Redox Properties of Grape Wine Skin Extracts from The Šumadija Region - An Electron Paramagnetic Resonance Study

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SUMMARY

Introduction: Comprising of a unique combination of bioactive polyphenol compounds, grapes are shown to have a beneficial effect on the skin as well as the whole body. They exhibit high antioxidative and antiradical activity through mechanisms of heavy metals chelation, scavenging reactive oxygen species, inhibiting lipid peroxidation, and preserving the integrity of the cell membranes. In the winemaking industry, after crushing and pressing, grape pomace (stems, seeds, pulp, and skin) is removed as a by-product. This valuable source of bioactive compounds is often treated as animal food, compost, raw material in the brewery, but unfortunately mostly disposed of as unusable waste.

Aim: The aim of this study was to compare redox properties of absolute EtOH, 50% EtOH, and H₂O grape wine skin extracts in a way of scavenging DPPH radicals, showing that bio-waste obtained from grape wineskins could be used as an immense source of bioactive compounds with high antiradical activity.

Material and Methods: Electron Paramagnetic Resonance (EPR) spectroscopy was used to detect the activity of grape skin extracts toward DPPH radicals and UHPLC-DAD MS/MS analysis to separate, identify and quantify their active components.

Results: The results show that Cabernet Sauvignon and Pinot Blanc grape skin extracts have unexpectedly large potential to remove DPPH radicals from the system. Having in mind the concentration of redox-active components obtained by UHPLC-DAD MS/MS analysis and presented EPR results, there is strong evidence that primarily quercetin 3-O-glucoside, as well as kaempferol 3-O-glucoside, isorhamnetin 3-O-glucoside, kaempferol 7-O-glucoside supplemented with catechin and rutin are responsible for the antioxidative capacity of extracts.

Conclusions: This study demonstrates that extracts obtained from grape skins, initially intended as biowaste, exhibit high antiradical activity. The largest quantity of the most redox-active components could be found in 50% EtOH extracts, which we propose to be the starting material for making potent redox-active cosmetic products and dietary supplements.

Keywords: Grape skin extracts, Antioxidative activity, Electron Paramagnetic Resonance (EPR) spectroscopy, UHPLC-DAD MS/MS analysis

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INTRODUCTION

Living in an unhealthy environment and leading a stressful life in demanding times, the modern individual is becoming interested in using plain natural resources to build up their immune system, skincare, and improve health.

The grape has been considered a divine fruit since ancient times. As mankind has acquired knowledge and experience, every constituent part of the grape plant has been expanded to its application in various areas of human activities: ecology [1], energetics [2-4], medicine [5-7], cosmetics [5,8], pharmacy [5,9], food industry [5,10,11], agriculture [5,12], etc. A wide range of nutritious compounds (carbohydrates, fibers, vitamins, minerals, fatty acids, and bioactive phytochemicals) make grapes the most exploited part of the whole grapevine. Bioactive compounds from grapes are proved to have beneficial effects when applied locally on the skin as well as systematically to the whole body [10]. The extraordinary reputation of wine is actually based on a unique combination and properties of the premium family of bioactive compounds, polyphenols [13]. They exhibit high antioxidative and antiradical activity through mechanisms of heavy metals chelation, scavenging reactive oxygen species, inhibiting lipid peroxidation, and the decrease in the fluidity of the membranes which hinders the diffusion of free radicals into the cell [14].

Polyphenol antioxidant activities in the human body occur in a wide range of preventive and therapeutic effects including anti-tumor, anti-microbial, cardioprotective, anti-mutagen, anti-proliferative, anti-aging, and anti-inflammatory effects. The bioactive grape compounds additionally improve appearance, health, and protective skin function, by acting locally as an anti-cellulite agent, improving circulation, preventing and reducing skin hyperpigmentation (freckles, age pigment, and melasma), collagen degradation and denaturation, etc. Furthermore, the influence of antioxidant defense at all levels (cell, tissue organs, system of organs) has also been studied concerning peristalsis in the digestive tract [15,16], myocardial infarction [17, 18], and reproductive organs [19, 20]. In other words, there is no cell, tissue or organ that does not enjoy the beneficial effects of natural non-toxic unique super-cocktails of grape bioactive substances.

In the winemaking industry, after crushing, pressing (and for black wines fermentation) until the must is formed, grape pomace (stems, seeds, pulp, and skin), which constitute around 20 w% of grapes, is removed as a by-product. This valuable source of bioactive compounds is often treated as animal food, compost, raw material in a brewery, but mostly disposed of as unusable waste [14].

The famous phenomenon of the „French paradox” [21], implies that the consumption of wine reduces the incidence of degenerative diseases (especially heart disease [22]), a strong synergetic activity of grape ingredients, which improves and intensifies the benefits of bioactive compounds. Consequently, the attention of the industry and scientific community has increased towards extraction, analyzing and application of the bioactive compounds [14].

EPR (Electron Paramagnetic Resonance) and UHPLC (Ultra-High Performance Liquid Chromatography) research are currently being undertaken to investigate the qualitative and quantitative composition of grape skin extracts and their antioxidant/antiradical activity. The raw materials (Cabernet Sauvignon, Pinot Blanc, Morava, and Muscat Ottonel) for the extractions in absolute EtOH, 50% EtOH, and H₂O were delivered from winery „Despotika”. Since the winery industry regularly disposes of huge quantities of grape pomace as an environmental burden, this study was oriented towards wine grapes. Instead of using artificially synthesized active compounds or isolating and concentrating from single to few naturally occurring active compounds (purified extracts), we decided to use grape skin extracts as originally obtained (complete/crude extracts). The main advantages of using complete grape skin extracts reflect in their ability to maintain biological activity in a similar form as in their natural environment, expressing synergistic effect and biocompatibility. For basic and instantaneous evaluation of the grape skin extracts activity, we have investigated the *in vitro* DPPH radical scavenging using EPR spectroscopy. Accordingly, we expect to develop low-priced, environmentally friendly, health-improving products from grape extracts.

AIM

The aim of this study was to compare redox properties of absolute EtOH, 50% EtOH, and H₂O grape wine skin extracts in a way of scavenging DPPH radicals, showing that biowaste obtained from grape wineskins could be used as an immense source of bioactive compounds with high antiradical activity.

MATERIAL AND METHODS

Reagents

Ethanol (EtOH), manufactured by Merck KGaA (Germany), and 2,2-diphenyl-1-picrylhydrazyl (DPPH), manufactured by Sigma (Germany), were of analytical grade. Solvents for LC-MS analyses (acetonitrile and formic acid), obtained from Fisher Scientific (Loughborough, UK), were HPLC or LC-MS grade. Ultrapure water was generated by deionization (Millipore, Billerica, USA). Standards of phenolics (synergic, protocatechuic, vanillic, cinnamic, 3-O-caffeoylquinic, caffeic, rosmarinic and ferulic acids; aesculin, luteolin, apigenin, chrysin, rutin, quercetin, kaempferol, galangin, naringenin, hesperidin and pinocembrin) were purchased from Sigma-Aldrich (Steinheim, Germany).

Sample preparation

Pressed marcs (pomace) were collected immediately after pressing and before vinification from a winemaking company near Smederevska Palanka, Serbia. Grape seeds were manually removed, and the samples were dried in the dehydrator at 60 °C up to reaching the moisture content of max 4% (determined by dry weight in oven at 105 °C until constant weight) [23]. Dried skins were milled by a grinder shortly before the extraction. Grinding was carried out in 10 s intervals followed by 30 s breaks, to avoid overheating [24]. The obtained powder was passed through the 100 mesh sieve. Hydroethanolic extraction was performed by mixing the grape skin powder with the 50% EtOH in 1/10 (w/v) ratio in an Erlenmeyer flask [23]. The flasks were capped and placed onto the orbital shaker (200 rpm), and the extraction was carried out in the incubator at 50 °C for 5 h [23,25,26]. The suspended solids were removed from the extracts by vacuum filtration through the qualitative

filter paper, additionally followed by centrifugation at 4500 g for 10 min and subsequent separation of the supernatant [23,25]. All of the extracts were passed through the 0.2 µm filter. The samples were kept in dark at -80 °C until further analysis. The same procedure was repeated for extraction in deionized water, as well as in absolute EtOH.

Determination of the scavenging activity toward the DPPH radicals

DPPH compound is a readily available stable free radical widely used in studying the antioxidant capacity of various compounds, especially by the means of UV-Vis spectroscopy. Standard DPPH assays are based on the reduction of the violet EtOH solution of its free radical form to the colorless (to yellow) form in the presence of antioxidant molecules [27]. On the other hand, DPPH radical is suitable for EPR assays as well, since its radical form is EPR active and the reduced form is EPR silent. EPR methods are the preferred ones, especially because they do not rely on the optical characteristics of the studied system. The distinctive EPR signal of DPPH radical enables the estimation of the initial radical concentration and the potential of the grape skin extracts to reduce its presence in the system [28].

Samples were studied by adapting the previously developed method [28,29] to detect the activity of grape skin extracts toward the DPPH radicals. In brief, 1 µl of grape skin extract was added to the 29 µl of the 210 µM DPPH solution in the appropriate solvent (absolute EtOH, 50% EtOH and H₂O). The mixture was transferred into the gas-permeable Teflon tube, and the EPR signal was recorded upon 2 min using Bruker ELEXSYS-II E540 spectrometer operating in X-band, under the following experimental settings: center field 3500 G, microwave power 10 mW, microwave frequency 9.85 GHz, modulation frequency 100 kHz, modulation amplitude 2 G. Control recording was made by substituting the samples with the same volume of the corresponding solvent.

Quantification of the polyphenolics using UHPLC-DAD MS/MS

Separation, determination, and quantifica-

1000 mg/l stock solution of a mixture of all phenolic standards was prepared in methanol. Dilution of the stock solution with methanol yielded the working solutions in following concentrations 0.025, 0.050, 0.100, 0.250, 0.500, 0.750, and 1.000 mg/l. Calibration curves were obtained by plotting the peak areas of the standards against their concentration. Calibration curves revealed good linearity, with r^2 values exceeding 0.99 (peak area vs. concentration).

This paper is part of Academic (non-commercial) study.

RESULTS

HPLC analysis

Results obtained using HPLC analysis show-

ing concentrations of detected components (in mg/ml) for selected H₂O, 50% EtOH and absolute EtOH extracts are presented in Figure 1.

EPR spectroscopy

EPR spectra obtained 2 minutes upon the interaction of DPPH with the grape skin extracts are shown in Figure 2.

After the initial experiments were performed, the results indicated that Cabernet Sauvignon (absolute EtOH and 50% EtOH), as well as Pinot Blanc (50% EtOH) grape skin extracts had an absolute DPPH radical scavenging potential for the selected concentration of DPPH. For this reason, these extracts were diluted 9 times, and the recordings were repeated. The anti-DPPH activity (AA) of the

Figure 2. EPR spectra of the control sample and the selected grape skin extracts obtained in absolute EtOH (A), 50% EtOH (B) and H₂O (C), recorded 2 min upon the addition of DPPH into the system. Cabernet Sauvignon (absolute EtOH and 50% EtOH) and Pinot Blanc (50% EtOH) grape skin extracts were diluted 9 × prior to the recordings.

Table 1. Anti-DPPH activity of the selected grape skin extracts calculated from the normalized double integral values. Results for 9 × diluted samples are designated by asterisk.

Grape skin extract	Anti-DPPH activity (%) in designated solvent		
	EtOH	50% EtOH	H ₂ O
Cabernet Sauvignon	31.26*	75.38*	19.41
Pinot Blanc	22.24	58.46*	15.10
Muscat Ottonel	18.78	96.28	17.67
Morava	7.30	85.66	15.06

Figure 3. Anti-DPPH activities of grape skin extracts scaled to the activity of Cabernet Sauvignon in 50% EtOH.

grape skin extracts was calculated using the formula [29]:

$$AA = \frac{I_c - I_a}{I_c} 100 (\%)$$

where I_c and I_a refer to the double integral values of the control and samples determined from the EPR spectra (using Xepr software), respectively. The calculated anti-DPPH activities of the extracts are presented in Table 1.

To present data more clearly (due to the dilution factor) anti-DPPH activities presented in Figure 3 were normalized to the highest value (Cabernet Sauvignon in 50% EtOH).

DISCUSSION

Previous studies indicate that grape pomace have remarkable antioxidant potential, strongly suggesting that they should be re-processed rather than put to biowaste. Red grape cultivars generally have high content of anthocyanins and flavonols and are proved to be highly active free radical scavengers [31]. Pomace from the red wine cultivars is proved to contain significant amount of polyphenols after the vinification process, sufficient to exert anti-hypertension effects *in vivo* in hypertensive rats [32]. Another study on rats showed that administration of grape pomace extracts

rich in anthocyanins, anthocyanidins, phenolic acids and flavonols actually induced oxidative stress, which could potentially trigger the response of body antioxidant mechanisms [33]. Most of the available studies are focused on red grape pomace polyphenols composition, while white varieties draw little attention [34]. While the entire grape mass is used for the fermentation of red wine, only the juice is used to produce white wines, which leads to significant grape pomace composition variations, making valorization rather difficult [35]. Pomace from the white grape cultivars is rich in dietary fibers and has a high total polyphenolic content [36]. Likewise, white grape pomace extracts are rich in phenols, flavanols, flavonoids and tannins, and show antioxidant, anti-tyrosinase and anti-inflammatory effects [34, 37]. Depending on the white grape variety, most of the pomace extracts have a high content of catechin, epicatechin and procyanidin B2 [34]. Both red and white grape pomace extracts showed the potential to suppress hyperglycemia in diabetic mice, with red grape pomace extracts exerting a higher suppression effect, but also being richer in total phenolic content [38]. Grape pomace can be incorporated in plant, meat, fish and dairy products, since dietary fibers and polyphenols could increase the nutritional value and oxidative stability of the final products [36, 39].

In this study, the obtained results indicate that hydroethanolic grape skin extracts possess the highest DPPH scavenging potential. This is in accordance with the fact that hydroethanolic extraction of phenolic compounds is more efficient compared to the extraction performed using corresponding mono-component solvents [23, 40]. As it could be observed, Cabernet Sauvignon grape skin extracts have overall the highest potential to remove DPPH radicals from the system. This finding correlates well with previous reports of red grape varieties having higher polyphenolic content compared to white grape varieties [41, 42].

Having in mind the concentration of redox-active components obtained by HPLC analysis and presented EPR results, there is strong evidence that primarily quercetin 3-O-glucoside as well as kaempferol 3-O-glucoside, isorhamnetin 3-O-glucoside, kaempferol 7-O-glucoside supplemented with catechin and rutin, are responsible for the antioxidative capacity of extracts. It could also be observed that the largest quantity of most redox-active components could be found in absolute EtOH extracts, and that H₂O extracts generally contain considerably lower amount of active constituents.

Even though anti-DPPH activity is up-to-date one of the most used indicators of free radical scavenging activity of target compounds, it should be noted that this approach has its limitations related to the ability of translation of obtained results to short-lived free radical species like ROS and RNS.

Although it is obvious that Cabernet Sauvignon is the most active towards elimination of stable DPPH radical, it would be interesting to identify how selected extracts are capable to remove biologically relevant short-lived radical species such as $\cdot\text{OH}$ and $\cdot\text{O}_2^-$, as well as how they perform in the vicinity of $\text{NO}^\cdot$ and $\text{Asc}^\cdot$ radicals.

CONCLUSION

50% EtOH, absolute EtOH and H₂O extracts, obtained from the grape skins, initially intended as biowaste, have shown high anti-radical activity. Among all investigated grape varieties from the Šumadija region, Cabernet Sauvignon and Pinot Blanc exhibit the highest potential to remove DPPH radicals, which sets them as effective natural resources for pharma

industry. HPLC analysis shows that primarily quercetin 3-O-glucoside as well as other detected redox-active components are responsible for the antioxidative capacity of extracts. EPR analysis shows that the largest quantity of the most redox-active components could be found in 50% EtOH extract, which we propose to be the starting material for making potent redox-active cosmetic products and dietary supplements.

CONFLICTS OF INTEREST

All authors declare no conflict of interest.

ACKNOWLEDGEMENT

The authors thank Veselin Despotović and Darko Pejović (Despotika wine-making factory, Vlački Do, Republic of Serbia) for kindly providing the pressed marcs required for this study. UHPLC/DAD/MS2 analysis was performed at the Department of Plant Physiology of the Institute for Biological Research “Siniša Stanković” - National Institute of the Republic of Serbia, University of Belgrade. The authors thank Dr. Danijela Mišić and Dr. Uroš Gašić for their professional help. The financial support for this project was provided by the Ministry of Education, Science and Technological Development of the Republic of Serbia Contract number: 451-03-9/2021-14/200146.

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Redoks svojstva ekstrakata pokožice vinskog grožđa iz šumadijskog kraja - Ispitivanje elektronskom paramagnetnom rezonacijom

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KRATAK SADRŽAJ

Uvod: Plodovi vinove loze obiluju jedinstvenim polifenolnim bioaktivnim jedinjenjima, a poznato je da grožđe ima blagotvorno dejstvo na celo telo. Ova jedinjenja pokazuju visoku antioksidativnu i antiradikalnu aktivnost posredstvom helacije teških metala, uklanjanja reaktivnih kiseoničnih vrsta, inhibicije lipidne peroksidacije i očuvanja integriteta ćelijskih membrana. U vinarskoj industriji, posle drobljenja i presovanja, komina grožđa (stabljike, seme, pulpa i pokožice) uklanja se kao nusproizvod. Ovaj dragoceni izvor bioaktivnih jedinjenja povremeno se koristi kao hrana za životinje, kompost, sirovina u pivari, ali se nažalost uglavnom odlaže kao neupotrebljiv otpad.

Cilj: Cilj ove studije je upoređivanje redoks svojstava ekstrakata pokožica vinove loze u apsolutnom EtOH, 50% EtOH i H₂O, ispitivanjem sposobnosti uklanjanja DPPH radikala, kako bi se pokazalo da se biootpad dobijen od pokožica grožđa može koristiti kao značajan izvor bioaktivnih jedinjenja sa visokom antiradikalnom aktivnošću.

Metodologija: Elektronska paramagnetna rezonantna (EPR) spektroskopija upotrebljena je za ispitivanje aktivnosti ekstrakata pokožica grožđa prema DPPH radikalima, a UHPLC-DAD MS/MS analiza za razdvajanje, identifikaciju i kvantifikaciju njihovih aktivnih komponenti.

Rezultati: Dobijeni rezultati pokazuju da ekstrakti pokožica grožđa Cabernet Sauvignon i Pinot Blanc imaju značajan potencijal za uklanjanje DPPH radikala iz sistema. Na osnovu koncentracija redoks-aktivnih komponenti dobijenih UHPLC-DAD MS/MS analizom i rezultata EPR merenja, može se zaključiti da su kvercetin-3-O-glukozid, kempferol-3-O-glukozid, izoramnetin-3-O-glukozid, kempferol-7-O-glukozid, katehin i rutin glavni nosioci antioksidativne aktivnosti ekstrakata.

Zaključak: Ova studija pokazuje da ekstrakti dobijeni iz pokožica grožđa (inicijalno namenjenih biootpadu) pokazuju visoku antiradikalnu aktivnost. Najveća količina redoks-aktivnih komponentata može se naći u ekstraktima 50% EtOH, za koje predlažemo da budu polazni materijal za izradu snažnih redoks-aktivnih kozmetičkih proizvoda i dodataka ishrani.

Ključne reči: Ekstrakti pokožice grožđa, antioksidativna aktivnost, elektronska paramagnetna rezonacija, UHPLC-DAD MS/MS analiza

Received: November 17, 2020
Accepted: December 09, 2020