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Green Extraction of Greek Propolis Using Natural Deep Eutectic Solvents (NADES) and Incorporation of the NADES-Extracts in Cosmetic Formulation

Andromachi Tzani [1,*], Ioanna Pitterou [1], Foteini Divani [1], Thalia Tsiaka [2,3], Georgios Sotiroudis [2], Panagiotis Zoumpoulakis [3] and Anastasia Detsi [1]

[1] Laboratory of Organic Chemistry, School of Chemical Engineering, National Technical University of Athens, Zografou Campus, Heroon Polytechniou 9, 15780 Athens, Greece
[2] Institute of Chemical Biology, National Hellenic Research Foundation, 48 Vas. Constantinou Avenue, 11635 Athens, Greece
[3] Department of Food Science and Technology, University of West Attica, Ag. Spyridonos, 12243 Athens, Greece
* Correspondence: atzani@mail.ntua.gr

Abstract: In this work, a greener approach for the extraction of Greek propolis using ultrasound-assisted extraction method in combination with Natural Deep Eutectic Solvents (NADES) is presented. Propolis is a natural material of outmost interest as it possesses various biological and pharmacological activities and is therefore used for the manufacturing of extracts useful to various fields, such as pharmaceutics, cosmetics etc. Herein, five NADES were task-specifically selected as appropriate extraction solvents since they provide important assets to the final NADES-extracts, comparing to the conventionally used organic solvents. The screening study of the prepared solvents indicated the NADES L-proline/D,L-Lactic acid as the most effective medium for the raw propolis extraction due to the extract's high total phenolic content as well as its' significantly higher antioxidant activity. Then, the extraction using the selected NADES, was optimized by performing Experimental Design to study the effect of extraction time, propolis-to-solvent ratio and the %NADES content in the NADES-water system. All the extracts were characterized regarding their antioxidant activity and total phenolic content. The optimum NADES-extract as well as an extract derived by extraction using a conventional hydroethanolic solution were further characterized by performing LC/MS/MS analysis. The results showed that the NADES-extracts composition was similar or superior to the hydroethanolic extracts regarding the presence of valuable phytochemicals such as apigenin, naringenin etc. A disadvantage that is usually mentioned in the literature regarding the extractions using NADES is that the extracted bioactive compounds cannot be easily separated from the NADES in order to obtain dry extracts. However, this drawback can be converted to an asset as the task-specifically designed NADES that are used in this study add value to the end product and the optimum as-obtained NADES-extract has been successfully incorporated in a cosmetic cream formulation. In this work, The antioxidant activity and organoleptic characteristics of the cream formulation were also determined.

Keywords: propolis; natural deep eutectic solvents; ultrasonic-assisted extraction; experimental design; cosmetic cream

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1. Introduction

Propolis is a resinous material produced by honeybees and used by them as a sealant to protect and insulate their hives. Propolis is a rich source of bioactive compounds whose composition varies based on factors such as the propolis origin (geographical and botanical), environmental factors etc. [1,2]. Over the centuries, propolis has been used in folk medicine due to its wide range of bioactivity and nowadays its extracts are used by the pharmaceutic, food and cosmetic industries. The chemical profile of the propolis extracts strongly depends on the used extraction solvent as well as the extraction procedures and this is a reason

why the selection of such parameters should be done carefully and task-specifically. Over the years, various extraction processes using different extraction methods and various extraction solvents have been used for the effective extraction of bioactive compounds by propolis [3].

The optimum extraction process must lead to extracts rich in bioactive compounds and at the same time is desirable to be cost-effective. This is not an easy goal to achieve and in the last three years, a few studies have been published in which the extraction of propolis is implemented using natural deep eutectic solvents (NADES) as effective extraction media [4–8]. The use of NADES in the propolis extraction has demonstrated promising potential and thus the research is turning to this direction.

The main advantages of NADES are their ease of preparation, their low cost as well as their ability to be specifically designed by the proper selection of starting materials [3]. The task-specific design of solvents can lead to desirable properties such as pH, polarity, viscosity, high solvation activity etc. Regarding their viscosity, most NADES, are more viscous at room temperature compared to other, commonly used organic solvents. The high viscosity may negatively affect an extraction process; however, this drawback can be overcome by adding water as a co-solvent. An important advantage regarding the use of NADES in extraction processes is that in many cases the extraction is more effective compared to the conventional extractions using organic solvents. Usually, the effectiveness is higher when the use of NADES is combined with the use of a high energy techniques (such as ultrasound assisted extractions (UAE) and microwave assisted extractions (MAE)) [9]. Moreover, the NADES-extracts are more stable over time possibly because the NADES shows a protective effect over the extracted bioactive compounds [10]. Finally, a significant advantage is the fact that the extract can be further used directly without any step of purification or solvent isolation, and this contributes to the reduction in the economic and energy cost of the overall process.

In the frame of research in which alternative and more effective extraction procedures are explored using green solvents and high energy techniques and in continuation of our work on the study of propolis extraction [4], we herein present an UAE of bioactive compounds from propolis using various NADES. The methodology was optimized using experimental design and the optimum extract was successfully incorporated as-obtained (that is without the isolation of bioactive from the NADES) in a cosmetic formulation. The phytochemical profile of the optimum NADES-extract was studied by performing the LC/MS/MS analysis and compared over the extract derived by the conventional extraction using a hydroethanolic solution.

2. Materials and Methods

2.1. Materials and Reagents

For the NADES preparation anhydrous betaine (Fluorochem, Hadfield, UK), D,L-lactic acid (80–85% aq. soln.) (Alfa Aesar, Ward Hill, MA, USA), D-(+)-glucose (Sigma-Aldrich, St. Luis, MO, USA, 97%), L-proline (Fluorochem Hadfield, UK), β-alanine (Merck Millipore, Darmstadt, Germany) and levulinic acid (Alfa Aesar, Kandel, Germany) were used. Folin-Ciocalteu reagent was purchased from Merck Millipore (Darmstadt, Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), sodium linoleate and catechin were purchased from Aldrich (St. Luis, MO, USA, 97%), Trolox was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and gallic acid monohydrate were purchased from Fluka (Dresden, Germany). For all the experiments double-deionized water was used. The raw propolis was obtained from the Peloponnese region (area of Epidaurus), Greece.

For the LC-MS analysis, methanol, acetonitrile, water, and formic acid, obtained from Thermo Fischer Scientific (Waltham, MA, USA), were of LC-MS grade. The phenolic compounds quercetin, kaempferol, eriodictyol, gallic acid, rosmarinic acid, taxifolin and caffeic acid were purchased from Extrasythese (Genay, France). Hesperitin, syringic acid, salicylic acid, naringenin and resveratrol were acquired from Alfa Aesar (Ward Hill, MA, USA).

Phenolic standards of coumaric acid, m-coumaric acid, (-)-catechin, vanillin, protocatechuic acid, pyrocatechol, syringaldehyde, p-hydroxy-benzoic acid, ferulic acid, ethyl vanillin, benzoic acid, apigenin, myricetin and chlorogenic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA).

For the cosmetic cream formulation, butylene glycol was purchased from Sigma-Aldrich (St. Louis, MO, USA), beeswax, vegetable-based emulsifier (Inci name:Potassium Palmitol Hydrolyzed Wheat protein Glyceryl Stearate Cetearyl Alcohol),almond oil, avocado oil and aloe jelly were purchased from a local pharmacy.

2.2. Sample Preparation

The raw propolis was obtained from the Eastern Peloponnese region, Greece. The samples were composed of a single batch and were maintained at 4 °C util their further use. Before their extraction the samples were chopped into small pieces of at least 1 mm due to their resinous nature.

2.3. NADES Preparation

The NADES were prepared using the heating and stirring method [10]. In a round-bottom flask, equipped with a magnetic stirrer, the appropriate amounts of the starting materials, in specific molar ratio, were added and the mixture was stirred at specific temperature until a homogenous and transparent liquid is formed. The equivalents of water included in some of the NADES correspond to the water contained in the reagent of D,L-lactic acid, as the reagent is available as an aqueous solution. Finally, the NADES were transferred in hermetically closed glass vessels and stored at room temperature in dark until their further use.

Betaine/D,L-Lactic acid/Water (Bet/LA/W) 1:2:2.5. According to the general procedure, 1 mmol of betaine was placed in a round-bottom flask and 2 mmol of D,L-lactic acid were added. Stirring and heating at 50 °C were performed for 1 h until a colorless transparent liquid was formed.

β-Alanine/D,L-Lactic acid/Water (Ala/LA/W) 1:2:2.5. According to the general procedure, 1 mmol of β-alanine was placed in a round-bottom flask and 2 mmol of D,L-lactic acid were added. Stirring and heating at 50 °C were performed for 3h until a yellowish viscous liquid was formed.

D-(+)-Glucose/D,L-Lactic acid/Water (Glu/LA/W) 1:5:6.5. According to the general procedure, 1 mmol of D-glucose was placed in a round-bottom flask and 5 mmol of D,L-lactic acid were added. Stirring and heating at 70 °C were performed for 1 h until a transparent colorless liquid was formed.

Betaine/Levulinic acid (Bet/Lev) 1:2. According to the general procedure, 1 mmol of betaine was placed in a round-bottom flask and 2 mmol of levulinic acid were added. Stirring and heating at 50 °C were performed for 3 h until a transparent light yellowish liquid was formed.

L-Proline/D,L-Lactic acid/Water (Pro/LA/W) 1:2:2.5. According to the general procedure, 1 mmol of L-proline was placed in a round-bottom flask and 2 mmol of D,L-lactic acid were added. Stirring and heating at 60 °C were performed for 4 h until a yellowish viscous liquid was formed.

2.4. Ultrasound-Assisted Extraction (UAE) Process Using NADES

The UAE process using NADES as extraction solvents was applied in order to obtain NADES-propolis extracts. In a glass vessel an appropriate number of NADES or NADES/water system was added in which an appropriate amount of propolis is then suspended. The extraction was performed in an ultrasonic probe (ultrasonic Vibrant Cell, model: VCX400, Probe Diameter 6 mm) while the vessel is immersed in an ice bath so as to avoid elevated temperatures. Then, centrifugation was performed at 7000 rpm for 15 min and the supernatants were stored at 4 °C until their further analysis. Total phenolic content (TPC) as well as the ability of the extracts to scavenge the DPPH radical were determined

by standard colorimetric procedures. The Solvent-to-propolis ratio (*w*/*w*), the ultrasonic power (W), the extraction time (min) and the % (*w*/*w*) NADES in the NADES-water solvents system (when water was used along with the NADES as a co-solvent) were fixed accordingly each time.

Firstly, a screening of the prepared NADES as extraction solvents was performed under the following conditions: Solvent-to-propolis ratio: 25 g/g, NADES-to-Water ratio: 80:20 (*w*/*w*), Power: 160 W, Pulse: 9″ on/3″ off and Time: 20 min. The TPC and the antioxidant activity of the extracts were measured and the optimum NADES for the propolis extraction was identified. The most important parameters to be examined as well as the boundaries of the system were predetermined via some preliminary extraction experiments using the indicated by the screening procedure NADES. Finally, the extraction process was optimized in terms of their TPC via a series of 15 experiments using the Response Surface Methodology (RSM).

2.5. Conventional Extraction Process

For comparison reasons, a conventional extraction of raw propolis using a hydroethanolic solution as extraction solvent was also implemented. In a round bottom flask the EtOH-Water system (70:30) is added and the appropriate amount of propolis is suspended in a Solvent-to-propolis ratio equal to 25 g/g. The mixture is stirred for 24 h, at room temperature. Then, filtration under vacuum and solvent evaporation follows and the dry extract stored at 4 °C until further analysis.

2.6. NADES-UAE Optimization Using Response Surface Methodology

The optimal conditions of the NADES-UAE for the extraction of phenolic compounds from Greek propolis were identified by response surface methodology (RSM). A three-level, 15-run Box–Behnken design (BBD) including three replicates at the center point was applied Design-Expert 12.0 software (Stat-Ease Inc., Minneapolis, MN, USA—Trial Version). The impact of the factors (A) extraction time (min), (B) Solvent-to-propolis ratio (*w*/*w*) and the (C) %NADES (in the NADES-to-Water system, *w*/*w*) was examined on the selected to be investigated response, namely total phenolic content (TPC). The level of statistical significance was set at $p < 0.05$. The levels, as well as the conditions of the conducted experiments can be seen in Table 1.

Table 1. Raw and coded values of independent variables of NADES-UAE.

Run	A: Time (min)		B: Solvent-To-Propolis Ratio (g/g)		C: % NADES	
1	25	0	30	+1	90	+1
2	10	−1	30	+1	55	0
3	40	+1	30	+1	55	0
4	40	+1	18	0	20	−1
5	25	0	18	0	55	0
6	10	−1	6	−1	55	0
7	10	−1	18	0	20	−1
8	40	+1	6	−1	55	0
9	25	0	6	−1	20	−1
10	25	0	30	+1	20	−1
11	10	−1	18	0	90	+1
12	25	0	6	−1	90	+1
13	25	0	18	0	55	0
14	40	+1	18	0	90	+1
15	25	0	18	0	55	0

Finally, an experiment inside the boundaries of the experimental design (and in the medium level (run 5, 13 and 15), was performed in triplicate in order to validate the obtained model.

2.7. Colorimetric Determination of Total Phenolic Content of Extracts (TPC)

TPC of the propolis extracts was determined through the reducing capacity of the Folin–Ciocalteu reagent, as described in our previously published research work [10]. The extracts used for the analysis were diluted either 10 or 20% in water depending on their bioactive compounds composition. All the experiments were performed in triplicate. The microplate spectrophotometer (BioTek Epoch 2) was used for the analysis.

The TPC of the extracts was calculated as gallic acid equivalents (GAE) from a calibration curve, using gallic acid as a standard polyphenolic compound and was expressed as mg GAE per g of propolis.

2.8. DPPH Radical Scavenging Ability of the Extracts

The antioxidant capacity of the ginger extracts was studied using the procedure described in our previously published research work [4]. For this method a 0.1 mg/mL stock solution of the stable DPPH radical in pure ethanol was used. The initial concentration of the stock solutions of the extracts was varying between 0.5–5% (*v*/*v*). All the experiments were performed in triplicate. The microplate spectrophotometer (BioTek Epoch 2) was used for the analysis.

The extracts' antioxidant activity was quantified by the IC_{50} value demonstrating the concentration of the extract required to inhibit 50% of the DPPH and the results were finally expressed as mg of the NADES-propolis extract per mL of solution.

2.9. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis of Propolis Extracts

The identification of phenolic compounds in the propolis samples was based on a developed in-house library of 26 phenolic reference standards. The analysis was performed by using an Agilent 1200 HPLC system (consisting of a G1379B micro vacuum degasser, a G1312A binary pump, a G1329 autosampler and a G1316A column compartment) (Agilent Technologies, Santa Clara, CA, USA) and a 3200 Q TRAP triple-quadrupole linear ion trap mass spectrometer fitted with a TurboIon Spray interface (Sciex, Framingham, MA, USA).

The chromatographic separation was carried out with an Agilent Eclipse Plus C-18 reversed-phase column (50 mm × 2.1 mm inner diameter, 3.5 μm particle size) combined with a RRLC in-line filter kit (2.1 mm, 0.2 μm filter) (Agilent Technologies, Santa Clara, CA, USA). The column and the autosampler temperature were set at 25 °C. The mobile phase consisted of water with 0.2% *v/v* formic acid (Solvent A) and acetonitrile with 0.1% *v*/*v* formic acid (Solvent B). The phenolic standards, at concentration of 10 μg/mL, were eluted by applying the gradient elution program, as published in previous work of our research group [11]: 0.0 min 10% B, linear 10–20% B at 0.5 min and then, linear 20–30% B at 4 min, with a flow rate at 300 μL/min. Next, the flow rate was set to 350 μL/min and the gradient was kept linear 30–50% B at 4.10 min, held constant for 0.40 min, then linear 50–65% B at 5.10 min and linear 65–100% B at 7 min. The gradient was kept steady for 1 min, and the flow rate returned to 300 μL/mL and 100% B for 1 more minute. Consequently, the gradient was linear 100–10% B at 9.10 min and to finish, the column was re-equilibrated at 10% B, from 9.10 to 15 min. For the LC-MS/MS analysis, 5 mg of conventional extract and 50 mg of NADES extract were diluted to 500 μL of methanol with 0.1% *v/v* formic acid. The injection volume was set at 5 μL.

The MS analysis was conducted using electrospray ionization (ESI) at the negative ionization mode. Regarding the optimization of the compound-specific MS/MS conditions, a mixture of phenolic standards, diluted, at concentration of 0.5 μg/mL (for each standard), in methanol with 0.1% *v/v* formic acid was directly infused in MS instrument using a syringe pump. In addition, a flow injection analysis (FIA) was performed in order to optimize the source parameters. Enhanced mass spectrum (EMS) survey scans and information dependent acquisition (IDA)-triggered MS/MS scans (EPI—enhanced product ion scans) were implemented for the MS elucidation of the reference standards. The EMS scan analysis was performed at a time of 0.1513 s, at a mass range from 100 to 700 amu, while the number

of scans to sum was settled to three and the scan rate was 4000 amu/s. The values of the remaining parameters for EMS were the following: curtain gas, 30; CAD gas, medium; temperature (TEM), 550; gas 1 (GS1), 45; gas 2 (GS2), 45; ion spray (IS), −4500; declustering potential (DP), −30; entrance potential (EP), −7.50; collision energy (CE), −10; C2B, −300.

Concerning the IDA analysis, the two most intense peaks for each cycle were chosen for acquiring an EPI scan, while the IDA threshold was set to 100,000 counts. For the EPI scans a linear ion trap (LIT) dynamic fill time was used (max fill 250 ms) and the scan mass range was performed from 50 to 700 amu, while the number of scans to sum was set to 1, the EPI scan rate at 1000 amu/s and the Q1 resolution to low. The values of the other EPI parameters were as follows: curtain gas, 30; temperature, medium; gas 1 (GS1), 45; gas 2 (GS2), 45; ion spray (IS), −4500; declustering potential (DP), −30 and entrance potential (EP), −7.50; CAD gas, high; collision energy (CE), −30; collision energy spread (CES), 10; C2B, −450.

The samples were analyzed in duplicate. All chromatographs and spectra were processed with the Analyst software program (version 1.4.2) (Sciex, Framingham, MA, USA). One-way ANOVA (Statistica package, trial version 12, TIBCO Software Inc., Palo Alto, CA, USA) was implemented for the statistical analysis of the samples. Confidence level was set at 95% (p-value ≤ 0.05).

2.10. Cosmetic Cream Preparation

Water-in-oil (W/O) emulsions with NADES and NADES-propolis-extract were prepared. The oil phase consists of beeswax, vegetable-based emulsifier (INCI name: Potassium Palmitol Hydrolyzed Wheat protein Glyceryl Stearate Cetearyl Alcohol), butylene glycol, almond oil, and avocado oil, and was heated up to 70 °C. The mixture was removed from the heat and the aloe jelly (water phase) was added slowly, with constant stirring. The formed cream was left to cool at room temperature. The use of the ultrasonic probe (ultrasonic Vibrant Cell, model: VCX400, Probe Diameter 6 mm) at 160W for a period of time of 4 min was performed in order to give to the formulation a smooth, uniform paste. In the next step, NADES, and NADES-propolis-extract (10% w/w) were added to the cooled emulsion. A control base cream without NADES and NADES-propolis-extract was also formulated with oil and water phases, as mentioned in Table 2.

Table 2. Composition of formulations.

Ingredients	Control Base Cream	NADES Cream	NADES-Propolis-Extract Cream
Almond oil	3 mL	3 mL	3 mL
Avocado oil	3 mL	3 mL	3 mL
Vegetable-based emulsifier	0.75 g	0.75 g	0.75 g
Beeswax	0.75 g	0.75 g	0.75 g
Aloe jelly	6 g	6 g	6 g
NADES	-	10% w/v	-
NADES-to- propolis-extract	-	-	10% w/v

2.11. Cosmetic Cream Characterization

2.11.1. Organoleptic Characteristics

The optimized formulations of the creams were characterized by various organoleptic characteristics, such as physical appearance, color, texture, phase separation, and homogeneity, by visual observation. The samples were placed between the thumb and index finger and then the homogeneity and texture characteristics of the developed formulation were assessed [12].

2.11.2. pH Measurement

The pH of the formulations was measured by a digital pH meter (Metrohm 744 pH Meter) at room temperature (25 ± 5 °C).

2.12. Physicochemical Stability of Cream

The optimized formulations of the cosmetic creams were subjected to a centrifugation test (5000 rpm for 30 min) and freeze-thaw cycle (3 alternate cycles at 25 °C, 40 °C and 4 °C for 24 h, respectively) to observe the changes of phase separation and change in appearance under stress conditions [13,14].

2.13. Propolis Extract Release Study from Cream

The release study of the propolis extract was conducted using the dialysis membrane method: 5 g of cream containing 500 μL propolis extract was loaded into a dialysis tubing (SERVA Electrophoresis GmbH, Heidelberg, Germany) with a MWCO of 12–14 kDa. The dialysis tubing was immersed in 25 mL of phosphate buffer solution, pH 5.5 at 32 °C with a magnetic stirring of 100 rpm. The amount of 1 mL of the dissolution medium was withdrawn at the time intervals 20, 60, 120, 180, and 240 min and replaced with an equal volume of fresh medium. The samples retrieved were evaluated for antioxidant activity.

2.14. In Vitro Evaluation of Antioxidant Activity of Cream Extract

2.14.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Scavenging Ability Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to measure the scavenging ability of the propolis extract and the cream formulation containing an amount of propolis extract. The DPPH test was performed using a 96-well plate and a microplate reader (BioTek Epoch 2). In a 96-well plate, 100 μL of released extract obtained at specific time intervals (20, 60, 120, 180, and 240 min) were added in 100 μL DPPH (0.1 mg/mL) solution. The samples were incubated for 30 min, in the dark at room temperature and then the absorbance was measured at 517 nm. The above-mentioned procedure was repeated for a control sample, which contained 100 μL water instead of propolis extract. All the experiments were performed in triplicate. The results of this study were finally expressed as % of DPPH radical inhibition and as Trolox equivalents.

2.14.2. Inhibition of 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) Induced Linoleic Acid Oxidation

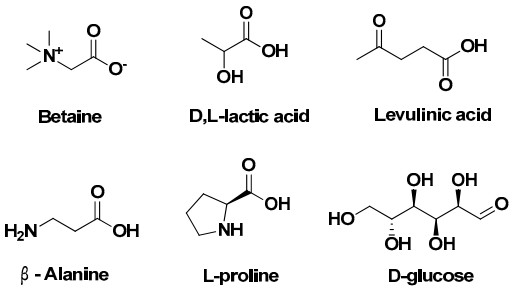
AAPH induced linoleic acid oxidation has been developed as a quick and reliable antioxidant activity assay. It is based on the inhibition of linoleic acid oxidation, initiated by the thermal free radical producer 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), providing a measure of how efficiently antioxidants protect against lipid oxidation in vitro.

This experimental procedure is also used for the evaluation of the antioxidant activity of the propolis extract. 14 μL of the 16 mM sodium linoleate solution were added to the UV cuvette containing 1.3 mL of a 0.05 M phosphate buffer, pH 7.4. The oxidation reaction was initiated at 37 °C under air by the addition of 70 μL of a 40 mM AAPH solution, which was used as a free radical initiator. Oxidation was carried out in the presence of the released extract from the cream (14 μL) in the assay and monitored at 234 nm. The procedure was repeated for the same volume of water and served as a negative control. Trolox was used as the appropriate standard [15] and the results were expressed as % Inhibition of linoleic acid peroxidation and as Trolox equivalents.

3. Results and Discussion

3.1. Task-Specific Design of NADES

Taking advantage of possibly one of the most important properties of the NADES, their ability to confer desirable properties on specific applications derived from the selection of their constituents, herein the hydrogen bond donors and the hydrogen bond acceptors for the NADES preparation were task-specifically selected. The components of the NADES (Scheme 1) were selected in terms of biocompatibility and safety so as they can be used directly in end user products such as cosmetic formulations. All of them are currently used by the food, pharmaceutical and cosmetic industries.



Scheme 1. Chemical structures of the NADES components.

More specifically, betaine is a quaternary ammonium salt of natural origin that is used in cosmetic and skincare products for its moisturizing properties. Lactic acid is an alpha-hydroxy acid that finds applications in food and beverages as an acidifier and flavor enhancer as well as in personal care products as a pH adjustive agent, a moisturizing agent etc. [16,17]. Levulinic acid is a chemical building block for a wide variety of other compounds and is used among with its derivatives, like sodium levulinate in cosmetics and personal care products, fragrances, cleaning products, as well as in the pharmaceutical and food industry [18]. β-Alanine and L-proline are amino acids and thus they could be considered as safe, and they have been used in the literature for NADES preparation. Specifically, L-proline is widely used in supplements and health foods [19] while β-alanine and its derivatives are used in medicine, feed, food etc. Finally, D-(+)-glucose was selected as one of the most representative monosaccharides, of low cost, that have been also studied lately and effectively used as a NADES constituent.

3.2. NADES Screening

All the task-specifically designed NADES were tested for their effectiveness as extraction solvents of bioactive compounds from Greek propolis using the UAE. The extraction conditions were selected based on our previous experience in propolis extraction process using a different extraction solvent and propolis of different origin [4] and are the following: Solvent-to-Propolis ratio: 25 g/g, NADES-to-Water ratio: 80:20, Power: 160 W, Time: 20 min and Pulse: 9″ on/3″ off. All the extracts were evaluated in terms of their TPC, and the antioxidant activity and the results are presented in Table 3.

Table 3. Screening of NADES as extraction solvents.

Extract	TPC ($mg_{GAE}/g_{propolis}$)	DPPH Scavenging Ability ($IC_{50\ (30min)}$, $mg_{\ of\ NADES\text{-}extract}/mL$) *
Bet/LA/W-extract	50.31 ± 0.85	8.01 ± 0.16
Ala/LA/W-extract	42.26 ± 0.75	5.66 ± 0.03
Glu/LA/W-extract	50.71 ± 1.30	5.89 ± 0.20
Bet/Lev-extract	66.37 ± 1.91	6.70 ± 0.05
Pro/LA/W-extract	55.61 ± 1.23	2.98 ± 0.08

* Trolox was used as a reference antioxidant compound: $IC_{50,t\,=\,30\ min}$ = 8.1 ± 0.02 μg/mL.

The obtained results indicated that the higher TPC value in combination with the higher antioxidant activity, is presented when the Pro/LA/W NADES was used as an extraction solvent. Thus, the Pro/LA/W NADES was selected to be further investigated as extraction media for the optimization of the proposed methodology.

3.3. Box–Behnken Experimental Design

For the optimization of the propolis extraction the response surface methodology (RSM) was implemented. More specifically, Box–Behnken design was employed to design the experiments and then ANOVA study was conducted. The TPC as well as the DPPH scavenging ability of the extracts were studied as dependent variables (responses) while

the independent variables (factors) were the extraction time, the solvent-to-propolis ratio (w/w) and the %NADES in the extraction solvent system (%NADES in the NADES-water system w/w).

3.3.1. Total Phenolic Content (TPC) of the NADES-Extracts

The 15 experiments indicated by the Experimental design were implemented and the results of the TPC of the obtained extracts are presented in Table 4.

Table 4. TPC results of the Box-Behnken Experimental Design.

Run	A: Time (min)		B: Solvent-To-Propolis Ratio (g/g)		C: % NADES		TPC ($mg_{GAE}/g_{propolis}$)
1	25	0	30	+1	90	+1	50.76
2	10	−1	30	+1	55	0	28.01
3	40	+1	30	+1	55	0	37.44
4	40	+1	18	0	20	−1	6.10
5	25	0	18	0	55	0	38.00
6	10	−1	6	−1	55	0	13.48
7	10	−1	18	0	20	−1	10.73
8	40	+1	6	−1	55	0	20.70
9	25	0	6	−1	20	−1	13.75
10	25	0	30	+1	20	−1	18.81
11	10	−1	18	0	90	+1	52.50
12	25	0	6	−1	90	+1	35.80
13	25	0	18	0	55	0	37.98
14	40	+1	18	0	90	+1	61.13
15	25	0	18	0	55	0	40.83

The presented results (Table 4) were statistically analyzed in order to determine the most important factors regarding the extraction process. The used model was the reduced cubic model in which some variables were not taken into consideration since according to the results, they do not truly affect the model. Calculated values of the regression coefficients for TPC are given in Table 5 while the statistical analysis revealed that the TPC is best described by the following actual and coded equations (Equations (1) and (2) respectively).

$$\begin{aligned}\text{TPC} = {} & 6.76907 + 1.041\ (\text{Time}) - 2.12071\ (\text{Solvent-to-propolis ratio}) - 0.295438\ (\%\text{NADES}) + 0.007114\ (\text{Time}) \\ & \times (\%\text{NADES}) + 0.085325\ (\text{Solvent-to-propolis ratio}) \times (\%\text{NADES}) - 0.025496\ (\text{Time})^2 \\ & + 0.064742\ (\text{Solvent-to-propolis ratio})^2 - 0.002206\ (\text{Solvent-to-propolis ratio})^2 \times (\%\text{NADES})\end{aligned} \quad (1)$$

$$\text{TPC} = 38.36 + 2.37\ \text{A} + 6.41\ \text{B} + 24.62\ \text{C} + 3.74\ \text{AC} + 2.48\ \text{BC} - 5.74\ \text{A}^2 - 8.15\ \text{B}^2 - 11.12\ \text{B}^2\text{C} \quad (2)$$

Table 5. Significance of each factor equation model terms for the TPC.

	Model	Lack-of-Fit	A	B	C	AC	BC	A^2	B^2	$C \times B^2$
p-value	<0.0001	0.2283	0.0364	0.0006	<0.0001	0.0507	0.1185	0.0077	0.0010	0.0014
F-value	65.44	3.61	7.19	44.45	316.55	5.94	3.32	15.42	35.23	30.94

Model: R^2 (%) = 98.87, $R^2_{adjusted}$ (%) = 97.36, $R^2_{predicted}$ (%) = 83.75.

The actual equation can be used for the predictions of the TPC while the coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

The proposed model is significant since it indicates a high F-value equal to 65.44 and a very low *p*-value (<0.0001). The total (R^2) and the adjusted ($R^2_{adjusted}$) determination coefficients in the regression equation are up to 98.87% and 97.36%, respectively, verifying

the adequacy of the model. The $R^2_{predicted}$ of 83.75% is in reasonable agreement with the $R^2_{adjusted}$ of 97.36% indicating the relevance between the experimental and the predicted values. Moreover, the *p*-values of 0.2283 and the F-value of 3.61 for lack of fit indicated the validity of the model. Thus, this model can be used to navigate the design space.

From the results above, it is clear that the %NADES (in the NADES-Water system, *w*/*w*) (factor C) is the factor that contributes the most to the efficient extraction of phenolic compounds followed by the Solvent-to-propolis ratio (*w*/*w*) (factor B). The 3D surface response plots depict the correlation between the studied factors and the selected response (Figure 1).

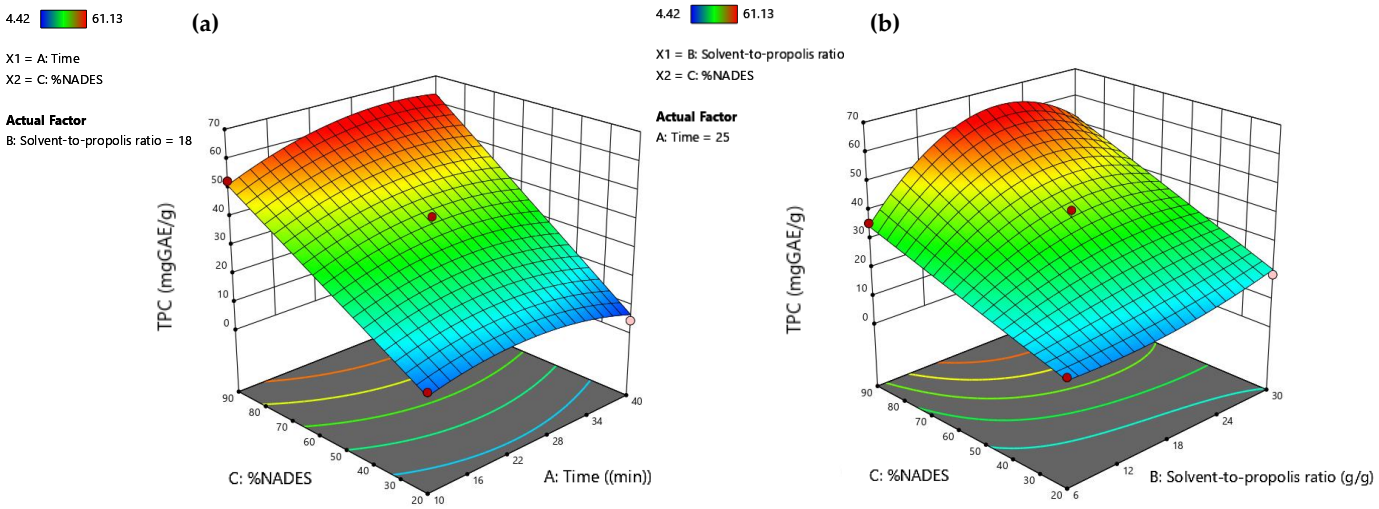


Figure 1. RSM plots for TPC response showing the correlation between TPC and the factors: (**a**) time and %NADES in the NADES-Water system (in a solvent-to-propolis ratio = 18 g/g); and (**b**) Solvent-to-propolis ratio and %NADES in the NADES-Water system (Time = 25 min).

Response surface analysis (Figure 1a) shows that when keeping constant the parameter B (Solvent-to-propolis ratio) at 18 g/g, the use of higher amounts of NADES (higher that ~75–80%) favors the extraction of phenolic compounds leading to higher TPC values.

Regarding the parameter of extraction time (for B = 18 g/g) it seems that extraction time higher than ~20 min leads to more effective extraction of phenolic compounds from propolis. Regarding the correlation between the parameters B and C (for extraction time 25 min) with the TPC of the extracts (Figure 1b), it seems that high percentages of NADES (in the NADES-water systems) are required when the Solvent-to-propolis ratio value is in the average area (15–25 g/g) of the experimental boundaries.

The optimization criterion of this study was set to be the maximization of the extracts' TPC, and the analysis indicated as optimum extraction conditions within the studied boundaries the following: 30.89 min, 22.57 g of solvents per g of propolis and 89.14% NADES. In order to confirm the validity of the proposed model, two additional experiments were conducted, and the results are presented in Table 6. It is observed that the TPC values are between the 95% low and high prediction intervals (PI) according to the estimated model and it could be concluded that experimental data within the boundaries of this design have a good fit to the model.

Table 6. Confirmation of the estimated model.

Time (min)	Solvent-to-Propolis Ratio (g/g)	% NADES	Experimental TPC ($mg_{GAE}/g_{propolis}$)	Predicted TPC ($mg_{GAE}/g_{propolis}$)	95% PI Low	95% PI High
33	20.5	90	73.4	65.28	57.11	73.45
31	22.57	89.14	67.0	64.17	56.18	72.16

3.3.2. DPPH Scavenging Ability of the NADES-Extracts

The 15 experiments indicated by the Experimental design were implemented and the results of the antioxidant activity of the obtained extracts are presented in Table 7.

Table 7. DPPH scavenging ability results of the Box-Behnken Experimental Design.

Run	A: Time (min)		B: Solvent-to-Propolis Ratio (g/g)		C: % NADES		DPPH Scavenging Ability ($IC_{50\ (30min)}$, mg $_{\text{of NADES-extract}}$/mL) *
1	25	0	30	+1	90	+1	2.10
2	10	−1	30	+1	55	0	5.20
3	40	+1	30	+1	55	0	4.08
4	40	+1	18	0	20	−1	12.0
5	25	0	18	0	55	0	3.78
6	10	−1	6	−1	55	0	1.65
7	10	−1	18	0	20	−1	8.30
8	40	+1	6	−1	55	0	2.53
9	25	0	6	−1	20	−1	6.00
10	25	0	30	+1	20	−1	9.60
11	10	−1	18	0	90	+1	3.79
12	25	0	6	−1	90	+1	1.10
13	25	0	18	0	55	0	2.60
14	40	+1	18	0	90	+1	1.50
15	25	0	18	0	55	0	2.79

* Trolox was used as a reference antioxidant compound: $IC_{50,t\,=\,30\ min}$ = 8.1 ± 0.02 μg/mL.

The experimental results of the extracts' DPPH scavenging ability (Table 8) were statistically analyzed, and the most important extraction parameters were determined. The quadratic model was selected as the most appropriate and the statistical analysis revealed that the selected response is best described by the following actual and coded equations (Equations (3) and (4), respectively).

$$\text{DPPH scavenging ability } (IC_{50\ (30min)}, \text{mg}_{\text{ of NADES-extract}}/\text{mL}) = 6.66951 - 0.006224\ (\text{Time}) + 0.429232\ (\text{Solvent-to-propolis ratio}) - 0.2086\ (\%\text{NADES}) - 0.002782\ (\text{Time}) \times (\text{Solvent-to-propolis ratio}) - 0.002852\ (\text{Time}) \times (\%\text{NADES}) - 0.001548\ (\text{Solvent-to-propolis ratio}) \times (\%\text{NADES}) + 0.004459\ (\text{Time})^2 - 0.004821\ (\text{Solvent-to-propolis ratio})^2 + 0.001908\ (\%\text{NADES})^2 \quad (3)$$

$$\text{DPPH scavenging ability } (IC_{50\ (30min)}, \text{mg}_{\text{ of NADES-extract}}/\text{mL}) = 3.06 + 0.1466\ A + 1.21\ B - 3.43\ C - 0.5007\ AB - 1.50\ AC - 0.65\ BC + 1.00\ A^2 - 0.6942\ B^2 + 2.34 \quad (4)$$

Table 8. Significance of each factor equation model terms for the extract's DPPH scavenging ability.

	Model	Lack-of-Fit	A	B	C	AB	AC	BC	A^2	B^2	C^2
p-value	<0.0004	0.5313	0.5434	0.0030	<0.0001	0.1764	0.0053	0.0965	0.0291	0.0902	0.0009
F-value	39.36	1.01	0.4246	29.03	231.94	2.48	22.15	4.17	9.18	4.40	49.83

Model: R^2 (%) = 98.61, R^2_{adjusted} (%) = 96.10, $R^2_{\text{predicted}}$ (%) = 85.32.

The proposed model is significant according to the F-value which is up to 39.36 and the *p*-value (<0.0004). Moreover, the predicted R^2 of 0.8532 is in a reasonable agreement with the adjusted R^2, thus, this model is appropriate to used.

From the presented results, factor C (the %NADES in the NADES-Water system) and factor B (the Solvent-to-propolis ratio) are the factors that contribute most to the selected response. In Figure 2, are presented the 3D surface response plots for the AC and BC correlation of factors A and B with factor C in respect of the extract's DPPH scavenging

ability. The parameters AC and BC affect the studied response more intensely comparing to the parameter AB as it is also indicated by their *p* values.

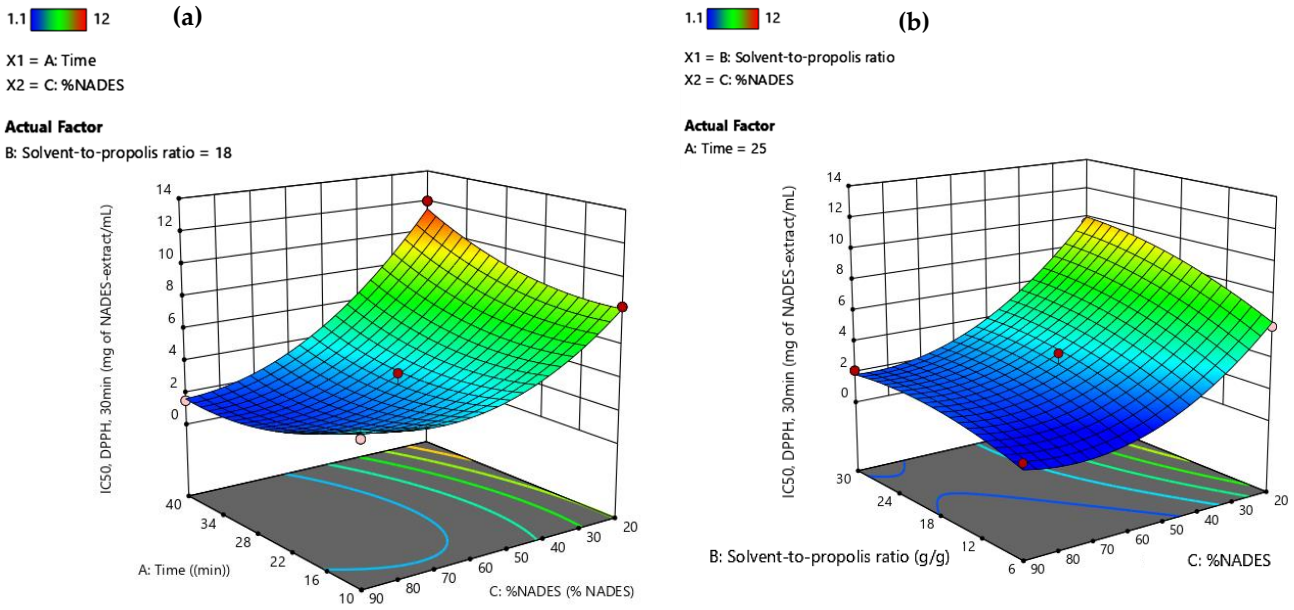


Figure 2. RSM plots. Correlation between the extract's DPPH scavenging ability response and the factors: (**a**) time and %NADES in the NADES-Water system (in a solvent-to-propolis ratio = 18 g/g); and (**b**) Solvent-to-propolis ratio and %NADES in the NADES-Water system (Time = 25 min).

Regarding the antioxidant activity of the NADES-extracts, the extracts with the higher antioxidant activity are those derived by extraction processes with higher percentages of NADES (blue areas in the RSM plots of Figure 2a,b). Regarding the extraction time parameter, higher extraction time values (>20 min) positively affect the overall antioxidant activity of the NADES-extract (Figure 2a) while regarding the parameter of solvent-to-propolis ratio, it seems that lower values (values close to 6 g/g) lead to higher antioxidant activity of the extract (Figure 2b).

The optimization criterion of this study is set to be the maximization of the antioxidant activity (i.e., the minimization of the IC_{50} value). The analysis indicated as optimum extraction conditions within the studied boundaries the following: 30.34 min extraction time, 6.322 g of Solvent per g of propolis and 75.71% NADES.

The proposed model was also confirmed by an additional experiment conducted under the following parameters: 33 min, 20.5 Solvent-to-propolis ratio, 90% NADES in the NADES-water system. The IC_{50} (DPPH scavenging ability assay) was found to be up to 2.59 $mg_{of\ extract}/mL$ while the predicted value regarding the proposed model was 2.44 $mg_{of\ extract}/mL$. The experimental results are close enough to those predicted by the proposed model and thus it can be concluded that the model has a good fit to the experimental data within the boundaries of the examined system.

3.4. Conventional Extraction Process

The application of ethanol-water mixtures predominates as conventional extraction solvent system for the propolis extraction and the optimal concentration of ethanol in water was found to be between 70–95% alcohol, most often 70–80% [3]. According to the results presented in Table 9, it can be concluded that the TPC assay gave higher values in the NADES-UAE indicating a more effective extraction of phenolic compounds comparing to the conventional extraction method (67 $mg_{GAE}/g_{propolis}$ comparing to 10 $mg_{GAE}/g_{propolis,}$ respectively).

Table 9. Total Phenolic Content and antioxidant activity of the extracts derived from NADES UAE and conventional extraction.

Time	Solvent-To-Propolis Ratio (g/g)	Extraction Solvent	TPC ($mg_{GAE}/g_{propolis}$)	DPPH Scavenging Ability ($IC_{50\ (30min)}$, mg $_{of\ extract}$/mL) *
31 min	22.57	89% NADES and 11% H_2O	67	3.34
24 h	20.79	70% EtOH and 30% H_2O	10	0.39

* Trolox was used as a reference antioxidant compound: Trolox = 8.1 μg/mL ± 0.02 μg/mL.

The antioxidant activity of the extract derived by the conventional extraction is lower (IC_{50} up to 0.39 mg $_{of\ extract}$/mL) than the antioxidant activity of the NADES-extract (IC_{50} up to 3.34 mg $_{of\ extract}$/mL). However, it must be mentioned that it is not possible to directly compare the obtained results of these two studied extraction processes since in the the case of the NADES-extract the amount of extract refers to the NADES-extract as obtained without removal of the NADES. On the other hand, in the conventional extraction, the amount of extract refers to the dry extract after the solvent (hydroethanolic solution) removal.

3.5. LC-MS/MS Analysis

The chromatographic and spectral information of the phenolic compounds, determined by the developed LC-MS/MS method, are presented in Table 10.

After generating the phenolic compounds' LC-MS/MS library, the propolis samples were analyzed in order to provide their phenolic profile. The assessment of LC-MS/MS chromatograms and spectra resulted in the annotation of 11 tentative phenolic compounds in the extracts of propolis (Table 11). As indicated in Table 11, the phenolic acids coumaric acid, caffeic acid and ferulic acid and the flavonoids apigenin, naringenin, kaempferol and quercetin were identified in both conventional and NADES extracts, while the phenolic aldehyde vanillin, the flavanone eriodictyol and the phenolic acids protocatechuic and gallic acid were only detected in the conventional extracts of propolis [1,20].

In particular, the content of phenolic acids was significantly higher (p-value ≤ 0.05) in the conventional extracts compared to the NADES samples. Specifically, coumaric acid, caffeic acid and ferulic acid levels were increased by 2.5-, 11- and 5.5-times in the samples extracted with conventional solvents (Figure 3). Furthermore, conventional extraction appeared to be more efficient for the recovery of kaempferol since its levels were 3-times lower in NADES extracts (Figure 3). On the contrary the aglycone flavonoids, apigenin and naringenin were present at higher concentrations (2-fold and 3.5-fold higher, respectively) in the NADES samples, while the content of quercetin was similar in both types of extracts (Figure 3). Thus, the results of the LC-MS/MS analysis highlighted the importance of the solvent in the extractability of individual phenolic compounds, since the use of NADES solvent favored the recovery of flavonoids in their aglycone forms [21], while the implementation of conventional solvents enhanced the extraction of phenolic acids [22].

3.6. Characterization of Cosmetic Cream

The results of the various organoleptic properties of the cream with propolis extract and blank cream are shown in Table 12. It was found that both formulations had a cosmetically appealing appearance, smooth texture, and homogenous dispersion, with no signs of phase separation (Figure 4).

Table 10. Chromatographic and spectral information of the phenolic reference standards.

Phenolic Compound	Retention Time (min)	Parent Ion [M-H]$^-$	Product Ions (MS/MS)
Apigenin	6.60	269.1	151.4, 149.3, **117.2** *
Benzoic acid	4.53	121.1	**93.4**, 77.5
Caffeic acid	2.75	179.1	**135.4**, 107.3
Catechin	1.39	289.2	**245.6**, 221.5, 203.5, 161.5, 151.4, 123.5, 109.3
Chlorogenic acid	1.72	353.2	**191.5**, 179.6, 161.5
Coumaric acid	3.85	163.1	**119.3**, 93.4
m-Coumaric acid	4.28	163.2	**119.3**, 118.5, 91.4
Eriodictyol	6.07	287.2	151.4, **135.4**, 107.3
Ethyl vanillin	4.82	165.1	**136.4**, 108.3, 92.3
Ferulic acid	4.35	193.1	178.4, 149.4, **134.3**
Gallic acid	0.65	169.1	**125.3**, 97.3, 79.3
Hesperitin	6.81	301.2	242.6, 199.4, 174.5, **164.3**, 136.3, 108.2
Kaempferol	6.66	285.3	**257.6**, 229.6, 187.5
Myricetin	5.26	317.1	**179.5**, 151.4, 137.3, 109.2
Naringenin	6.64	271.1	177.4, 151.4, **119.3**, 107.3, 93.2
Protocatechuic acid	1.10	153.1	**109.3**, 153.4
Pyrocatechol	1.20	109.0	**91.3**, 81.3, 65.2
Quercetin	6.20	301.1	273.5, 179.3, **151.4**
Resveratrol	5.40	227.1	**185.5**, 159.5, 143.5
Rosmarinic acid	4.95	359.3	197.5, 179.5, **161.5**, 135.4
Salicylic acid	5.13	137.1	**93.2**, 65.2
p-Hydroxy-benzoic acid	1.96	137.0	**93.3**, 65.3
Syringaldehyde	3.79	181.1	166.3, **151.3**, 123.2
Syringic acid	2.62	197.0	**182.5**, 167.5, 123.2, 95.2
Taxifolin	4.42	303.1	285.5, 217.6, 177.5, **125.3**
Vanillin	3.53	151.1	**136.3**, 108.3, 92.3

* The fragments in bold are the most intense MS/MS fragments and were used for the peak area determination of each phenolic compound.

Table 11. Phenolic compounds detected in the propolis extracts.

Phenolic Compound	Conventional Propolis Extract	NADES Propolis Extract
Vanillin	√	
Protocatechuic acid	√	
Coumaric acid	√	√
Gallic acid	√	
Caffeic acid	√	√
Ferulic acid	√	√
Apigenin	√	√
Naringenin	√	√
Kaempferol	√	√
Eriodictyol	√	
Quercetin	√	√

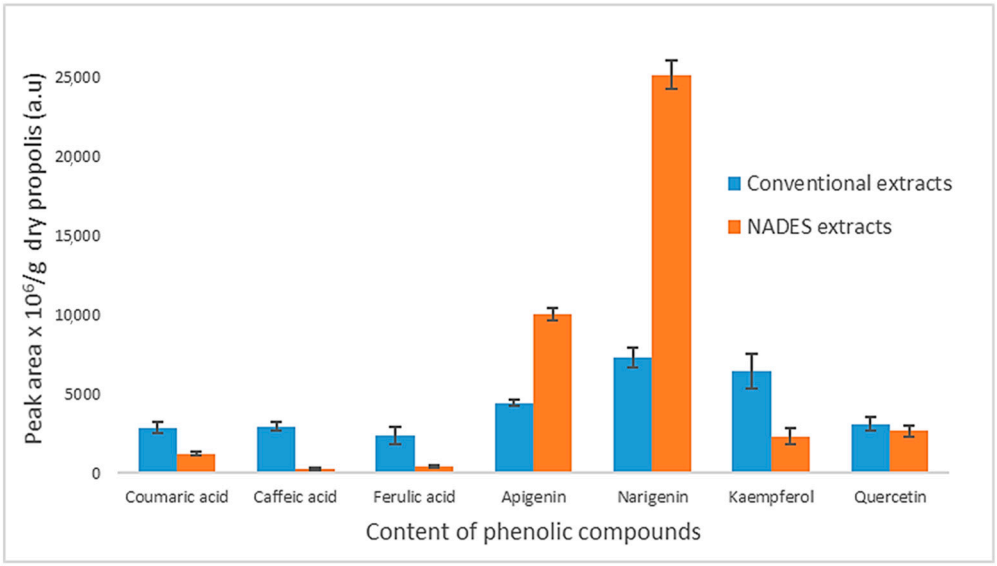


Figure 3. Comparison of the content of individual phenolic compounds in conventional and NADES extracts, expressed as fold changes.

Table 12. Organoleptic characteristics of the cream with propolis extract and blank cream.

Formulation	Color	Odor	Appearance	Consistency	pH
Cream with propolis extract	White	Pleasant	Semi-solid	Smooth	6.5
Blank Cream	White	Pleasant	Semi-solid	Smooth	7.0

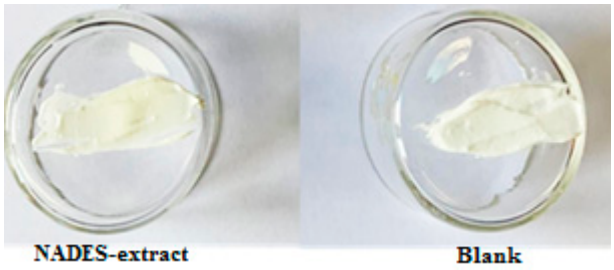


Figure 4. Organoleptic characteristics (physical appearance, color, texture) of cream with propolis extract and blank cream.

The pH of normal skin is usually between 4.5 and 6.5. Therefore, the pH of skin care products should be included in this range to allow a formulation to be approved for industrial use. The pH of the cream with propolis extract just after the preparation was found to be more acidic than that of the pH of normal skin. The pH of the developed cream has been adjusted to 6.5 by adding NaOH solution (1N) drop by drop under continuous mixing.

3.7. Physicochemical Stability of Cosmetic Cream

The produced formulations remained homogeneous after 5000 rpm centrifugation (30 min). Thus, the prepared creams were considered stable under this experimental condition.

Freeze-thaw testing was conducted by exposing the product to freezing temperatures (approximately −18 °C) for 24 h, and then allowing it to thaw at room temperature for 2 h. The sample was then placed in a higher temperature (approximately 40 °C) for 24 h and then placed at room temperature again for 24 h. After three cycles of freeze-thaw testing, no significant changes were observed.

3.8. In Vitro Evaluation of Antioxidant Activity of the Cosmetic Cream Incorporating the NADES-Propolis-Extract as Obtained

In order to investigate the effect of the incorporated NADES-propolis-extract on the antioxidant activity of the cosmetic cream, a release experiment was performed as described in Section 2.12. In certain time intervals, a sample was withdrawn from the release medium and the antioxidant activity was evaluated using the DPPH and AAPH assays.

The blank cream and the cream containing only the NADES were also tested with the same assays and found to possess no antioxidant activity.

The bioactive components of the extract are slowly released from the cream formulation during a period of 4 h. This is indicated by the increasing antioxidant activity of the samples obtained over time. In particular, the strongest inhibition of DPPH radical was recorded for propolis extract released from the cream after 2 h (72%) and 3 h, 4 h (74%) (Table 13).

Table 13. Antioxidant activity of the released propolis NADES-propolis extract.

NADES-Propolis-Extract Release Time (min)	DPPH Radical Scavenging Activity (%)	Inhibition of Linoleic Acid Peroxidation (%)	Trolox Equivalent Antioxidant Capacity/DPPH Assay (μg/mL)	Trolox Equivalent Antioxidant Capacity/AAPH Assay (μg/mL)
20	45.18 ± 0.19	86.25 ± 0.16	7.32	24.03
60	63.47 ± 0.09	90.41 ± 0.22	10.28	25.19
120	72.26 ± 0.37	91.09 ± 0.52	11.71	25.39
180	73.71 ± 0.18	91.12 ± 0.38	11.94	25.42
240	74.17 ± 0.28	91.17 ± 0.22	12.01	25.44

Note: Trolox was used as a reference antioxidant compound: % DPPH inhibition 88.5 ± 0.74 (concentration of Trolox solution 14.47 μg/mL).; % lipid peroxidation inhibition: 89.73 ± 0.90 (concentration of Trolox solution 25.02 μg/mL).

In this test, linoleic acid was oxidized in a chain reaction initiated by peroxy radicals generated by the thermal decomposition of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). This chain reaction can be slowed down by an antioxidant donor of hydrogen atom, which scavenges the peroxy radical. The inhibition percentage of linoleic acid peroxidation by the NADES-ptopolis extract is given in Table 13. There is a strong dependance between the antioxidant activity of the released extract and the release time. According to the results of this study, the highest inhibition of linoleic acid peroxidation (up to 91%) is presented after of 2 h release of the NADES-propolis extract from the cream.

The % inhibition of linoleic acid oxidation and the DPPH radical scavenging activity of the NADES-propolis extract are also expressed as Trolox equivalent antioxidant capacity (TEAC). Regarding the DPPH assay, the TEAC is up to 7.32 μg/mL in the first 20 min and gradually increases to 12.01 μg/mL (after 4 h) while the TEAC in the AAPH assay is up to 24.03 μg/mL in the first 20 min and gradually increases to 25.44 μg/mL (after 4 h).

The antioxidant activity of the cosmetic cream (in which the NADES-propolis extract incorporated) is time dependent and shows an increase even after 120 min, proving that this formulation can act as sustained release agent for the extracted compounds. Thus, the findings of this study indicate the prolonged antioxidant activity of the NADES-extract since the extracted bioactive compounds from propolis are gradually released to the cream.

4. Conclusions

In the present work, the ultrasound assisted extraction of bioactive compounds from Greek propolis (Peloponnese region) using Natural Deep Eutectic Solvents (NADES) were investigated and optimized. Among the tested solvents, the L-Proline/D,L-Lactic acid/Water (Pro/LA/W) was selected as optimum in terms of the total phenolic content and the antioxidant activity of the extracts. The extraction process was optimized implementing an experimental design and three of the most important extraction parameters were investigated about their impact in the studied process. Extraction time, solvent-to-propolis ratio as well as the amount of water and NADES in the NADES-water solvents system were selected as parameters for the optimization process. The amount of water in

the final solvent system is an important parameter and was selected because the viscous nature of NADES is known to negatively affect some extraction processes. The addition of an appropriate amount of water consists of a well-known technique to overcome such a drawback. The Box-Behnken Experimental Design resulted in a prediction model for the studied extraction process that found to be reliable after the confirmation experiments. This study indicated that the optimum extraction conditions (within the studied boundaries) in order to obtain an extract with maximum TPC are the following: 31 min extraction time, 22.57 g of NADES per g of propolis and 89.14% NADES. In terms of the maximum DPPH scavenging ability, the optimum conditions are the following: 30 min extraction time, 6.32 g of Solvent per g of propolis and 75.71% NADES.

For comparison reasons, a conventional extraction using stirring at room temperature for 24 h in a hydroethanolic solution as extraction solvent was also investigated. The optimum NADES-extract derived by the UAE and the extract derived by the conventional extraction were evaluated regarding their antioxidant activity, and their phytochemical profile using the LC-MS/MS analysis. This study showed that the NADES as extraction solvent leads to extracts with higher amounts of naringenin and apigenin, two very important bioactive flavonoids.

The optimum NADES-propolis extract was incorporated, as obtained, to a cosmetic cream and sensory analysis was carried out to evaluate organoleptic characteristics such as color, aroma, and texture. Real storage conditions confirmed the stability prediction performed using the combination of accelerated stability tests such as centrifugation, sonication, freeze-thaw cycles. The antioxidant activity of the NADES-propolis extract alone and after incorporation in the cream was assessed using different in vitro methods. The strongest inhibition of DPPH radical was presented for propolis extract released from the cream at 2 h (72%) and 4 h (74%). Furthermore, the results revealed that the highest inhibition of linoleic acid peroxidation was recorded for the spropolis extract released from the cream at 2, 3 and 4 h (91%). According to these findings, the antioxidant activity of the cosmetic cream incorporating the NADES-propolis extract is time dependent showing an increase even after 120 min. Thus, it is shown that the use of NADES-extracts leads to prolonged antioxidant activity due to the gradual release of extracted bioactive compounds to the cream. In view of the encouraging results concerning the antioxidant activity and TPC content of the NADES-propolis extract as well as its successful incorporation in a cosmetic formulation, it is our priority to thoroughly evaluate the toxicity and antimicrobial activity of the NADES-extract. Moreover, the spectrum of potential applications of the NADES-propolis extract will be further expanded in areas such as wound healing and biomedical applications.

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