.7 ^{ac}	8.7 $\pm$ 1.2 ^{ac}
70% EtOH	7.0 $\pm$ 0 ^a	7.0 $\pm$ 0.0 ^a	7.0 $\pm$ 0 ^a	6.7 $\pm$ 0.6 ^a
Gentamicin	15.0 $\pm$ 0.0 ^{ac}	11.0 $\pm$ 0.0 ^{ac}	11.0 $\pm$ 0.0 ^{ac}	15.0 $\pm$ 0.0 ^{ac}

* Different letters in the columns denote significant differences between the inhibition zones of plant extracts and negative control (solvent) values according to one-way ANOVA and LSD tests ($ab\ p \leq 0.05$; $ac\ p > 0.05$), ** no activity (IZ = 6.0 mm).

In this study, NADES1 showed higher activity against Gram-positive bacteria (*S. aureus* and *B. cereus*) compared to Gram-negative ones (*E. coli* and *P. aeruginosa*). Concerning the antibacterial potential of NADESs, it is essential to outline the specific characteristics of the structure of Gram-negative and Gram-positive bacteria. In this respect, Gram-negative bacteria have an additional outer layer made of lipopolysaccharides, which Gram-positive bacteria lack. This structural difference makes Gram-negative bacteria less sensitive to the damage of NADESs [67]. On the other hand, 70% ethanol showed low antibacterial activity, similar to the experimental results of Lim et al. [68]. It is noteworthy that NADES1 extracts showed similar activity to ethanolic extracts against the Gram-positive bacteria, as well as higher activity against the Gram-negative bacteria compared to the negative control. These differences in the activity against Gram-negative bacteria can hardly be explained

by the data in Table 2 regarding the content of biologically active substances, because, in general, NADES1 extracted more polyphenols, but less flavonoids and tannins than ethanol, and all these compounds are known to have antibacterial activity [69,70]. However, the interesting fact that the acidity of NADES1 decreases sharply in the extracts where it is used as a solvent indicates that probably most of the antibacterial activity of NADES1 extracts is due to the biologically active compounds extracted, rather than the high acidity. This hypothesis is supported by the fact that NADES2 (with $\text{pH} = 2.63 \pm 0.02$) did not exhibit antibacterial activity, whereas NADES1 leaf extracts (with $\text{pH} = 1.14 \pm 0.02$) had very high activity, with inhibitory zones ranging from 27.3 to 33.3 mm. Jenny et al. found that the antibacterial effect caused by extracts prepared in citric acid (such as NADES1 extracts) can be partly attributed to the pH alteration they cause [71]. The NADES1 root extracts displayed the highest activity among all NADES1 extracts against *P. aeruginosa* (33.3 mm IZ) compared to the negative control (28.3 mm IZ).



Scheme 1. Antimicrobial mechanisms of NADESs.

The ethanolic extracts of *M. sylvestris* exhibited low antibacterial activity against *S. aureus*, *P. aeruginosa*, and *B. cereus*, and no activity regarding *E. coli*. The activity against *S. aureus* was slightly higher than that against *P. aeruginosa* and *B. cereus*. These findings mostly coincide with the experimental results of Yousefi, who found higher antimicrobial activity of ethanolic extracts of *M. sylvestris* against *S. aureus* and *B. cereus* than against *P. aeruginosa* and *E. coli* [72].

The fact that NADES2 (choline chloride + glycerol, 1:1 mol/mol + 30% *w/w* water) extracts displayed almost no antibacterial activity provoked our scientific interest. The probable reasons for the observed lack of potential are the relatively low acidity as well as the low yield of biologically active compounds (Table 2).

The previous studies on the antimicrobial activity of NADESs showed that fungi such as *Candida albicans* are much less affected by NADESs than bacteria. Their cell walls are made of two layers rich in chitin and glucans, which are harder for NADESs to penetrate. As a result, mixtures like choline chloride/oxalic acid/glycerol and choline chloride/citric

acid/glycerol exerted damage to bacteria but not to fungi [73]. These findings coincide with the experimental results of our study, where NADESs exhibited much less activity against fungi compared to against bacteria.

According to the data in Table 6, NADES1 showed high antifungal activity against *A. niger* and *P. chrysogenum*, which was higher than the potential of the positive control amphotericin B. NADES1 exhibited moderate activity against *A. flavus*, and low activity against *A. ochraceus*, *F. oxysporum*, and *A. parasiticus*. No activity was observed against *A. carbonarius*. These experimental results are in line with the data reported by Hassan et al., who found that 10% citric acid inhibited 17.71% of *A. flavus* and 20.16% of *Penicillium purpogenum* growth [74]. Amphotericin B has low to moderate activity against *A. carbonarius*, *A. flavus*, and *A. parasiticus*, low activity against *A. niger*, and no activity against *P. chrysogenum*, *F. oxysporum*, and *A. ochraceus*. These findings partly coincide with the results of other authors who reported mostly low to moderate activity of amphotericin B against *Penicillium* spp., *Fusarium* spp., and *Aspergillus* spp. [75,76]. The antifungal activity of NADES1 is much higher than that of 70% ethanol. The latter showed low activity only against *P. chrysogenum*, while in the other cases, traces of activity or no activity were observed. These results are partly in line with the data reported by Sequeira et al., who found that 70% ethanol had fungicidal properties against both *P. chrysogenum* and *A. niger* [77]. As in the case of antibacterial activity, neither NADES2 nor its extracts exhibited antifungal activity.

Table 6. Antifungal activity of crude extracts of *M. sylvestris* L. determined by measuring the diameter of inhibition zones in mm (mean $\pm$ SD) *.

ID	Diameter of Inhibition Zones (mm)						
	<i>P. chrysogenum</i>	<i>F. oxysporum</i>	<i>A. parasiticus</i>	<i>A. niger</i>	<i>A. flavus</i>	<i>A. carbonarius</i>	<i>A. ochraceus</i>
L1	12.7 $\pm$ 1.1 ^{ac}	11.7 $\pm$ 0.6 ^{ab}	9.7 $\pm$ 0.6 ^{ab}	- **	10.0 $\pm$ 0.0 ^{ac}	- **	10.3 $\pm$ 0.6 ^{ab}
F1	14.0 $\pm$ 1.0 ^{ab}	12.3 $\pm$ 0.6 ^{ab}	9.7 $\pm$ 0.6 ^{ab}	- **	11.0 $\pm$ 0.0 ^{ab}	- **	10.0 $\pm$ 0.0 ^{ab}
R1	14.7 $\pm$ 0.6 ^{ab}	13.0 $\pm$ 1.0 ^{ac}	- **	- **	13.0 $\pm$ 0.0 ^{ab}	- **	11.3 $\pm$ 0.6 ^{ab}
NADES1	15.3 $\pm$ 0.6 ^a	10.0 $\pm$ 0.0 ^a	9.0 $\pm$ 0.0 ^a	17.7 $\pm$ 0.6 ^a	12.0 $\pm$ 0.0 ^a	6.0 $\pm$ 0.0 ^a	10.0 $\pm$ 0.0 ^a
L2	- **	- **	- **	- **	- **	- **	- **
F2	- **	- **	- **	- **	- **	- **	- **
R2	- **	- **	- **	- **	- **	- **	- **
NADES2	6.0 $\pm$ 0.0 ^a	6.0 $\pm$ 0.0 ^a	6.0 $\pm$ 0.0 ^a	6.0 $\pm$ 0.0 ^a	6.0 $\pm$ 0.0 ^a	6.0 $\pm$ 0.0 ^a	6.0 $\pm$ 0.0 ^a
L3	9.0 $\pm$ 0.0 ^{ab}	10.0 $\pm$ 0.0 ^{ac}	7.3 $\pm$ 0.6 ^{ab}	- **	7.7 $\pm$ 0.6 ^{ab}	- **	8.7 $\pm$ 0.6 ^{ab}
F3	10.0 $\pm$ 0.0 ^{ac}	- **	7.3 $\pm$ 0.6 ^{ab}	- **	7.7 $\pm$ 0.6 ^{ab}	- **	8.7 $\pm$ 0.6 ^{ab}
R3	10.0 $\pm$ 0.0 ^{ac}	9.7 $\pm$ 0.6 ^{ac}	8.3 $\pm$ 0.6 ^{ab}	- **	9.7 $\pm$ 0.6 ^{ac}	- **	9.0 $\pm$ 1.0 ^{ab}
70% EtOH	8.0 $\pm$ 0.0 ^a	7.7 $\pm$ 0.6 ^a	7.0 $\pm$ 0.0 ^a	6.0 $\pm$ 0.0 ^a	6.0 $\pm$ 0.0 ^a	6.0 $\pm$ 0.0 ^a	7.7 $\pm$ 0.6 ^a
Amphotericin B	6.0 $\pm$ 0.0 ^{ab}	6.0 $\pm$ 0.0 ^{ab}	11.0 $\pm$ 0.0 ^{ac}	9.0 $\pm$ 0.0 ^{ab}	11.5 $\pm$ 0.3 ^a	13.8 $\pm$ 0.3 ^a	6.0 $\pm$ 0.0 ^a

* Different letters in the columns denote significant differences between the inhibition zones of plant extracts and negative control (solvent) values according to one-way ANOVA and LSD tests (ab $p \leq 0.05$; ac $p > 0.05$), ** no activity (IZ = 6.0 mm).

When comparing the activity of NADES1 extracts of *M. sylvestris* against different fungal strains, the interesting trend of the decreasing activity of NADES1 extracts compared to the negative control (NADES1) is noteworthy. This is particularly pronounced against *A. niger*, but is also clearly observed with regard to *P. chrysogenum* and *A. flavus*. A possible explanation for this trend is the decrease in acidity of NADES1 extracts compared to NADES1 (Table 2), which significantly influences the antifungal effect of NADES1 extracts [74]. In the case of the activity of NADES1 plant extracts against *P. chrysogenum* and *A. flavus*, although the activity of these extracts was lower than that of the negative control (NADES1), it can be considered that they show antifungal activity, as evidenced by the sometimes significant differences between the activities of the leaf, flower and root extracts (Table 6). This activity is probably due to both the biologically active compounds extracted using NADES1 and the high acidity of the extracts [62,63]. From the experimental data,

we can conclude that the extracts of NADES1 probably exhibited the highest antifungal activity against *F. oxysporum*, since there is only a steady trend of increasing values of leaf, flower, and root extract inhibition zones against this fungal strain compared to NADES1. In the case of *A. ochraceus*, this tendency is less pronounced, and with respect to *A. parasiticus*, the trend is weak and inconsistent. NADES1 extracts are not active against *A. niger* or *A. carbonarius* (Table 6).

With regard to the ethanolic extracts of *M. sylvestris*, they exhibited low activity against *P. chrysogenum*, *F. oxysporum*, *A. flavus*, and *A. ochraceus*, insignificant antifungal potential against *A. parasiticus*, and a complete lack of activity against *A. niger* and *A. carbonarius* (Table 6). These findings are similar to the results of Mihaylova et al., who found low antifungal activity of aqueous extracts of *M. sylvestris* against *Penicillium* spp., as well as no activity against *A. niger* [78].

It is noteworthy that regardless of the type of solvent, the highest antifungal activity was exhibited by root extracts of *M. sylvestris* compared to the leaf and flower extracts (Table 6). As for the antibacterial activity, such a clear trend was absent (Table 5).

4. Conclusions

The comparative analyses of the extraction potential of natural eutectic solvents NADES1 and NADES2 compared to classic solvent ethanol in *M. sylvestris* established that the ethanolic extracts were characterized by the highest TPC, TFC, and TCT. Ethanolic and NADES flower extracts were the richest in the tested antioxidants (phenols, flavonoids, condensed tannins, and anthocyanins). Alkaloids were extracted in low quantities, which is expected due to the edible character of *M. sylvestris*. The experimentally determined antioxidant potential of the extracts proved the highest DPPH scavenging activity of the 70% ethanol extracts and the lowest of NADES1 extracts. The ABTS scavenging capacity of NADES1 and ethanolic extracts displayed comparable results, while NADES2 extracts were characterized by the highest FRAP activity. NADES1 extracts showed pronounced antibacterial activity, partially due to the low pH of the pure solvent, as well as the inconsistent antifungal activity—from moderate to a complete lack of activity. Generally, the antibacterial potential of NADES1 extracts was higher than the potential of ethanolic extracts. Such a clear tendency regarding the antifungal potential was not observed. NADES2 extracts displayed almost no antibacterial activity, as well as a lack of antifungal activity. This shows that the “classic” green solvent 70% ethanol is also a good choice for the preparation of plant extracts with antimicrobial potential. A strong positive correlation was reported between the DPPH radical scavenging capacity and phenolic compound contents (TPC, TFC, TCT, and TAntC). Future detailed investigations on the mechanisms of antimicrobial activity of NADES1 extracts are necessary to outline and clarify the observed phenomenon of the decreased antifungal potential of NADES1 extracts compared to the pure solvent NADES1.

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