**READY-TO-USE GREEN POLYPHENOLIC EXTRACTS FROM FOOD BY-PRODUCTS**

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**Abstract**

To establish environmentally friendly polyphenolic extracts from grape and olive pomace, natural deep eutectic solvents (NADES) were used coupled with alternative energy sources – ultrasound and microwave irradiation. Obtained extracts were characterized by HPLC analysis, while antioxidant capacity was determined by ORAC method. Furthermore, *in vitro* cytotoxicity of prepared extracts was assessed by antiproliferation assay on two tumour cell lines, whereas for investigation of type of cell death or cell cycle arrest a flow cytometric analysis was applied. In addition, a detection of compounds with DNA/RNA-binding affinity in extracts was investigated by UV/Vis and circular dichroism spectroscopy. Grape pomace extract in NADES showed to be the best of all extracts tested, with regard to extraction efficiency of polyphenolic compounds and related biological activities such as antioxidant and antiproliferative activity. Prepared polyphenolic extracts in NADES could be considered as ready-to-use in food and pharmaceutical industry without demanding and expensive downstream purification steps.

**Keywords:** biological activities; grape pomace; natural deep eutectic solvents; olive pomace; polypolyphenolic compounds

1. **Introduction**

Food processing generates a substantial volume of solid organic by-products. Only small amounts of these by-products are up-graded or recycled and used usually for composting or even discarded in open areas potentially causing environmental problems (Chemat et al., 2012; Russo et al., 2015). The large quantity of waste generated from agricultural and food production remains a great challenge and an opportunity for the food industry. The valorisation of this waste may include several options including extraction and isolation of bioactive compounds for utilisation in the food, cosmetic and pharmaceutical industries. The utilisation of bioactive compounds isolated from waste could reduce the risks and the costs of waste treatment, but moreover could potentially add more value in agricultural and food production (Kumar et al., 2017; Ravindran and Jaiswall, 2016). Among food industry, wine and oil by-products are the most abundant worldwide and have been lately often evaluated as a source of natural bioactive compounds NBCs and/or natural antioxidants due to high content of polyphenolics (Cioffi et al., 2010). Polyphenolic compounds are the most abundant secondary metabolites of plants, and have drawn increasing attention due to marked effects in the prevention of various oxidative stress associated diseases such as cancer, Alzheimer, sepsis, diabetes, and cardiovascular disease (Xia et al., 2010; Manton et al, 2004).

Bioactive components present in agro-industrial waste can be recovered using various techniques but nowadays, this should be based on green and sustainable technologies by following the principles of green extraction. This can be achieved by: (*i*) improvements and optimization of existing processes; (*ii*) use of non-dedicated equipment; and (*iii*) innovations in the processes and procedures used, including the application of alternative solvents. Various environment-friendly, tuneable and smart solvents including natural deep eutectic solvents (NADES) have been considered (Cvjetko Bubalo et al., 2018). Actually, NADES fully fits the principles of green chemistry and offers many advantages including low cost, readily available component, simple preparation, and a good ecotoxicity profile. NADES are prepared simply by mixing two or three component such as organic salts (quaternary ammonium or phosphonium salt) and hydrogen bond donor (HBD) including choline, amines, sugars, polyalcohol and carboxylic acids indicating that NADES with different physicochemical characteristic could be prepared (Paiva et al., 2014). Another interesting feature of NADES is its potential biological activity since it was proposed that is possible to design NADES with specific biologically activity. For example, if you need solvent with antioxidative or/and antitumor activity you could simply prepare NADES with compounds that possess desired biologically active. Previously, it was demonstrated that NADES used for extraction purposes could enhance the antioxidative activities of obtained plant extracts, which could be explained by the reactive oxygen species scavenging activities of the NADES itself or NADES forming compounds (Nam, et al., 2015; Radošević et al., 2016). Furthermore, since the components of NADES are abundant in the nature and are part of our daily diet, it is suggested that extract obtained by NADES may be directly used in food, pharmaceutical, cosmetical and agrochemical products without the need for expensive downstream purification steps (Dai et al. 2013). Also, if we take into account that nowadays many people are already taking food supplements containing NADES-forming compounds (e.g. choline, citic acid, betaine, amino acids, etc.) it could be anticipated that NADES will be used in the future as safety solvent for food supplement preparation or as nutriceuticals (Silva et al., 2018).

Based on aforementioned, potential biologically activity of polyphenolic extracts prepared from grape and olive pomace according to the green chemistry principles were evaluated. Biological activities of extracts prepared in NADES were valorised and compared to extracts obtained by ethanol, as a referent conventional solvent. Determination of biological activities included measurement of the antioxidant capacity by ORAC (oxygen radical absorbance capacity), *in vitro* analysis of cell populations undergoing oxidative stress and *in vitro* cytotoxicity on two tumour cell lines (HeLa and MCF-7). Also, to investigate possible mechanism of action related with observed cytotoxicity of prepared extracts a flow cytometric analysis was applied to determine type of cell death or cell cycle arrest. Furthermore, since DNA and RNA are the molecular targets for many drugs that are used in cancer and antimicrobial therapy (Hurley, 2002), extracts from grape and olive pomace were additionally screened for presence of compounds with DNA/RNA-binding affinity by using thermal melting of double-stranded (ds-) polynucleotides and circular dichroism spectroscopy (Vujčić et al., 2017). Beside extracts, we also investigated interactions of DNA and RNA with each of DES constituents (choline chloride and citric acid) to evaluate their individual effect on the structure of those biomacromolecules. It is expected that based on all obtained results, the possible application of NADES as a good solvent for the preparation of a food supplement could be substantially discussed.

1. **Materials and Methods**
	1. *Chemicals and materials*

All chemicals and standards were purchased from Sigma (St. Louis, MO, USA), Polypolyphenols AS (Sandnes, Norway), Gibco Invitrogen Corporation (Paisley, UK) and Gibco Invitrogen Corporation (Auckland, New Zealand). CellTiter 96® AQueous One Solution Cell Proliferation assay was purchased from Promega (USA), while measurement was done on the microplate reader (Tecan, Switzerland). The MuseTM Annexin V & Dead Cell Kit, Cell Cycle Kit and Oxidative Stress Kit was purchased from Merck KGaA (Darmstadt, Germany), while related analyses were done using Muse® Cell Analyser (EMD Milipore Corporation, Massachusetts, USA). Calf thymus DNA (ctDNA) and poly A – poly U (RNA) were purchased from Sigma Aldrich.

Two used adherent human cancer cell lines (HeLa cell line derived from the cervical adenocarcinoma (ATCC No. CCL-2) and MCF-7 cell line derived from breast adenocarcinoma (ATCC No. HTB-22) were obtained from the Ruđer Bošković Institute, (Zagreb, Croatia). Cells were cultured in BioLite petri dishes (Thermo Fisher Scientific, USA) in humidified atmosphere with 5% CO2 at 37°C obtained by the incubator. Individual experiments were performed in 6-, 12- or 96- wells plates (Thermo Fisher Scientific, USA).

Grape pomace was obtained from the Croatian native red grape cultivar, *Vitis vinifera* cv. Plavac mali, originating from Dalmatia (Croatia southern vine- growing region), freeze-dried (Alpha 1-2 LD plus Christ, Germany) for two days -, milled and stored at 25°C in desiccator until preparation extracts

Olive pomace was obtained from the biggest Croatian olive mill company Agrolaguna d.d., Poreč, Croatia. Olive pomace from two phase extraction process was collected and immediately placed into the oven at 60°C and dried for 7 days. Dried olive pomace was grinded using disc mill and stored at -20°C until analysis.

Software Statistica V.12 (Statsoft Inc., Tulsa, OK, USA) was used for statistical analysis of all experimental results.

* 1. *Preparation of NADES*

Choline chloride (ChCl) was dried in a vacuum concentrator (Savant SPD131DDA SpeedVac Concentrator) at 60 °C for 24 hours before use. Choline chloride:citric acid (ChCit) was prepared by mixing ChCl and Cit in molar ration 2:1 with 30% of water (v/v) and stirred in a flask at 50 °C for 2 hours until a homogeneous transparent colourless liquid was formed.

* 1. *Preparation of extracts*

Extraction was performed in an ultrasonic – microwave cooperative reactor (Lab Kits, MW-ER-01, Hong Kong). All extraction was conducted under microwave power of 300 W, ultrasound power 50W during 10 min. Solid-liquid ratios of 0.05 g of freeze dried grape or olive pomace permL of ChCit or aqueous ethanol (70% of ethanol, v/v) were used for extraction. Then, extracts were centrifuged for 15 min at 5000 rpm and the supernatant was decanted and adjusted to a final volume of 10 mL (0.05 mg mL-1).

* 1. *HPLC analysis of polyphenolic compounds*

HPLC analyses were performed on the Agilent 1200 Series HPLC system (Agilent, San Jose, CA, USA) equipped with a diode array detector (DAD) and Phenomenex C18 column (Kinetex 150 mm × 4.6 mm, 2.6 µm, 100 Å).

Mobile phases for the separation of polyphenols were water/formic acid (99.9:0.1, v/v) (solvent A) and methanol/formic acid (99.9:0.1, v/v) (solvent B). Prior to analysis, samples were filtered through 0.22 µm PTFE (polytetrafluoroethylene) filters and analysed by a HPLC with following elution gradient according to solvent B: 0–3 min, 10%; 3–15 min, 10%–50%; 15–20 min, 50%-60%; 20–25 min, 60%-100%; 25-26 min 100%. Flow rate was 0.9 mL min-1, The column temperature was kept at 30°C and the autosampler at 4°C. The retention times and spectral data of polyphenolic compounds were compared with external standards. Gallic acid, hydroxytyrosol, tyrosol, catehin, vanillic acid, vanillin and pinoresinol were identified at 280 nm and quercetin-3-*O*-glucoside at 360 nm. Polyphenols were quantified considering calibration curves of authentic external standards (3-300 mg L-1) at the wavelength of maximum absorbance.

In filtered prepared grape pomace extracts anthocyanins were also analysed. Mobile phases were water/formic acid (95:5, v/v) (solvent A) and acetonitrile/formic acid (95:5, v/v) (solvent B). Analyses were performed with the following elution gradient conditions, according to solvent B: 0-15.5 min, 10-35%; 15.5-16 min, 35%-100%, 16-17 min, 100%, 17-18 min, 100%-10%. Flow rate was 0.9 mg mL-1. The column temperature was 40°C, and the autosampler temperature 4°C. Anthocyanins (delphinidin-3-*O*-monoglucoside, petunidin-3-*O*-monoglucoside, peonidin-3-*O*-monoglucoside, malvidin-3-*O*-monoglucoside, peonidin-3-acetylmonoglucosides, malvidin-3-acetylmonoglucosides, cyanidin-3-(6-*O*-p-coumaroyl)monoglucosides, peonidin -3-(6-*O*-p-coumaroyl)monoglucosides and malvidin-3-(6-*O*-p-coumaroyl)monoglucosides) were detected and identified comparing their retention times and spectral data with authentic external standards, and were quantified with external standard calibration curve (3-300 mg L-1) of malvidin-3-*O*-monoglucoside, at the wavelength of maximum absorbance (520 nm).

HPLC analyses were conducted in triplicate. Content of anthocyanins and polyphenols were expressed as mg of compound per kg of dry weight (DW).

* 1. *Oxygen Radical Absorbance Capacity Assay (ORAC)*

ORAC was determined according to the Ninfali et. al, 2005 and results were expressed as relative ORAC values (µmolTE g-1 dw). The assay was conducted in a quartz stones with 3 mL of reaction mixture with 2.25 mL of fluorescein sodium salt (0.04 μmol L-1) in sodium phosphate buffer (0.075 M, pH 7.0), and 0.375 mL diluted NADES, Trolox (25 μmol L-1) as standard or 0.075 M sodium phosphate buffer (pH 7) as blank control. The reaction mixtures were incubated for 30 min at 37°C, and after incubation for 30 min at 37°C, 0.375 mL of AAPH was added. Fluorescence was recorded every minute up to value zero by a Varian Cary Eclipse Spectrofluorimeter (Palo Alto, CA, USA) with 485 nm excitation and 520 nm emission.

* 1. *Determination of antiproliferative activity by cytotoxicity assay*

Antiproliferative activity of the grape and olive pomace extracts prepared in NADES and ethanol were evaluated *in vitro* against two adherent human tumour cell lines by the CellTiter 96® AQueous One Solution Cell Proliferation (MTS) assay. HeLa and MCF-7 cells were cultured in DMEM supplemented with 5% FBS and maintained in BioLite petri dishes in the incubator with humidified atmosphere and 5% CO2 at 37 °C. Individual experiments to test cytotoxicity of the prepared extracts were performed in BioLite 96-wells plates seeded with exponentially growing cells at the concentration (~3x104 cells per well in 100 µL of media) and incubated for 24 h, after which the treatment was done. Grape and olive pomace extracts (GPCCit, OPCCit, GPEtOH and OPEtOH), extraction solvents (ChCit, EtOH) and NADES-forming compounds (Ch, Cit) were diluted in the culture medium when applied to the cells so the final volume ratio was 1%, 5% and 10% (v/v), while control cells were non-treated cells. Upon 72 hours of treatment, the CellTiter 96® AQueous One Solution Cell Proliferation assay was preformed according to the manufacturer's instructions with minor modification. Briefly, 10 µL of the CellTiter 96® AQueous One Solution Cell Proliferation reagent was added to each well, and cells were incubated for further 3h, after which absorbance at 490 nm was measured on the microplate reader. Cell viability was expressed as percentage of treated versus control cells. The experiments were performed three times with five parallels for each volume ratio and data were expressed as the means ± S.D.

* 1. *Evaluation of cell death and cell cycle arrest by flowcytometric analysis*

Quantitative analysis of live, apoptotic and death cells treated with extracts obtained in NADES and ethanol was done by Muse® Cell Analyser using MuseTM Annexin V & Dead Cell Kit according to the manufacturer’s specifications. Briefly, HeLa cells were seeded into a 6-well culture plates at a density of 5 x 104 cells mL-1 (2 mL per well) and after overnight growth cells were treated with 5% (v/v) of extracts for 72 h. After treatment, both floating and adherent cells were collected, centrifuged (600 g min-1) and suspended in cell culture medium to adjust the cell concentration according to the manufacturer’s protocol. Then, a 100 µL aliquots of cell suspension were added to 100 µL of MuseTM Annexin V & Dead Cell Reagent and incubated in the dark for 20 minutes at room temperature. The cells were then analysed using the Muse® Cell Analyser. Each sample was tested in a duplicate and each experiment was performed twice. The MuseTM Annexin V & Dead Cell Assay detect phosphatidylserine on the external membrane of apoptotic cells by Annexin V-PE binding, while 7-aminoactinomycin D (7-AAD) is used as a dead cell marker. Based on that, this assay detects four distinctive populations of cells: live (Annexin V negative and 7-AAD negative), early apoptotic (Annexin V positive and 7-AAD negative), late stage apoptotic (Annexin V positive and 7-AAD positive) and dead cells, mostly nuclear debris (Annexin V negative and 7-AAD positive).

The Muse™ Cell Cycle Kit coupled with the Muse®Cell Analyzer enables quantitative information on cell cycle distribution. The assay utilizes PI-based staining of DNA content to discriminate and measure the percentage of cells in each phase of cell cycle (G0/G1, S, and G2/M). Analysis was done according to the manufacturer’s specifications. HeLa cells were seeded and treated as described for cell death analysis. After 72 hours of treatment, adherent cells were harvested using trypsin-EDTA solution to dissociate the cells from the culture plates and obtain single-cell suspensions. Further step, after adjusting the cell concentration according to the manufacturer’s protocol, was fixing of samples by ice-cold 70% ethanol. Such fixed cells are stable for 2 to 3 months at –20°C. At the day of analysis, staining protocol was done, which ends up with suspending of the cell pellet in 200 μL of Muse™ Cell Cycle Reagent and incubation of samples for 30 minutes at room temperature, protected from light. After that sample is ready for analysis on Muse™ Cell Analyzer.

* 1. *Evaluation of oxidative stress by flowcytometric analysis*

The Muse® Oxidative Stress Kit was used in here to measure percentage of ROS(+) cells in order to determine antioxidative potential of extracts toward HeLa cells, in which oxidative stress was induced by H2O2. The Muse® Oxidative Stress Reagent is based on dihydroethidium (DHE), which is often used for the detection of reactive oxidative species in cellular populations. HeLa cells were seeded into a 12-well culture plates at a density of 5 x 104 cells mL-1 (1 mL per well) and after overnight growth pre-treated with 5% (v/v) of extracts for 24 h. After pre-treatment oxidative stress was induced by 100 µM H2O2 for further 24 hours, after which preparation of samples for flowcytometric analysis was done according to the manufacturer’s specifications. Briefly, adherent HeLa cells were dissociated from the flask by trypsine-EDTA solution after which cell concentration was adjusted and cell samples were prepared in 1X Assay Buffer before proceeding with staining protocol. Mixture of cells and Muse® Oxidative Stress Reagent was incubated for 30 minutes at 37°C, after which samples were analysed on the Muse® Cell Analyzer.

* 1. *Spectroscopic experiments*

The UV/Vis and circular dichroism (CD) experiments were conducted on Varian Cary 100 Bio and on JASCO J815 spectrophotometers, respectively. DNA and RNA were prepared in sodium cacodylate buffer, *I*=0.05 mol dm-3, pH 7.0 and their concentrations were determined at the UV/Vis spectrophotometer (Chargaff et al., 1953). CD experiments were carried out at 25°C in buffer solution by adding aliquots of DMSO stock solutions of extract or compound into the polynucleotide solution (Supplementary). Melting (denaturation) curves for complexes of DNA, RNA and studied extracts or compounds were determined (Mergny and Lacroix, 2003) by monitoring the absorption change at 260 nm as a temperature function (from 25 – 100°C). Extract or compound absorbance was subtracted from each curve. Tm (melting temperature) values were determined from the maximum of the first derivative. ΔTm value is a difference between melting temperature values of free polynucleotide and a complex with compound (the average of two measurements).

1. **Results and discussion**
	1. *Green polyphenolic extracts preparation and characterisation*

Considering the principles of green extraction (Chemat et al., 2012), polyphenols from food-by-products (principle 1) were extracted with NADES (principle 2) under simultaneously microwave and ultrasound condition (principle 3). Based on our preliminary work and the literature (Radošević et al., 2016.; Cvjetko Bubalo et al., 2018), ChCit was selected as exaction solvent for extraction of polyphenolics from grape and olive pomace. ChCit possess physicochemical characteristic desirable for extraction processes such as low pH value as well as polarity similar to polarity of water and polar organic solvents commonly used for extraction of polyphenolic compounds (ethanol and methanol) (Ruesgas –Ramón et al., 2017). Extraction was performed by ChCit with 30% of water added, what leads to decreased viscosity of NADES, enhancement of the mass transfer from plant matrices to a solution and consequently increase in the extraction efficiency (Cvjetko Bubalo et al., 2018). Furthermore, selected choline based NADES having organic acid as HBD is interesting due to biological activity of forming compounds itself (choline and citric acid) which probably could enhance biological activity of plant extracts as well (Radošević et al., 2016; Nam, et al., 2015). Choline is essential component of the human diet that is necessary for synthases of acetylcholine, membrane and signalling phospholipid, and functions as important methyl donor (Awwad et al., 2012). A few studies observed positive association between the dietary choline intake or plasma concentration and risk of some types of cancer (Xu et al., 2009; Zeisel, 2012). The recommended daily requirement for choline has been set to 550 mg /day and 425 mg /day for non-pregnant women. The daily upper limit for adults is 3,500 mg per day which is the highest level of intake that is unlikely to cause harm (U.S. Food and Drug Administration, 2018). Similar, citric acid also possess various interesting biological activity including antioxidant, anti-inflammatory and antitumor effects (Chen et al., 2017). Citric acid is found in large quantity in many fruits and vegetables especially in citrus fruits and it is common food and drink additive widely used by food industry as chemical acidifier, flavouring agent and a preservative (Yoshikawa et al., 2011). It is general consider natural and healthy and for example, the Food and Drug Administration (FDA) does not pose limit for citric acid addition in food and drinks. However, to evaluate the NADES as possible replacements for conventional solvents, the ChCit extracts from grape and olive pomace were characterized and compared with those obtained with an aqueous solution of ethanol (70%, v/v) (Table 1) (Teixiera et al., 2014).

[Table 1 near here]

In general, considering solvent extractibility, better extraction efficiency of polyphenols from both pomaces was obtained with NADES ChCit. In grape pomaces extracts several polyphenolic compounds were identified and quantified including anthocyanins, gallic acid, (+)-catechin and quercetin-3-*O*-glucoside, with the highest content of anthocyanins. Obtained polyphenol profile in grape pomace extracts was in good agreement with previous studies (Teixiera et al., 2014). In GPChCit extract significantly higher content of all anthocyanin -3-*O*-monoglucosides and -3-acetylmonoglucosides was noticed in comparison to GPEtOH while content of three anthocyanin -3-(6-O-p-coumaroyl)monoglucosides were higher in aqueous solution of ethanol. Similar with previous studied (Paini et al., 2016), in olive pomace-prepared extracts gallic acid, hydroxytyrosol, tyrosol, catechin, vanillic acid, vanillin and pinoresinol were identified, and the dominant compound was vanillin in OPEtOH. The content of polyphenols was different in extracts prepared with different solvents (ChCit and EtoH). Thus, pinoresinol content in NADES extract was significantly lower than in ethanolic extract, while tyrosol content was in completely opposite manner.

Furthermore, in both pomace-prepared extracts with NADES and aqueous ethanol, phenolic pattern was similar with some exception. For example, in olive pomace-prepared extracts, gallic acid and hydroxytyrosol were identified only in extract prepared with NADES. However, in the case of total polyphenols content, grape pomace is containing ≈ 5 times more polyphenolic compounds than olive pomace (Table 1).

* 1. *Biological evaluation of polyphenolic extracts*

Biological evaluation of grape and olive pomace polyphenolic extracts, was conducted on two human tumour cell lines. Such *in vitro* preliminary screen of natural extracts is simple, inexpensive and rapid method for cytotoxicity assessment for a large number of samples in the range of different concentrations. Pure NADES and their forming compounds were also evaluated with regard to its *in vitro* cytotoxicity, having in mind that they also possess certain biological activities which could contribute the biological activities of plant extract. Ethanol extracts were also tested, as well as ethanol itself, since it is being used as referent solvent for polyphenolic extraction. Cytotoxicity of extracts (GPChCit, GPEtOH, OPChCit, OPEtOH), extraction solvents (ChCit and EtOH) and NADES-forming compounds (ChCl and Cit) was evaluated by CellTiter 96® AQueous One Solution Cell Proliferation assay on HeLa and MCF-7 cells. All samples were applied to cells in different volume ratio 1%, 5% and 10% (v/v) during 72 hours (Figure 1), where it was observed that inhibitory effect of GPChCit, OPChCit, ChCit and Cit is almost the same no matter of applied volume ratio, while GPEtOH, OPEtOH and Ch showed dose-dependent effect. EtOH as a solvent did not had impact on cell viability of HeLa and MCF-7 cells.

[Figure 1 near here]

Extracts obtained by ethanol (GPEtOH and OPEtOH) have not had strong impact on cell viability of HeLa and MCF-7 cells, while rather low cell viability (from 12.19% to 37.61%) was observed in both cell lines treated with GPChCit, OPChCit, ChCit and Cit. Such grouping of results is probably related to low pH values of those samples (from 0.6 for ChCit to 1.33 for Cit), for which it is all in common to contain citric acid. Citric acid as a HDB is surely responsible for these low pH values of NADES itself as well as extracts obtained by ChCit. The puffer system of used culture medium could not buffer it and, for example, the pH of the DMEM after addition of 5% of GPChCit was 2.83 what is still much lower than the optimal pH for cell culture cultivation. But, it is known that *in vivo* many tumours have relatively acidic extracellular pH, while the intracellular pH of tumour cells remains normal due to the efficient maintenance of a large proton gradient across the membrane (Zanke et al., 1998). Considering that observed cytotoxicity of GPChCit and OPChCit in tumour HeLa and MCF-7 cells is probably mostly related to polyphenolic compounds in extracts, although synergistic effect of polyphenolics and low pH is possible, since there are data suggesting a link between induced cellular acidification and cell death (Harguindey et al., 2017). Furthermore, the comparison between extracts depending on type of extraction solvent used goes in support of the fact that polyphenols are most likely to be responsible for the notable antiproliferative effect of NADES-extracts. Grape pomace and olive pomace extracts in ChCit had stronger cytotoxic effect on HeLa and MCF-7 cells than those obtained by EtOH, what is in good correlation with HPLC analysis of individual polyphenolic compounds, measured TP and ORAC values (Table 1). TP and ORAC values were higher for GPChCit and OPChCit than for GPEtOH and OPEtOH, respectivly. Such result is in agreement with our previously published results on polyphenolic grape pomace extracts prepared by five choline chloride based NADES, where it was showed that the best performance concerning extraction efficiency and biological activities was obtained by ChMa i.e. NADES containing organic acid as HDB, which by itself possessed biological activity (Radošević et al., 2016).

When it comes to observed significant antiproliferative effect of GPChCit and OPChCit, we wanted to elucidate whether inhibition of cell growth was related to cell cycle arrest or cell death and therefore we employed flow cytometry by Muse® Cell Analyzer and optimized assays to obtain quantitative data at the single cell level. Since there were no significant differences in cytotoxicity results between two cell lines (Figure 1) further experiments were done only on HeLa cells treated with middle volume ratio (5%, v/v). According to the results presented on Figure 2a observed inhibition of cell growth by GPChCit and OPChCit could not be primary related to induction of apoptosis in HeLa cells, since there is no notable increase in number of apoptotic cells (early, late, total) when compared to control, untreated cells.

Apoptosis as a way of cell death has taken a central position in cell death investigations, especially those related with cancer research, although it becomes more and more evident that cells can also die by non-apoptotic mechanisms, such as autophagy, mitotic catastrophe and necrosis. Herein, significant difference between treated and control cells was observed only for those treated with GPChCit and GPEtOH, where percentage of dead cells is increased to 21.38% and 40.58%, respectively, meaning that those cells probably died by necrosis. Besides so-called accidental necrosis, newer literature also reports about necrosis-like programmed cell death which has been considered as a type of cell death, although its role in tumorigenesis and cancer treatment is still unclear (de Bruin and Medema, 2008). The background of observed inhibitory effect in tumour cell lines could also be a delay in cell cycle, which is the most significant and fundamental processes in eukaryotic cells. As it could be seen at Figure 2b treatment of HeLa cells with both extracts prepared with NADES (GPChCit and OPChCit) significantly affected the distribution of cells in G0/G1 phase and reduced number of cells in S phase when compared to control cells. A slightly increased G2/M cell was observed in the case of GPChCit and GPEtOH. These data suggest that inhibition of cell proliferation by NADES-extracts in HeLa cells is associated mainly with the induction of G0/G1 arrest, which allow cells to have time to trigger repair mechanisms or induce cell death pathways. Combined with results on cell death this may indicate that observed G0/G1 arrest over time lead to necrosis. It is reported that the mechanisms of polyphenols activities are comprised of many different pathways from induction of apoptosis and cell cycle arrest, scavenging of free radicals, regulation of gene expression, and stimulation of the immune system (Dziedzic et al, 2017). Also, since it is known that different cell death mechanisms may lead one to another, it is rather difficult to determine the one dominantly responsible for certain observed outcome, like inhibition of cell growth, especially when dealing with natural plant extracts, which are mixtures of pro- and anti-apoptotic compounds, pro- and anti-oxidant compounds, etc.

[Figure 2 near here]

As already mentioned, the most studied biological activity of polyphenolic compounds is their antioxidant activity, which surely contributes to the antioxidant capacity of related plant extracts (Xia et al., 2010). Since the major biological activity of polyphenolic compounds is related to its antioxidative activity, the antioxidative capacity of obtained plant extracts was also measured by ORAC methods (Xia et al., 2010; Cioffi et al., 2010) and the obtained ORAC values of the extracts varied widely from 351.58 to 2189.97 µmol TE g-1 dw, with the highest value obtained for the GPChCit following by GPEtOH>OPEtOH>OPChCit (Table 1). Furthermore, we wanted to test the ability of grape and olive pomace extracts to protect cells from induced oxidative stress. HeLa cells were pre-treated with extracts for 24 hours and afterwards oxidative stress was induced by 100 µM H2O2 for further 24 hours. Then the cell viability was determined and relative percentage of ROS positive cells was measured at the Muse® Cell Analyzer.

Results presented at Figure 3. showed significant increase in HeLa cell viability when pre-treatment was done by GPChCit (63.88%) compared with H2O2 alone (44.87%). Increase in cell viability is in good correlation with significant decrease in the percentage of ROS(+) cells obtained in GPChCit pre-treated cells. Reduction of ROS(+) cells from 11.77% in H2O2 only sample to 1.63% in sample pre-treated with GPChCit confirms its antioxidant potential measured by ORAC method. OPChCit extract and both ethanol extracts have not shown ability to protect cells from oxidative stress. Several studies tried to answer which polyphenolic compound is mainly responsible for antioxidant activities of specific plant, but consistent relationship between polyphenolic compounds and antioxidant capacity was not found. Besides, many studies indicate synergistic effects between individual classes of polyphenolics which could be related to their antioxidant capacity, rather than individual polyphenolic compound by itself (Teixiera et al., 2014) so considering that plant extracts are probably better choice than pure polyphenolic compounds. Furthermore, observed G0/G1 arrest in HeLa cells treated with GPChCit, together with detected interaction of compounds from extracts with both DNA and RNA (result showed in next section), probably enables time and way to repair DNA damage induced by oxidative stress (Pandya et al, 2012). Therefore, that may be a background of GPChCit antioxidant activity, which resulted with increased cellular viability after oxidative stress (Figure 3).

[Figure 3 near here]

Considering all data presented, antioxidative activity of GPChCit extract is advantageous for many possible applications in food and probably pharmaceutical industry. Having in mind that it was showed that formulation of antioxidants in NADES could greatly improve their activity for ROS inhibition (Durand et al., 2017), it could be anticipated that such natural extract can serve as food preservative even better than currently known antioxidants. Also, it is reported that NADES improve absorption and bioavailability of active compounds (Faggian et al., 2016) and therefore could serve as a delivery agent or administration vehicle, what opens new possibilities for application of extracts obtained by NADES. Furthermore, it is known that grapes and its products have anticancer activity (Xia et al., 2010) so results obtained herein on human tumour cell lines confirms antitumor potential of polyphenolic extracts, but since there is still a debate should cancer treatments focus on lowering ROS levels to prevent signalling or increasing ROS to selectively kill cancer cells (Schieber and Chandel, 2014), further studies in that direction are needed.

* 1. *Interactions of extracts with DNA and RNA in aqueous medium*

Noncovalent binding interactions with DNA and RNA like intercalation, groove and electrostatic binding can be investigated by variety of methods (Demeunynck et al., 2002). In order to detect possible presence of DNA/RNA active compounds in extracts, we utilized the most common methods in DNA binding studies: CD spectroscopy and the thermal denaturation of polynucleotides (Mergny and Lacroix, 2003, Eriksson et al., 2001). While the former method monitors the impact of studied compounds on CD spectra of polynucleotides, the latter provides the information about the polynucleotide stabilization driven by compounds (ΔTm value). We investigated interactions of compounds in extracts and NADES constituents with ctDNA and poly A – poly U in buffer solutions (see Materials and Methods, section 2.9).

[Figure 4 near here]

Only extracts from grape pomace induced stabilization of both DNA and RNA (Table 2, Figure 4a) indicating the presence of DNA/RNA active compounds in these extracts. We also investigated interactions of choline chloride and citric acid with DNA and RNA to see whether the solvents used for the extraction from grape and olive pomace have an influence on interaction with these biomacromolecules. Neither choline chloride or citric acid showed significant stabilization or destabilization of studied polynucleotides (Supplementary).

[Table 2 near here]

The most reliable information on compounds’ interaction with polynucleotides can be obtained in CD region at λ > 300 nm, where polynucleotides do not absorb while compounds are CD-active. Grape pomace extract exhibited CD bands around 330 and 390 nm, smaller in case of ctDNA and greater in case of poly A – poly U (Figure 4b, Supplementary). Unlike grape pomace, the olive pomace extract as well as choline chloride and citric acid displayed only negligible changes of CD spectra of both polynucleotides (Supplementary). Significant stabilization effects of DNA and RNA as well as the presence of CD signals at wavelengths above 300 nm are in line with findings of higher percentage of polyphenols with larger aromatic surfaces like catechins and anthocyanins in grape pomace (Table 1). Recently it was reported by two different methods (surface plasmon resonance assay and cold spray ionization-mass spectrometry) that catechins from green tea bind to both DNA and RNA molecules (Kuzuhara et al, 2006). Thermal melting experiments could not be carried out in NADES of the relevant ionic strength (*I* = 0.05 mol dm-3) due to high total absorption of NADES and polynucleotide which exceeded the instrument detection limit (Supplementary). Unlike ΔTm experiments, CD experiments were possible to perform in NADES. Since only grape pomace extract exerted the significant CD changes in the cacodylate buffer in the relevant CD region (above 300 nm), we performed CD experiments with that extract in 50 mM NADES to see the effect of NADES on interaction of extract and DNA. Addition of grape pomace extract to NADES solution containing polynucleotide resulted in an appearance of the positive CD bands near 330 nm.

CD changes in NADES similar to those in cacodylate buffer suggested that replacement of media (cacodylate buffer with NADES) did not have effect on dominant interaction of grape pomace extract compounds' with DNA (Supplementary, Figure S11).

In conclusion, a binding study with DNA and RNA suggested that grape pomace, despite being a by-product, still contains compounds with DNA/RNA-binding affinity while NADES constituents do not have harmful effects on DNA and RNA biomacromolecules (Figures S5, S9-10, Supplementary).

1. **Conclusion**

Taken all together, presented results indicate that selected environmentally friendly extraction method gives better yield of polyphenolics from food by-products than classical extraction with EtOH as a solvent. Good extractability of polyphenolic compounds in NADES, especially herein from grape pomace, supports the use of NADES for the extraction of polyphenols from different plant material and/or food by-products. Furthermore, determined antiproliferative and antioxidative activity of NADES-extract, together with the fact that grape pomace contains DNA/RNA active compounds, highlights the great potential of extracts obtained by NADES. However, obtained NADES extracts possess low pH value, so further pharmacokinetic and *in vivo* studies are needed to answer whether such polyphenolic extracts could be used in food and pharmaceutical industry without demanding and expensive downstream purification steps.

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1. **Conflict of Interest**

The authors declare no conflict of interest.

1. **References**

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**Figure 1.** Effect of prepared extracts, solvents and NADES-forming compounds on HeLa and MCF-7 cell viability determined by the MTS assay was assessed in volume ratio 1%, 5% and 10% (v/v). Cell viability (%) was expressed as percentage of treated cells versus control cells and the data were expressed as the means (n=5) ±S.D.

**Figure 2.** Cell dead (a) and cell cycle (b) analysis of HeLa cells after treatment with 5% (v/v) grape and pomace extracts in NADES and conventional solvent. Distribution of cell populations (%) were expressed as the means (n=3) ±S.D. Non-identical lower-case letters in every group of results (a-c) indicate significant difference (P < 0.05) determined by Tukey’s HSD test.

**Figure 3**. Cell viability (%) of HeLa cells measured by MTS assay and percentage of ROS (+) cells pre-treated with 5 % (v/v) grape and pomace extracts in NADES and conventional solvent. Oxidative stress was induced with H2O2 (100 µM) after 24h pre-treatment. Cell viability was expressed as percentage of treated cells *vs*. control cells and the data were expressed as the means (n=5) ±S.D., as well as % of ROS (+) cells. Each sample followed by non-identical letters (a-b and A-B for cell viability (%) and % of ROS (+) cells respectively) indicate significant difference (P < 0.05) determined by Tukey’s HSD test.

**Figure 4.** (a) Denaturation curve of poly A - poly U upon addition of different concentrations of GPEtOH and OPEtOH ((2a and 2b) = 0.4 mg mL-1, (4a and 4b) = 0.8 mg mL-1) at pH 7.0 (buffer sodium cacodylate, I = 0.05 mol dm-3). (b) CD titrations of poly A - poly U (*c* = 3 × 10-5 mol dm-3) with grape GPEtOH (γ(G) = 0.01; 0.02; 0.03; 0.1 mg mL-1) in sodium cacodylate buffer (pH 7.0, *I* = 0.05 mol dm-3).