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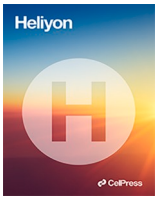

Research article

Insight into the influence of natural deep eutectic solvents on the extraction of phenolic compounds from poplar type propolis: Composition and *in vitro* biological activity

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ABSTRACT

Natural deep eutectic solvents (NADESs) have been considered promising to replace traditional volatile and toxic organic solvents for the extraction of biologically active substances from natural sources. This work applied an efficient and ethanol-exclusion strategy for extraction of phenolic compounds from poplar type propolis using five known NADESs (lactic acid:1,2-propanediol 1:1, lactic acid:fructose 5:1, choline chloride:1,2-propanediol 1:3, choline chloride:1,2-propanediol: water 1:1:1 and betaine:malic acid:water 1:1:6). The selected NADESs' extractability was evaluated by measuring the concentrations of total phenolics and total flavones and flavonols in the propolis extracts obtained, which qualitative chemical composition was further determined in detail by gas chromatography-mass spectrometry (GC-MS) analysis. It demonstrated that the chemical profiles of NADES and 70% ethanolic propolis extracts are similar. To expand the knowledge about the role of the applied solvents in the poplar propolis extraction process, the *in vitro* antimicrobial, cytotoxic and genotoxic activity of both NADESs and liquid NADES extracts were evaluated. The results revealed that the use of the selected NADESs as an extraction media for phenolic compounds from poplar propolis not only delivered a good extraction yield in some cases, but generally led to the preservation of propolis extracts' biological activity and even to the enhancement of their antimicrobial effect in comparison with the hydroethanolic one. Besides, the tested NADESs except for lactic acid:fructose and betaine:malic acid:water exerted low to negligible toxicity against normal cells treated and apart from lactic acid:fructose the remaining solvents demonstrated concentration-dependent moderate to subtle genotoxicity. There is a probability that not the supramolecular structure of the NADESs, but their components, played a key role for the observed biological effects. The present study has demonstrated an alternative

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approach for extracting the biologically active complex from poplar type propolis using NADESs, which could be useful for further pharmaceutical and cosmeceutical applications.

1. Introduction

Propolis (bee glue) is a bee product of plant origin. In general, propolis is a mixture of resinous plant secretions that the bees collect from various parts of plants without changing them and beeswax. The bees use propolis to construct and adapt their nests and above all to protect them against disease-causing microorganisms [1]. The bee glue has been known to humankind for its healing properties since ancient times. The modern science has confirmed its diverse biological activities such as antimicrobial, antiviral, antioxidant, anti-inflammatory, immunostimulating, antitumor [2–4] and thus, propolis is widely used in phytoproducts, food additives, healthy foods, biocosmetics, etc. [5].

It is generally accepted that due to the synergistic action of individual components, propolis has a broad-spectrum pharmacological value as a natural mixture [6]. However, its components are hydrophobic specialized plant metabolites with a protective function and minimal water solubility. Therefore, one of the most commonly used forms of propolis, containing all its biologically active compounds, is obtained by extraction with ethanol or with an ethanol/water mixture (70% ethanol), the so-called tincture. The use of alcohol, however, limits the application of bee glue, as the direct consumption or topical application of liquid alcoholic extracts is not always recommended. At the same time, the traditional extraction technique is not economical as it consumes an excessive amount of energy, time and solvent. Considering environmental protection, it is necessary to look for alternative ways to extract this valuable biologically active complex which will help make the extraction process more environmentally friendly and possibly increase the sustainability of a future industrial application [7].

Nowadays, one of the main issues in the field of chemistry is the development of "green technologies", which aims to preserve the environment and reduce the negative impact of the human involvement. One of the key approaches is the discovery and application of ecological "green" solvents to replace the use of harmful organic ones. Not far ago (dating back to the 1990s), as such, the deep eutectic solvents (DESs) were discovered [8,9]. DESs are mixtures of two or more organic compounds (solid or liquid) with a melting point significantly lower than that of each component. They are liquid even at very low temperatures [10]. The individual components in these mixtures have strong hydrogen bond acceptor (HBA) or hydrogen bond donor (HBD) characteristics; they are connected by intermolecular hydrogen bonds and form a liquid with a supramolecular structure [11,12]. The interest in them is constantly growing, as they act effectively to dissolve entirely and extract a wide range of non-polar and polar compounds. Therefore, they have been proposed as an alternative to some traditional but toxic organic solvents [13]. In a specific combination and ratio, natural compounds (primary metabolites) can also turn into liquids and form the so-called natural deep eutectic solvents (NADESs), which are present in nature and play a role during some cellular processes in living organisms [12].

The NADESs have some advantages as solvents: their preparation is easy and cheap, they are biodegradable, and the precursors used for their preparation are natural, non-volatile, non-toxic (unlike ionic liquids) and renewable compounds. NADESs have been found to possess several interesting applications in electrochemistry, functional materials, organic synthesis, catalytic conversions, and pre-treatment processes [14]. Recently, the interest in NADESs has been growing due to their successful application in the extraction, separation and stabilization of biologically active plant metabolites (flavonoids, catechins, phenolic acids, terpenes, saponins). In some cases, this method has been proven more effective than the traditionally used alcoholic extraction method [14–18]. However, in addition to the good extraction yields, the advantages of NADESs over the conventional organic solvents desired by the researchers include the obtaining of a final extract with preserved biological effects, which does not exhibit toxicity when used, as well as the option to utilize the NADESs as a part of the final formulations [19]. Still, insufficient data on toxicology and the biological activity of both NADESs and NADES extracts currently limits their commercial and industrial application.

Different chemical types of propolis have been registered according to their plant source. The poplar type propolis, obtained from *Populus* spp., is the best studied and the most widespread. It is distributed in the temperate zone countries (in Europe, North America, non-tropical regions of Asia and New Zealand), and its major constituents are flavonoid aglycones, and phenolic acids and their esters [20]. Poplar propolis is also one of the most commonly applied types of propolis in food, pharmaceutical and cosmetic formulations existing in the market. The extraction process of this natural raw material is of great interest, as it gives a product of medicinal and economic importance. Our previous investigation on applying some NADESs as alternative solvents for the extraction of poplar propolis has displayed promising results. Among the studied NADESs the best extraction yield was achieved using citric acid:1, 2-propanediol 1:4 [21]. This gave us a reason to continue the research by including other NADESs evaluating their extraction capacity and the antioxidant activity of NADES propolis extracts. The results revealed that NADES extracts of poplar propolis have good antioxidant potential providing natural antioxidants without applying organic solvents [22]. However, to find the NADESs with good extraction profiles for industrial utilization, the studies on their application have to be accompanied by an evaluation of safety and biological properties i.e. cytotoxicity and genotoxicity of both NADESs and extracts, although NADESs are a class of solvents based on substances that are safe for human consumption and are often presumed to be non-toxic [23]. In addition, data on the toxicity profile of NADES propolis extracts are still lacking.

Based on the above discussion and as a continuation of our previous research [21,22], the overall goal of the present study is to investigate the possibility to obtain an ethanol free natural-based poplar propolis extract enriched with bioactives and having a satisfactory toxicity profile with a potential to be incorporated in different formulations basically for topical application without removing the NADES. Simultaneously, the study aimed to expand the knowledge about the role of five selected NADESs in the

extraction of biologically active compounds from poplar propolis and especially to demonstrate their influence on the biological activity of the extracts obtained. Thereby, in this work a method of extracting phytochemicals from poplar propolis using five selected NADESs was applied. The content of total phenolics (tP) and total flavones and flavonols (tFF) in the extracts were measured, and their chemical composition was determined in detail by GC-MS. The antimicrobial, cytotoxic and genotoxic activity of the pure NADESs and their liquid extracts were evaluated. A comparison was also made with extraction using 70% ethanol as a solvent. This is the first report on the cytotoxicity and genotoxicity of NADES propolis extracts and the first in-depth study on applying the selected NADESs for poplar propolis extraction, including both an estimation of the efficiency of the extraction process and an evaluation of the biological activity and the toxicity profile of the pure NADESs used and their propolis extracts. The data obtained from the latter would serve to fill the existing gaps in the knowledge about the environmental fate of the selected NADESs and the respective NADES propolis extracts and could be used to predict their effects on human health and environment.

2. Material and methods

2.1. Chemicals and reagents

Ethanol (absolute) was obtained from Alkaloid (Skopje, Macedonia). 1,2-propanediol (p.a., >99%) was purchased from Valerus (Sofia, Bulgaria); galangin (≥95%), choline chloride (≥98%) and Nile Red from Sigma Aldrich (Switzerland); methanol (HPLC grade, ≥99.8%), D/L-lactic acid (≥88%), D (−) fructose (≥99%) and betaine (98%) from Fisher Chemical (Loughborough, UK); D/L-malic acid (>99%) from Acros Organics (Geel, Belgium); Folin-Ciocalteau reagent, dry pyridine and N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) from Merck (Darmstadt, Germany); aluminium chloride anhydrous from Fluka (Buchs, Switzerland) and pinocembrin (≥95%) from Extrasynthese (Genay, France). The chemicals 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT, #M2128-1G), L-glutamine (#G7513) and Dulbecco's phosphate buffered saline (PBS, #D8537) were purchased from Merck (Sigma-Aldrich, Steinheim, Germany). HCOOH originated from Chimspektar OOD, Bulgaria. The media, sera, enzymes and antibiotics for culturing of the cell lines were delivered from Capricorn®, Germany: MEM (#MEM-A), DMEM (#DMEM-HPA), horse serum (#HOS-1A), fetal bovine serum (#FBS-HI-12A), CM-1 (#800125), Trypsin (#TRY-1B10, # TRY-2B10), Accutase® (#ACC-1B), and Pen/Strep 100 × (#PS-B).

2.2. Propolis sample

The raw propolis (a single batch consisting of 550 g of sample) used in this study was supplied by a beekeeper. The sample was collected by scraping in 2019 from several hives in an apiary located in the area of the town of Montana, Bulgaria. The poplar origin of the sample (the bud exudates of black poplar *Populus nigra* L.) was confirmed by screening the composition using TLC [24]. The sample was stored in a refrigerator at 4 °C until use.

2.3. NADESs preparation

The five NADESs were prepared according to the procedure described by Dai et al. [12]. Briefly, the NADES components were mixed in appropriate amounts in a 100 mL Erlenmeyer flask and subsequently stirred in a water bath (300 rpm) at mild heating of 50 °C until a homogeneous liquid was formed (about 60–90 min). The final volumes of the prepared NADESs were 40–50 mL.

2.4. NADESs physicochemical properties measurements

The NADESs polarity was measured with the solvatochromic dye Nile red [12,25]. The dye was dissolved in each NADES in the concentration range 0.01–0.1 mM, and the absorption spectra were recorded on a Helios Gamma UV–vis spectrophotometer at room temperature. The λ_{max} was determined and used to calculate the molar transition energy E_{NR}, based on the equation: $E_{NR} = hcN_A/\lambda_{max} = 28\ 591/\lambda_{max}$, where E_{NR} is in kcal/mol and λ_{max} in nm.

The viscosity was measured using a Thermo Haake rheometer connected with the temperature control unit Thermo Haake Peltier TC81. A cone and plate configuration was used (d = 35 mm and angle = 2°) at a gap of 0.103 mm. Each sample was sheared at 40 °C in the shear rate range of 0.1–100 s^{-1}. For each NADES, three replicates were performed.

2.5. Extraction

First the raw propolis sample was frozen and immediately after that it was ground using a coffee mill, the average particle size was 0.75 mm. The extraction of the ground material was performed as 50 mg of raw propolis and 1.5 mL solvent (NADES or 70% ethanol) (solid/liquid ratio 1:30 w/v) were placed in a 2 mL Eppendorf tube and afterwards were treated with ultrasound (US) in an ultrasonic bath (Elmasonic S 30 H, Singen, Germany, ultrasonic frequency 37 kHz, tank volume 2.75 L, power consumption 280 W), without heating, for 1 h. The mixture was then centrifuged at 13 000 rpm for 40 min (Sigma 1–14, Osterode am Harz, Germany) and filtered through cotton whereby a liquid extract was obtained. To eliminate the variability from the heat generated from the sonication all extraction procedures (using five NADESs and 70% ethanol) have been performed simultaneously and repeated three times, each time starting with fresh materials and room temperature (20 °C) of the ultrasound bath. The liquid extracts thus obtained were combined, respectively and further analysed to determine their chemical composition, and used for biological activity evaluation.

2.6. Quantitative determination of total phenolics (tP) and total flavones and flavonols (tFF)

The test solution of each liquid extract used in these analyses was prepared as follows: 3 mL from the initial NADES or 70% ethanol extract were diluted to 25 mL with 70% ethanol in a volumetric flask. An aliquot (2 mL) of the same solution was transferred into a volumetric flask and diluted to 25 mL with 70% ethanol. This solution was further analysed to determine the concentration of total phenolics, and total flavones and flavonols using the previously reported validated spectrophotometric methods, described in Ref. [26] with minor modifications.

2.6.1. Total phenolics content

For total phenolics determination the Folin-Ciocalteu method was applied. In brief, 0.5 mL of the test solution was transferred to a 25 mL volumetric flask, containing 7.5 mL distilled water, and 2 mL of Folin–Ciocalteu reagent and 3 mL of 20% sodium carbonate solution in distilled water (w/v) were added. The volume was made up with distilled water to 25 mL. After 2 h, the absorbance was measured at 760 nm. For blank: solution of respective NADES diluted in the same way described above instead of the test sample (diluted NADES propolis extract) was used in analogous procedure. Every assay was carried out in triplicate. Total phenolics content was estimated using a calibration curve of a standard pinocembrin-galangin 2:1 mixture with a concentration range 6–240 μg/mL in methanol. The initial concentration of tP has been calculated considering the dilution factor.

2.6.2. Total flavones and flavonols content

The total flavones and flavonols content was measured by spectrophotometric assay based on aluminium chloride complex formation. In brief, to 10 mL methanol in 25 mL volumetric flask, 1 mL of the test solution, and 0.5 mL 5% $AlCl_3$ (w/v) in methanol were added and the volume was made up to 25 mL with methanol. After 30 min the absorbance was measured at 425 nm. For blank: solution of respective NADES diluted in the same way described above instead of the test sample (diluted NADES propolis extract) was used in analogous procedure. Every assay was carried out in triplicate. The tFF content was estimated using a calibration curve of galangin in a concentration range of 4–44 μg/mL in methanol. The initial concentration of tFF has been calculated considering the dilution factor.

2.7. Chemical characterization of propolis extracts by GC-MS

2.7.1. Recovery of compounds from NADES extracts

Prior to GC-MS analysis the NADES propolis extracts were subjected to solid-phase extraction as 1 mL of each NADES propolis extract obtained as described in section 2.5 was mixed with 1 mL of distilled water and then passed through an extraction tube LiChrolut® RP-18 (40–63 μm, 2000 mg, 6 mL) pre-saturated with distilled water. The tube was washed with 50 mL of distilled water and then eluted with 20 mL ethanol. The ethanolic fraction was collected, evaporated to dryness and analysed by GC-MS. The experiments were performed in duplicate.

2.7.2. GC-MS analysis of propolis extracts

For determination of each recovered extract's composition (together with 70% ethanolic extract just evaporated to dryness), a small part of it was subjected to GC-MS analysis after silylation (5 mg dry extract was mixed with 50 μL of dry pyridine and 75 μL of N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and heated at 80 °C for 20 min). The GC-MS analysis was performed with a gas chromatography instrument coupled to a tandem mass spectrometer GC-MS-TQ 8050 NX Shimadzu (Tokyo, Japan), operating in Q3 scan mode and equipped with a 30 m long, 250 μm i. d., and 0.25 μm film thickness SAPIENS-5MS capillary column (Teknokroma, Spain). The oven temperature was programmed from 75 to 300 °C at a rate of 5 °C/min, with 5 min hold at 75 °C and 25 min hold at 300 °C. Helium was used as a carrier gas at a constant flow rate of 0.8 mL/min. The pressure was 45.9 kPa, the split ratio was 75:1 with injection volume 1 μL, the injector temperature was 280 °C, the interface temperature was 310 °C, and the ionization voltage was 70 eV. The compounds were identified using comparison with spectra of authentic samples, computer searches on commercial libraries (>95% match), and literature data [27]. The percentage values from the GC-MS analysis refer to the percent of the Total Ion Current (TIC) and are semi-quantitative. Semi-quantification was carried out by internal normalization with the area of each compound. The addition of individual areas of the compounds corresponds to 100% area.

2.8. Antimicrobial activity

2.8.1. Test microorganisms

For the antimicrobial susceptibility testing, the following microorganisms were used: *Staphylococcus aureus* ATCC 29213 (American Type Cell Culture Collection, USA), *Bacillus cereus* ATCC 9634, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* SAIMC C12 (The Stephan Angeloff Institute of Microbiology Collection, Bulgaria), *Streptococcus pyogenes* SAIMC 10535 and *Candida albicans* SAIM 562.

2.8.2. Culture medium and growth conditions

Muller Hinton agar (MHA) and broth (MHB) (CM0337B, resp. CM0405B, Thermo Scientific-Oxoid, UK) were applied for *S. aureus*, *B. cereus* and *E. coli*. Brain Heart Infusion (BHI) and agar (BHIA) (GM210, resp. M211, HiMedia, India) were used as a culture medium for the remaining bacteria and *C. albicans*. All microorganisms were grown at 37 °C overnight, except *B. cereus*, grown at 30 °C.

2.8.3. Minimal inhibitory (MIC) and bactericidal/fungicidal (MBC/MFC) concentrations

The antimicrobial activity was estimated by the broth microdilution method according to ISO 20776–1:2006 [28,29]. Briefly, 50 μL microorganism inoculums with a concentration of 10^5 CFU/mL (in MHB or BHI) were added to 96-well plates containing 50 μl of two-fold serially diluted solutions of pure NADESs or NADES propolis extracts. Plates were incubated at 37 °C for 18 h. Experiments were performed in triplicate. MICs were determined visually as the lowest concentration without visible growth. Gentamicin was used as a reference antibiotic for *B. cereus*, *S. aureus, E. coli* and *P. aeruginosa*, penicillin – for *S. pyogenes*, benzylpenicillin – for *L. monocytogenes*, amphotericin B – for *C. albicans* following the requirements of EUCAST [30]. A hundred μL from the untreated control and samples treated with ½ × MIC, MIC and 2 × MIC were plated on MHA/BHI agar and incubated overnight for determination of MBCs/MFCs. MBCs/MFCs were read as concentrations where no microbial growth occurred on the agar plates. All microbiological studies were performed under sterile conditions in a Class II laminar box (FASTER BH-EN 2003, Ferarra, Italy).

2.8.4. Dehydrogenase (DEHA) activity

The DEHA activity of the test microorganisms was performed by an MTT test, as described before [29]. The method is based on the reduction of the MTT dye by the membrane-located bacterial enzyme NADH: ubiquinone reductase (H + -translocation) to insoluble formazan crystals. Briefly, the treated and untreated bacterial/fungal cells were incubated for 2 h with MTT dye (final concentration 0.05 mg/mL). Then, the formed formazan crystals were dissolved with 5% HCOOH in isopropanol. Absorption was measured on an ELISA reader (BioTek Elx800, Instruments, Inc., Winooski, VT, USA) at 550 nm (reference 690 nm) against a blank solution.

2.9. In vitro cytotoxic activity

2.9.1. Cell lines and culture conditions

The following four cell lines (two non-tumorigenic and two tumorigenic) were used as *in vitro* cell models in this study: CCL-1™ (transformed mouse fibroblasts, American Type Culture Collection ATCC, Manassas, Virginia, USA, NCTC clone 929), HaCaT (immortalized human keratinocytes), A-431 (non-melanoma epidermoid squamous skin carcinoma) and A-375 (malignant melanoma). HaCaT, A-431 and A-375 were obtained from CLS Cell Lines Service (GmbH, Eppelheim, Germany). Cells were maintained in sterile cell culture flasks at 37 °C and a humidified atmosphere containing 5% CO_2 in a CO_2 incubator (Panasonic MCO-18AC, Japan). CCL-1 was cultured in MEM enriched with 10% heat-inactivated horse serum and 2 mM L-glutamine. The other three cell lines were cultured in DMEM-HG enriched with 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum and 4 mM L-glutamine. In addition, Pen/Strep (Units/L penicillin G sodium and 100 mg/L streptomycin sulphate) was added to all cell lines to avoid contamination during experiments. All experiments were performed in the log phase of the cell growth. The cells were sub-cultivated at a seeding density of 1.0×10^4 cells/cm^2 1–3 times per week after the monolayer reached 80–90% confluence according to the protocols of the biobanks. Trypsin/EDTA 0.1%/0.05% was used for the detachment of CCL-1 and HaCaT and Accutase® - for A-431 and A-375 lines. All cells were frozen in CM-1 medium for long-term storage at −80 °C and −150 °C.

2.9.2. Cell viability assay

The cell viability test followed [31] and Annex C of ISO 10993–5 [32]. The results were used to determine the solvents and extracts' median inhibitory (IC_{50}) concentrations. Briefly, cell suspension with a density of 1×10^5 cells/mL for the non-tumorigenic and 0.7 x 10^5 cells/mL for the tumorigenic cell lines was distributed in 96-well flat-bottom plates in the volume of 100 μL per well. The cells were first incubated for 24 h until entering the log phase of their growth. After that, they were treated with the solvents and extracts in concentrations ranging from 0.026 to 2% volume fraction. The result was measured after 24 and 72 h of exposure by using the ability of the living cells to reduce the yellow MTT dye to insoluble violet crystals formazan. The latter were dissolved with 2-propanol supplemented with 5% formic acid. The same solvent served as blank, whereas untreated cells were considered as negative control and normalized for 100%. The absorption was measured at 540 nm (reference filter 690) on a microplate reader ELx800 (BioTek Instruments, Inc., USA). The IC_{50} values (mean inhibitory concentration 50, which reduces vital cells by half) were calculated using the GraphPad Prizm software (Version 6.00, for Windows, San Diego, CA, USA) with a non-linear regression analysis (inhibition dose–response model, variable slope). Statistical analysis for each extract and incubation period was performed using One-way ANOVA (GraphPad Prizm software). Difference between the samples with p-value <0.05 was considered statistically significant.

2.10. In vitro genotoxicity evaluation

A test for the presence of genotoxic activity was performed using Comet assay under neutral conditions. The protocol was executed as described in Ref. [33]. In brief, CCL-1 cells were treated with relevant volume of the selected NADESs and their propolis extracts diluted in sterile distilled water to achieve the final concentration of IC_{50}, $1/2IC_{50}$, and $1/4IC_{50}$, determined in the MTT test analyses, for 24 h, together with the untreated control. Subsequently, the cells were mixed with 1.4% low melting agarose to form microgels onto microscopic slides, pre-coated with 0.5% standard agarose. The slides were then lysed with a lysis buffer (146 mM NaCl, 30 mM EDTA, pH 8; 10 mM Tris–HCl, pH 8; 0.1% *N*-lauroyl sarcosine) for 20 min. This was followed by washing of the slides in 0.5 × TBE buffer, a 20 min electrophoresis of 0.45 V/cm in the same buffer, and subsequent ethanol dehydration. The probes were stained with SYBR Green (Roche Diagnostics GmbH) and visualized using an epifluorescent microscope Leitz—Orthoplan, Vario Orthomat 2 (450/490 nm). CometScore software was used for data analysis by measuring Olive Moment. Results are represented as mean ± SD from triplicates.

2.11. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's post hoc test at significance levels of $p < 0.01$ and $p < 0.05$ were performed with Excel (ChemOffice 2019).

3. Results and discussion

3.1. Extractability of natural deep eutectic solvents (NADESs) for bioactive compounds in poplar propolis

In this work five known hydrophilic NADESs were selected to test their extraction efficiency for isolation of biologically active metabolites from poplar propolis. These NADESs were chosen since according to bibliographic research their polarity was close to that of 70% ethanol and they were successfully applied in the extraction of phenolic compounds and flavonoids [12,34,35]. There are also hydrophobic NADESs (HDESs) [36], but in order to avoid the extraction of wax from the raw propolis we focused on hydrophilic ones. Then the selected NADESs were prepared and additionally physicochemically characterized (Table 1). The results from viscosity measurements showed that all examined NADESs were Newtonian fluids.

To evaluate the extractability of the prepared NADESs they were applied as extraction solvents for the isolation of bioactive compounds from Bulgarian poplar propolis. In parallel, under the same conditions, extraction with 70% ethanol was carried out as a reference solvent. Due to the significant viscosity of the NADESs, ultrasound-assisted extraction was applied to accelerate the process. The NADESs' extraction capacity was evaluated by measuring the tP and tFF concentrations in the extracts. The results obtained are presented in Table 2.

While there were statistically significant differences between the concentrations of tFF and tP extracted with the different solvents, respectively, the ratio between these two groups of compounds (tFF/tP) remained relatively constant: it was close to 0.3 for all solvents used including 70% ethanol.

Further, GC-MS analysis was performed to investigate the detailed qualitative chemical composition of the extracts. Besides, it was essential to find out if there was any selectivity in the extraction with different NADESs. Prior to GC-MS analysis, solid phase extraction was performed to remove the NADESs, and the recovered components were then derivatized. The chemical composition of NADES and 70% EtOH propolis extracts as per GC-MS analysis is presented in Table 3. In addition, the chemical profiles of the extracts by classes of compounds are represented in Fig. 1.

The GC fingerprints of the NADES extracts showed all the peaks present in the ethanol extract and no significant qualitative differences were observed in their composition. According to the GC-MS data the chemical profiles of NADES and 70% ethanol propolis extracts were similar (Fig. 1 and Table 3). The percentage of total ion current (% of TIC) for cinnamic acids in all NADES extracts, except BMAW/pro, was lower than in the ethanolic extract. BMAW/pro also stands out with the highest relative content of chalcones, flavanones and dihydroflavonols. A tendency of the relative flavone and flavonol content in relation to the polarity of the solvent used was observed: with an increase in the polarity of the extractant (a decrease in E_{NR}), the relative content of flavones and flavonols decreases (Table 1 and Fig. 1).

Regarding the individual compounds, the GC-MS analysis revealed that the main biologically active components of the poplar type propolis - caffeic acid phenethyl ester (CAPE), pinocembrin, pinobanksin-3-acetate, chrysin and galangin (Fig. 2), all of them chemical markers of this type of propolis, are also major constituents of the NADES extracts. As it is known that certain flavanone and flavanol aglycones can cause chalcone formation during the silylation procedure [37], in the relative amounts of pinocembrin and pinobanksin-3-acetate the amounts of the respective chalcones must be taken into consideration.

According to the results in Table 2, the lowest extractability was achieved with BMAW, followed by LAFr, while CCPDW, LAPD and CCPD, possess extraction capacity similar to that of 70% ethanol. Moreover, the yield of the extracted phenolics in CCPDW/pro and LAPD/pro slightly exceeded that of the classical extraction. The GC-MS analysis data showed no substantial difference in the qualitative chemical composition of the propolis extracts obtained with NADESs and 70% ethanol. However, the LAFr and BMAW solvents do not extract as comprehensively as 70% ethanol (Table 2) and a significant difference at $p < 0.01$ was found between them as well as

Table 1
List of NADESs explored in this study.

NADES Abbreviation	Components			Molar ratio	E_{NR}[a], kcal/mol	η[b], Pa.s, at 40°C	Appearance at room temperature
	1	2	3				
LAPD	D/L-Lactic acid	1,2-Propanediol	–	1:1	49.59	0.021	Transparent liquid
LAFr	D/L-Lactic acid	D-Fructose	–	5:1	47.97	0.497	Transparent liquid
CCPD	Choline chloride	1,2-Propanediol	–	1:3	50.69	0.019	Transparent liquid
CCPDW	Choline chloride	1,2-Propanediol	Water	1:1:1	50.29	0.019	Transparent liquid
BMAW	Betaine	D/L-Malic acid	Water	1:1:6	48.87	6.278	Transparent viscous liquid

[a] Molar transition energy, an indicator of the polarity of the corresponding NADES, defined with Nile Red (NR) as a solvatochromic dye; E_{NR} (70% ethanol) = 50.87 kcal/mol.
[b] Dynamic viscosity.

Table 2
Concentration of extracted propolis components in the extracts, expressed as mean ± SD (n = 3) (solid/liquid ratio of 1:30, without heating, US 1 h).

Extract	tP, mg/mL	tFF, mg/mL	tFF/tP
LAPD/pro	12.8 ± 0.2[a]	3.7 ± 0.0[a]	0.29
LAFr/pro	10.3 ± 0.2[b]	2.9 ± 0.1[b]	0.28
CCPD/pro	12.3 ± 0.2[a]	3.5 ± 0.0[ad]	0.28
CCPDW/pro	13.3 ± 0.2[a]	3.8 ± 0.1[ae]	0.29
BMAW/pro	6.7 ± 0.7[c]	1.8 ± 0.1[c]	0.27
70% EtOH/pro[a]	12.4 ± 0.4[a]	3.8 ± 0.1[ae]	0.31

Values in the same column with different superscripts are significantly different: a-c at $p < 0.01$ and d-e at $p < 0.05$ by analysis of variance followed by Tukey's test.
[a] 70% ethanol extraction was applied under the same conditions as a reference.

with the other four solvents. A possible explanation for the different extraction yields of the defined groups of compounds in the different NADES extracts is that the spatial supramolecular structure of LAFr and BMAW is such that it probably hinders to a different extent the interaction of their components with the phenolic compounds in the propolis matrix. The effect of solvent viscosity should not be neglected either. Thus, LAPD, CCPD, and CCPDW solvents are the most promising for extracting bioactive compounds from poplar propolis. Furthermore, propanediol as a HBD in NADES and the lower viscosity of NADES are favorable in the propolis extraction. All these findings demonstrated that some NADESs could potentially replace conventionally used ethanol as an extraction media for poplar type propolis. The present outcomes are in a good accordance with our previous results [21,22] and those reported by some authors [38–41]. Funari et al. (2019) found that choline chloride:propylene glycol 1:2 and choline chloride:lactic acid:water 1:1:1 extracts appeared as promising candidates for replacing hydroethanolic extract of green propolis [38], and a little later Contieri et al. found that betaine:citric acid 1:2 with 50 wt % of water (v/v) was the most efficient in selectively extracting green propolis polyphenols, with a maximum extraction yield of 100 mg polyphenol/g green propolis at the optimum conditions [41]. Koutsoukos et al. (2019) investigated and established that choline chloride:tartaric acid 2:1 could be used as a greener alternative solvent for the extraction of Greek brown propolis [39], and Tzani et al. succeed to incorporate optimum as-obtained NADES extract from Greek propolis in cream using L-proline:D,L-lactic acid:water as the most effective medium for the raw propolis extraction [40].

3.2. *In vitro biological activity*

In the literature, there are already numerous studies showing the good extractability of the NADESs for various constituents of natural origin [42]. But still, the data on their biological properties and how they affect those of the obtained extracts are insufficient [43]. This limits the incorporation of NADES extracts in therapeutic and cosmetic products because it raises the question of their efficacy, safety, and health impact. Therefore, we aimed to study the antimicrobial, cytotoxic and genotoxic activity of both NADESs and their propolis extracts, as the NADESs were used as a part of the extracts tested, bypassing the problem of solute recovery, and to compare the results with the activity of 70% ethanolic extract. The literature lacks data on the cytotoxicity and genotoxicity of NADES propolis extracts.

3.2.1. *In vitro antimicrobial activity*

NADESs, their extracts and 70% ethanolic extract of poplar propolis were tested *in vitro* for antimicrobial activity, a major driving bioactivity in propolis usage. The pure NADESs and extracts' MIC as % v/v and extracts' MIC and MBC/MFC as μg tP/mL were measured. In addition, the metabolic activity of the microorganisms after the treatment with NADES extracts was studied by measuring the dehydrogenase activity (DEHA) with the MTT test. The results are shown in Tables 4 and 5. The notation n. a.-not active indicates no difference between the effect of the liquid extract and the pure solvent.

None of the extracts, including hydroethanolic one, showed activity against *E. coli* and *P. aeruginosa* in the tested concentration range, which is not unusual, since it is well known that propolis extracts are more effective against Gram-positive bacteria in comparison to Gram-negative ones [44]. For the other tested microorganisms, except for *B. cereus*, the MIC and MBC/MFC values for the NADES extracts were lower than that of the conventional ethanolic extract (Tables 4 and 5). The most potent antimicrobial activity was observed for BMAW/pro against *S. aureus* and *S. pyogenes* with MICs = MBCs = 13.1 μg tP/mL. Therefore, the metabolic activity (DEHA) of cells is zero. The LAFr/pro extract was the most prospective, with MIC = MBC = 10.04 μg tP/mL against *S. aureus* and MICs = MBCs slightly above 20 μg tP/mL against *L. monocytogenes* and *C. albicans*. LAPD/pro was active against *S. aureus*, *L. monocytogenes* and *S. pyogenes* but did not have an antifungal effect. At the same time, the MICs (% v/v) of BMAW/pro, LAFr/pro and LAPD/pro against the mentioned microorganisms are slightly lower than those of the corresponding pure solvents (Table 4). Conversely, CCPD/pro and CCPDW/pro possessed effects against *L. monocytogenes*, *S. pyogenes* and *C. albicans* with MICs varying from 96.41 to 385.62 μg tP/mL, but the extracts' MICs (% v/v) were significantly lower than those of the corresponding solvents.

Practically all studied NADESs showed antimicrobial properties against all tested pathogens, with some of the solvents (LAFr and BMAW) having significant effects (Table 4). Alongside in all cases when NADES propolis extracts displayed antimicrobial activity, it was higher than the hydroethanolic one and such improving of antimicrobial effect was already observed [33,45]. Most probably, this is due to the antimicrobial activity of the NADESs themselves, and the composition of the solvent used for extraction plays a role in the activity of the liquid extract. In support of this, the conducted correlation analysis between tP and tFF content in the extracts and the

Table 3
Chemical composition of propolis extracts by means of GC-MS (percent of Total Ion Current, TMS derivatives)[a].

RT, min	Compound	LAPD/pro	LAFr/pro	CCPD/pro	CCPDW/pro	BMAW/pro	70% EtOH/pro
28.9	*p*-Methoxycinnamic acid	0.1	–	0.1	0.1	0.1	0.1
31.2	*p*-Coumaric acid	0.1	tr	0.1	0.1	0.8	0.7
32.9	Dimethoxycinnamic acid	0.4	0.3	0.3	0.4	1.2	0.7
33.1	Palmitic acid	0.3	0.2	0.1	0.1	0.1	0.2
33.8	Ferulic acid	0.1	tr	0.1	0.1	0.9	0.6
34.1	Isoferulic acid	0.1	0.1	0.1	0.1	0.6	0.5
34.9	Caffeic acid	0.3	0.7	0.2	0.2	1.5	1.7
35.2	Isopentenyl *p*-coumarate	tr	–	tr	0.1	tr	0.1
36.1	Oleic acid	0.6	–	0.4	0.1	–	0.4
36.6	Stearic acid	0.1	–	0.1	tr	–	0.1
37.9	3-methyl-3-butenyl ferulate	0.5	0.2	0.3	0.3	0.1	0.3
38.7	3-methyl-3-butenyl caffeate	1.2	1.1	1.3	1.6	1.7	1.2
38.8	Dimethylallyl ferulate	0.9	0.5	0.8	0.8	0.4	0.8
39.4	2-methyl-2-butenyl caffeate	0.6	0.5	0.7	0.8	0.8	0.6
39.5	Dimethylallyl caffeate	1.5	1.4	1.8	2.2	2.4	1.6
40.6	Isosakuranetin chalcone	1.0	0.3	0.7	0.4	0.2	0.7
40.9	Pinostrobin chalcone	tr	0.1	0.8	0.9	–	0.3
41.2	Pinostrobin	1.3	0.8	0.6	0.5	0.7	0.7
41.3	Pinocembrin chalcone	0.4	0.7	8.8	12.8	0.6	2.4
41.6	Pinocembrin	10.2	7.6	3.8	2.1	15.9	7.4
41.9	Pinobanksin chalcone	–	–	1.5	2.8	–	0.1
42.1	Pinobanksin methyl ether	0.3	0.2	0.5	0.5	0.2	0.3
42.4	Pinobanksin	2.3	1.6	3.2	3.2	8.4	5.4
43.0	Pinobanksin-3-acetate chalcone	–	tr	1.9	1.4	–	0.6
43.4	Pinobanksin-3-methyl ether	1.0	2.1	1.6	2.0	1.9	2.1
43.6	Benzyl ferulate	1.0	0.6	0.7	0.8	0.7	0.7
43.7	Pinobanksin-3-acetate	8.6	8.8	5.9	2.8	11.8	7.0
44.2	Benzyl caffeate	4.2	3.8	4.8	5.7	5.5	3.9
44.6	Chrysin	12.6	6.9	13.3	11.2	6.9	9.8
44.8	Galangin	8.6	5.7	7.5	6.7	9.4	6.6
45.0	Pinobanksin-3-isobutanoate	1.0	0.7	1.3	1.0	0.4	0.7
45.3	CAPE	2.6	1.9	3.4	3.7	3.7	2.6
45.5	Galangin methyl ether	0.5	0.4	0.6	0.6	0.5	0.6
45.6	Lignoceric acid	0.2	0.3	0.1	0.3	–	0.5
45.8	Pinobanksin-3-butanoate	0.8	0.4	0.5	0.2	0.3	0.6
46.3	Pinobanksin-3-pentanoate	0.8	1.7	1.6	0.5	1.2	1.8
46.4	Naringenin	–	–	–	–	0.1	0.2
47.3	Pinobanksin-3-pentenoate	0.5	0.3	0.3	tr	0.3	0.3
47.7	Cinnamyl ferulate	0.4	0.3	0.4	0.4	–	0.4
48.3	Pinobanksin-3-hexanoate	0.2	0.2	0.1	–	–	0.2
48.5	Cinnamyl caffeate	1.0	0.7	1.0	1.2	0.7	0.9
48.9	Sakuranetin chalcone	–	–	–	0.2	–	0.2
49.2	Kaempferol methyl ether	0.1	0.2	0.3	0.5	0.5	0.3
49.2	Kaempferol	0.6	0.2	0.3	0.5	0.7	0.8
49.6	Kaempferol methyl ether (isomer)	0.2	0.1	0.2	0.2	0.3	0.4
49.9	Apigenin	0.4	0.2	0.4	0.6	0.5	0.9
50.4	Quercetine dimethyl ether	0.2	0.1	0.2	0.4	0.2	0.3
50.5	Quercetine methyl ether	0.2	0.1	0.2	0.4	0.4	0.4
50.7	Quercetine	0.3	0.1	0.3	0.7	0.9	2.5
50.8	Quercetine methyl ether (isomer)	1.2	0.8	1.3	1.5	2.2	1.7
51.1	Quercetine dimethyl ether (isomer)	0.3	0.1	0.4	0.4	0.2	0.5
51.2	Quercetine dimethyl ether (isomer)	0.1	0.1	0.7	0.6	0.2	0.7
51.5	Quercetine methyl ether (isomer)	0.1	–	tr	0.5	0.5	1.5
51.7	Quercetine dimethyl ether (isomer)	0.2	0.1	0.5	0.5	0.4	1.0
	Unidentified	29.8	46.8	23.9	24.3	13.0	22.4

tr.- traces.
[a] Two replicates, relative SD < 10%.

antimicrobial activity, respectively, showed no statistically significant Pearson correlations ($p > 0.05$) between the concentration of the groups of compounds and the MICs. The individual NADES constituents could also contribute to the propolis solution's overall effect, as adding more than 50% of water to NADES during the antimicrobial tests could break the NADES supramolecular complex [46]. The latter could be considered, especially in the case of the most active extracts LAFr/pro and BMAW/pro, which contain the least amount of phenolics, flavones and flavonols (Tables 2, 4 and 5). Their enhanced antimicrobial effect compared to the other extracts can be partly explained by the effect of the presence of lactic and malic acids, respectively, as elements of the NADESs. The literature indicates that NADESs containing organic acids possess higher antimicrobial activity since some organic acids present many pharmacological effects [47]. Some authors suggested that the main cause for the inhibition of microbial growth by pure NADESs was

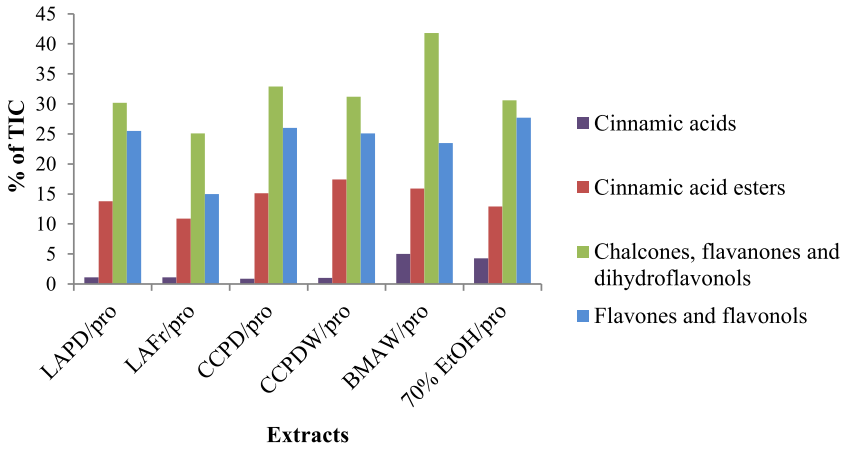


Fig. 1. Chemical profiles of the propolis extracts by classes of compounds according to the GC-MS analysis after silylation. By the ordinate percent of Total Ion Current (% of TIC) is displayed.

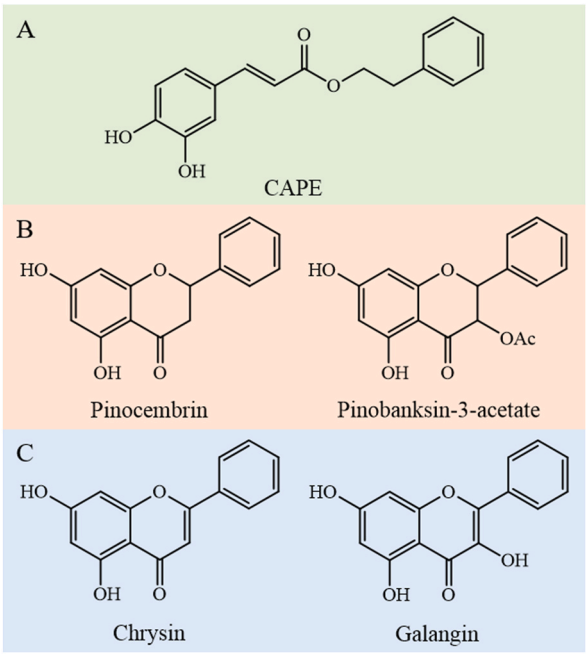


Fig. 2. Structures of the main biologically active components of 70% ethanolic and NADES extracts of poplar propolis divided by classes of compounds: A) cinnamic acid esters, B) flavanones and dihydroflavonols and C) flavones and flavonols.

the low pH due to acid components: when the pH of NADES was readjusted to the optimal bacteria growth range, no inhibitory effects of NADESs on bacteria were observed [48]. More investigations on this matter are required.

Since the phenolic components of poplar propolis also possess antimicrobial activity, expectedly the MICs (% v/v) of some NADES propolis extracts were lower than the MICs (% v/v) of corresponding pure solvents (Table 4). The most significant differences were in CCPD/pro and CCPDW/pro against *S. pyogenes* and *L. monocytogenes*, and in CCPDW/pro against *C. albicans*. Most probably the extracted phenolic constituents in the CCPD/pro and CCPDW/pro liquid extracts are of foremost importance to their antimicrobial abilities against these pathogens.

3.2.2. In vitro cytotoxic activity

Since most of the starting components used in preparing NADESs are natural compounds, the resulting eutectic mixtures are often considered non-toxic. However, this assumption should not be made without thoroughly investigating their toxicity [23]. In addition, to incorporate NADES extracts in finished products, the study of their toxicity profile is also required to assess safety and health and environmental impact. The cell lines serve as a good biological system for toxicity testing of novel chemicals and estimating their

Table 4
Minimal inhibitory concentrations of pure NADESs and their propolis extracts (MICs are expressed in % v/v and in μg of tP in the extracts per mL).

Sample	*S. aureus*		*B. cereus*		*E. coli*		*P. aeruginosa*		*L. monocytogenes*		*S. pyogenes*		*C. albicans*	
	% v/v	μg tP/mL	% v/v	μg tP/mL	% v/v	μg tP/mL	% v/v	μg tP/mL	% v/v	μg tP/mL	% v/v	μg tP/mL	% v/v	μg tP/mL
LAPD	1.563		0.391		0.781		0.391		0.781		1.563		0.781	
LAPD/pro	0.391	49.84	0.391	n.a.	0.781	n.a.	0.391	n.a.	0.195	24.92	0.781	99.69	0.781	n.a.
LAFr	0.195		0.195		0.195		0.195		0.391		0.195		0.391	
LAFr/pro	0.098	10.04	0.195	n.a.	0.195	n.a.	0.195	n.a.	0.195	20.08	0.195	n.a.	0.195	20.08
CCPD	3.125		0.781		25		1.563		6.25		12.5		6.25	
CCPD/pro	3.125	n.a.	0.781	n.a.	25	n.a.	1.563	n.a.	0.781	96.41	0.781	96.41	3.125	385.62
CCPDW	6.25		0.781		3.125		3.125		6.25		12.5		6.25	
CCPDW/pro	6.25	n.a.	0.781	n.a.	3.125	n.a.	3.125	n.a.	0.781	103.83	0.781	103.83	0.781	103.83
BMAW	0.391		0.195		0.391		0.391		0.391		0.391		0.391	
BMAW/pro	0.195	13.10	0.195	n.a.	0.391	n.a.	0.391	n.a.	0.391	n.a.	0.195	13.1	0.391	n.a.
70% EtOH/pro		443.88		443.88		>887.75		>887.75		887.75		443.88		887.75
Antibiotic	Gentamicin								Benzylpenicillin		Penicillin		Amphotericin B	
MIC, [μg/mL]	0.125								0.5		0.063		0.125	

n.a. – not active.
LAPD, LAFr, CCPD, CCPDW and BMAW are the pure solvents.
LAPD/pro, LAFr/pro, CCPD/pro, CCPDW/pro and BMAW/pro are the respective propolis extracts.

Table 5
MBC/MFC of total phenolics in different solvent extracts of poplar propolis and DEHA (MBC/MFC are expressed in μg tP/mL in the extracts; DEHA data are expressed in % as the mean ± SD of three measurements).

Parameters	LAPD/pro	LAFr/pro	CCPD/pro	CCPDW/pro	BMAW/pro	70% EtOH/pro
Staphylococcus aureus ATCC 29213						
MBC	49.84	10.04	n.a.	n.a.	13.10	443.88
DEHA	–	–			–	–
Bacillus cereus ATCC 9634						
MBC	n.a.	n.a.	n.a.	n.a.	n.a.	443.88
DEHA						–
Escherichia coli ATCC 35218						
MBC	n.a.	n.a.	n.a.	n.a.	n.a.	>887.75
DEHA						–
Pseudomonas aeruginosa ATCC 27853						
MBC	n.a.	n.a.	n.a.	n.a.	n.a.	>887.75
DEHA						–
Listeria monocytogenes SAIMC C12						
MBC	49.84	20.07	192.81	207.66	n.a.	>887.75
DEHA	0.41 ± 0.02	–	7 ± 2	6 ± 1		7.50 ± 0.01
Streptococcus pyogenes SAIMC 10535						
MBC	99.69	n.a.	96.41	103.83	13.1	>887.75
DEHA	–		–	–	–	7.32 ± 0.02
Candida albicans SAIMC 562						
MFC	n.a.	20.08	385.62	103.83	n.a.	887.75
DEHA		–	–	–		–

n.a. – not active.

potential biological activity. Given this, the cytotoxicity of pure NADESs and their extracts, as well as 70% ethanol propolis extract towards two non-tumorigenic cell lines CCL-1 and HaCaT and two tumorigenic cell lines A-431 and A-375 was studied by MTT test for two exposure times—24 and 72 h. The results are shown in Fig. 3 and Appendix A (Tables A.1-A.8).

As expected, with some exceptions, the IC_{50} values of studied samples after 72 h of exposure were (to a different extent) lower than those at 24 h – time-dependent cytotoxicity. The results showed that the pure NADESs affected the viability of the four treated cell lines. The most cytotoxic NADES was LAFr, but BMAW and LAPD also strongly affected the cell lines used. In contrast, CCPD and CCPDW exhibited extremely low cytotoxicity compared to the others. The IC_{50} values of these solvents on all cell lines at 72 h were in the range of 0.52–2.02% v/v (or μL in 100 μL), while the other solvents had IC_{50} of 0.026–0.19 % v/v (Fig. 3 and Tables A.2, A.4, A.6 and A.8). Allowing being comparable with literature data, the mean values of IC_{50} of the studied NADESs on the cells at 72 h were recalculated to g/L and mM and are presented in Table 6.

Based on the data in Table 6, generally, for all treated cell lines, LAFr can be classified to possess moderate cytotoxicity (0.1 mM < IC_{50} < 5 mM), while LAPD, CCPD and CCPDW possess low to negligible cytotoxicity (IC_{50} > 5 mM) [49]. In turn, BMAW can be classified as possessing moderate toxicity to HaCaT and A-375 cells and weak but close to moderate toxicity against CCL-1 and A-431 cells. The data obtained indicated that the inclusion of organic acid in the composition of the NADESs seems to increase their overall cytotoxic effect, which is consistent with other reports on the cytotoxicity of NADESs [13,49,50].

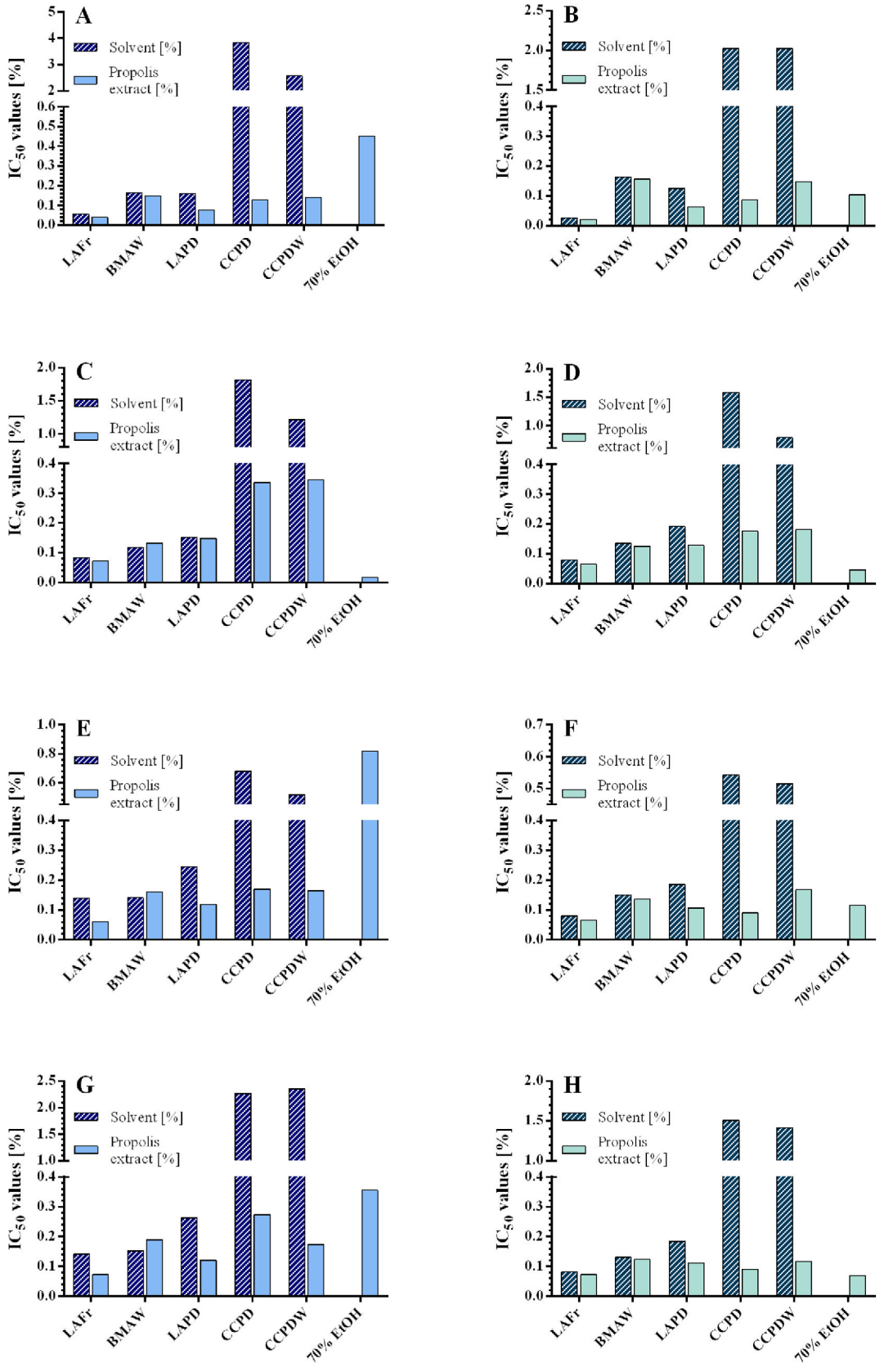
Although the cytotoxic effect of the NADES propolis extracts varied for the cell lines treated, in general after 72 h, it was comparable to that of the ethanolic extract. With some exceptions, the NADES extracts were more cytotoxic than the corresponding pure solvents. The difference was significant in the CCPD/pro and CCPDW/pro, in other cases, ignorable. This is expected, since the phenolic components of propolis are more or less cytotoxic. But no statistically significant Pearson correlation was observed between the IC_{50} value and the phenolic concentration of the extracts ($p > 0.05$). Probably the intrinsic cytotoxicity of NADESs contributed to the higher cytotoxicity of some extracts, regardless of their lower concentration of phenolic compounds. The results pointed again to the organic acids' role as a major cytotoxicity enhancer [13,50]. Zhao et al. (2015) [48] observed that the NADESs containing organic acids have a low pH (<6.5), when the optimal growth range for mammalian cells is 7.0–7.4. This change in the cells' environmental conditions is partially responsible for the higher toxicity of LAFr, BMAW and LAPD compared to the others. Conversely, the cytotoxicity of the CCPD/pro and CCPDW/pro extracts, similarly to the 70% EtOH/pro, is mainly due to their phenolics, because ethanol alone like CCPD and CCPDW solvents is practically harmless to the treated cells with IC_{50} >2% v/v [51].

For most of the samples, there was no particular selectivity towards the tumour cell lines. However, CCPD and CCPDW are exceptions again but only in terms of the A-431 tumour cells. While these two NADESs had relatively high IC_{50} values after 72 h for CCL-1 (2.02% v/v) and HaCaT (1.59 and 0.79% v/v) cells, these parameters for A-431 cells were only 0.54 and 0.52% v/v, respectively (Fig. 3 and Tables A2, A4 and A6).

The results highlighted the CCPD and CCPDW as NADESs with exceptionally low toxicity for normal cells and intrinsic selective cytotoxic effects for non-melanoma skin cancer, capable of extracting phenolics from poplar type propolis in high yield.

3.2.3. In vitro genotoxicity evaluation

The five used NADESs - LAPD, LAFr, CCPD, CCPDW and BMAW, as well as 70% ethanol (Fig. 4), and the propolis extracts (Fig. 5) were tested for the presence of genotoxicity utilizing Comet Assay [52]. In addition, CCL-1 cells (normal mouse fibroblasts) were used,



(caption on next page)

Fig. 3. Median inhibitory concentrations of the NADES solvents and extracts, as well as the ethanol extract, expressed as percentages on a panel of four cell lines – comparison between the selected cell lines, solvents, and extracts after incubation of 24 and 72 h. (**A**) and (**B**) CCL-1 cells (transformed mouse fibroblasts) after 24 and 72 h of exposure, respectively; (**C**) and (**D**) HaCaT cells (immortalized human keratinocytes) after 24 and 72 h of exposure, respectively; (**E**) and (**F**) A-431 cells (non-melanoma epidermoid squamous skin carcinoma) after 24 and 72 h of exposure, respectively; (**G**) and (**H**) A-375 cells (malignant melanoma) after 24 and 72 h of exposure, respectively.

which were treated with concentrations of IC_{50}, ½ IC_{50}, and ¼ IC_{50}, determined by the MTT test. The results were analysed using Comet Score software, measuring the parameter Olive Moment. The values for each solvent and the corresponding propolis extract were calculated as a fraction according to their untreated control, which was accepted as 100%.

Generally, a genotoxic effect was observed in all the tested NADESs, including the 70% EtOH, relevant to the increasing concentrations (Fig. 4). The highest genotoxic effect among the NADES solvents was observed for LAFr, which, even at ¼ IC_{50}, demonstrated high genotoxicity. On the other hand, CCPD proved to be the least toxic among all the tested solvents, having only a moderate genotoxic effect at concentrations of IC_{50} and no presence of a genotoxic effect in the other two used concentrations. The remaining solvents demonstrated a primarily concentration-dependent moderate to subtle genotoxicity.

The obtained results for the propolis extracts (Fig. 5) showed a relatively similar picture. LAFr/pro had a powerful genotoxic effect in all the used concentrations. Among the rest of the NADES extracts, the CCPD/pro and BMAW/pro had the lowest genotoxic effect when used at concentrations of ¼ IC_{50} compared to the other. It should be noted that the value for the IC_{50} concentration of the BMAW/pro is missing from the graph because this extract at this concentration exerted high genotoxicity with comets extending outside the visible camera field, which prevented its data quantitation. Regarding the 70% EtOH/pro extract, even though it experienced relatively high genotoxicity at the concentration of IC_{50} when used at a lower concentration of ¼ IC_{50}, it did not demonstrate any presence of a genotoxic effect. From the correlation analysis performed, it was found that there is no statistically significant Pearson correlation between the concentration of tP and tFF, respectively, and the genotoxic effect of the extracts ($p > 0.05$).

Overall, the lowest to highest genotoxicity can be classified among the pure solvents and their propolis extracts. The solvents can be classified as CCPD < BMAW and 70% EtOH < LAPD < CCPDW < LAFr. The propolis extracts can be classified as 70% EtOH/pro < CCPD/pro < BMAW/pro < LAPD/pro < CCPDW/pro < LAFr/pro.

Table 6
Mean values of IC_{50} of the studied pure NADESs on the treated cell lines at 72 h

Solvent	CCL-1		HaCaT		A-431		A-375	
	IC_{50}		IC_{50}		IC_{50}		IC_{50}	
	g/L	mM	g/L	mM	g/L	mM	g/L	mM
LAPD	1.39	8.35	2.12	12.76	2.06	12.4	2.04	12.2
LAFr	0.33	0.53	1.01	1.60	1.02	1.62	1.05	1.66
CCPD	21.44	58.29	16.81	45.70	5.75	15.6	16.00	43.48
CCPDW	21.65	92.61	8.43	36.07	5.51	23.58	15.16	64.87
BMAW	2.04	5.66	1.69	4.70	1.89	5.25	1.63	4.52

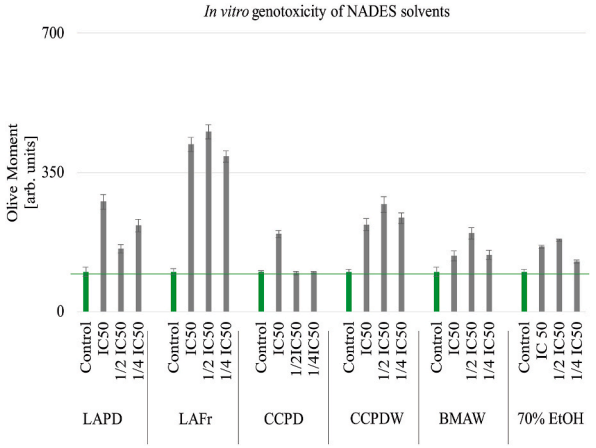


Fig. 4. *In vitro* genotoxicity assessment of the tested NADESs on CCL-1 cells by Comet Assay. Three repetitions of the experiment were done, and results are displayed as mean values of the parameter Tail Olive Moment ± SD. The green line marks the threshold above which the genotoxicity was detected and evaluated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

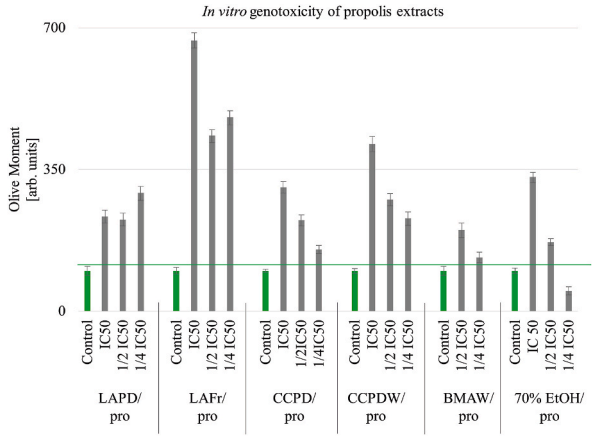


Fig. 5. *In vitro* genotoxicity assessment of the tested NADES propolis extracts on CCL-1 cells by Comet Assay. Three repetitions of the experiment were done, and results are displayed as mean values of the parameter Tail Olive Moment ± SD. The green line marks the threshold above which the genotoxicity was detected and evaluated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusions

This research confirmed that some NADESs are suitable and promising solvents for extraction of phenolic compounds from poplar type propolis. Good extractability was achieved with CCPDW, LAPD and CCPD, possessing extraction capacity similar to that of 70% ethanol. The GC-MS analysis data showed no significant qualitative differences in the chemical composition of the propolis extracts obtained with the used NADESs and 70% ethanol. The results from antimicrobial tests confirmed that NADESs could improve the antimicrobial effect of propolis extracts against pathogenic microorganisms. The cytotoxicity assessment of the pure NADESs revealed that for the normal CCL-1 and HaCaT cell lines LAFr and BMAW can generally be classified as possessing moderate cytotoxicity (IC_{50} after 72 h exposure 0.026–0.16% v/v), which ranks them among the solvents with an unsatisfactory toxicity profile. In contrast, LAPD, CCPD and CCPDW possess low to negligible cytotoxicity (IC_{50} after 72 h exposure 0.125–2.02% v/v). With some exceptions, the NADES extracts were more cytotoxic than the corresponding pure solvents and generally after 72 h the NADES extracts' cytotoxicity towards CCL-1, HaCaT, A-431 and A-375 cells was comparable to that of the ethanolic extract. It seems that the intrinsic cytotoxicity of some NADESs contributes to the lower IC_{50} values of the propolis extracts, regardless of their low concentration of phenolic compounds, as was the case with LAFr/pro and BMAW/pro. None of the propolis extracts, including ethanolic, demonstrated selectivity towards the treated tumour cell lines. The results highlighted the CCPD and CCPDW as NADESs with very low toxicity for normal cells and intrinsic selective cytotoxic effects for non-melanoma skin cancer. Overall, the lowest to highest genotoxicity can be classified among the pure solvents and their propolis extracts. The solvents can be classified as CCPD < BMAW and 70% EtOH < LAPD < CCPDW < LAFr and the propolis extracts can be classified as 70% EtOH/pro < CCPD/pro < BMAW/pro < LAPD/pro < CCPDW/pro < LAFr/pro. It should be noted that due to the specificity of the conducted biological experiments, there is a probability that not the supramolecular structure of the NADESs, but their components, played a key role for the observed biological effects.

The present study has some limitations. The data from the GC-MS analysis provide information on the qualitative composition of the studied extracts and the relative content of the individual components, but not on their actual quantitative content, which necessitates additional analyses. The biological tests conducted are only *in vitro*, and we must keep in mind that *in vitro* results do not often translate into the same or even similar *in vivo* results. Cell lines lack the protective and buffering barriers and systems of a multicellular organism. Furthermore, along with evaluating the safety of the used NADESs and their propolis extracts for human health, careful and critical assessment of the environmental impact to different trophic levels should also be performed.

Notwithstanding, the reported investigation is a step forward in developing an ethanol free natural-based poplar propolis extract enriched with bioactives and having a satisfactory toxicity profile with numerous potential applications. Considering the extraction efficiency, the extract's chemical composition, the results from *in vitro* cytotoxicity evaluation and the low genotoxicity of both NADES and propolis extract, choline chloride:1,2-propanediol 1:3 (CCPD) appeared as the best candidate for replacing 70% EtOH in the hydroethanolic solution of poplar propolis incorporated in different formulations for topical application in cases where the presence of ethanol must be avoided. Of course, further studies are needed to establish the optimal extraction conditions and to elucidate *in vivo* the CCPD/pro bioactivity and safety for the health.

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Data availability

The data presented in this study will be available upon reasonable request.

CRediT authorship contribution statement

Boryana Trusheva: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **Hristo Petkov:** Investigation. **Ralitsa Chimshirova:** Investigation. **Milena Popova:** Writing – review & editing, Visualization, Methodology, Investigation. **Lyudmila Dimitrova:** Writing – review & editing, Methodology, Investigation. **Maya M. Zaharieva:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Yana Ilieva:** Writing – review & editing, Investigation, Formal analysis. **Bela Vasileva:** Investigation. **Iva Tsvetkova:** Investigation. **Hristo Najdenski:** Supervision, Project administration, Methodology, Conceptualization. **George Miloshev:** Supervision, Methodology, Conceptualization. **Milena Georgieva:** Writing – review & editing, Supervision, Project administration, Methodology, Formal analysis, Conceptualization. **Vassya Bankova:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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