

Spectroscopic characterization of some grape extracts with potential role in tumor growth inhibition

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Some varieties of grapes, namely, Burgund Mare (BMR), Cabernet-Sauvignon (CSR), Muscat-Hamburg (MHR), Merlot (MR), Merlot Dealu-Mare (MDM) from a wine production area in Romania, Recas, were investigated in order to find optimal efficiency of certain extraction procedures for satisfactory polyphenolic composition and to evaluate their antioxidant activities. Total polyphenols content of studied extracts, was determined according to Folin-Ciocalteu methodology. The antioxidant capacity (reducing power) was performed by ESR spectroscopy and spectrophotometry with DPPH as radical scavenger. The antioxidant activity was found following order: seeds>skins>clusters; that might be explained by a corresponding decrease of activity with decrease of total phenolic contents.

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1. Introduction

Reactive oxygen species (ROS) including hydroxyl radicals ($\bullet\text{OH}$), superoxide radicals ($\text{O}_2^{\bullet-}$) and singlet oxygen ($^1\text{O}_2$) are generated as by-products of normal human metabolism. However, increased concentration of ROS induce the undesired oxidative stress causing various oxidative damages in main biological molecules (lipids, proteins and DNA), thus participating in many pathogenesis processes (cancer, coronary diseases etc) and ageing [1, 2].

The harmful action of these reactive species can be diminished or even blocked by an array of natural compounds, among those, polyphenols, due to their antioxidant properties, play a major role as potential free radicals scavengers. Polyphenols are multifunctional derivatives and their antioxidant activity is mainly due to their redox properties which contribute to absorption and neutralization of free radicals [1, 3]. A potential source of polyphenolic compounds are the grapes (particularly red) and their derivate products (wines). However, data of such products antioxidant properties related to their polyphenolic composition are quite limited.

In this study, we investigated the efficiency of certain extraction procedures for satisfactory polyphenolic composition in five grapes varieties and the extracts obtained were subjected to some "in vitro" tests in order to evaluate their antioxidant activity and correlate with the polyphenolic content.

2. Materials and methods

The grape varieties for red wine production: Burgund Mare (BMR), Cabernet-Sauvignon (CSR), Muscat-Hamburg (MHR), Merlot (MR), Merlot Dealu Mare (MDM) from a wine production area in Romania, (Recas, harvest from the 2005), were investigated. Three solid parts of grape: seeds, skins and clusters were used as materials for extraction production.

Extraction procedures: a) at room temperature: 1g of well grounded product was treated with 20 ml solvent (water, respectively ethanol: water 50/50, v/v) and mixture was subjected for 24 h to maceration with occasional stirring. The resulting crude material was filtered and volume adjusted to 20 ml with the corresponding solvent. b) at refluxing: the mixture vegetal-solvent was boiled one water bath, for 30 min. After cooling, the product was filtered, centrifuged, and volume adjusted to 20 ml.

Determination of total polyphenol content (TPF). Total polyphenols content was determined according to Folin-Ciocalteu methodology [4]. All samples extract were diluted at a ratio of 1:10 or more (as a function of their phenolic compound contents) before measuring. One ml of diluted sample was treated with 7.5 ml Folin-Ciocalteu reagent (Merck). After 5 min. of agitation, the mixture was treated with 4 ml 7.5% Na_2CO_3 solution. After standing 2 hrs at room temperature in dark, the samples absorbance was measured at 740 nm (Spectrophotometer UV-Vis, Jasco, V-530) vs. a blank. The absorbance values were multiplied by 10 or the appropriate dilution factor in order to obtain the polyphenols concentration. This was expressed as gallic acid mg/l solution, used as standard for calibration.

Determination of antioxidant activity (AO).

- a) *Spectrophotometry.* The DPPH antioxidant test was carried following the Brand-Williams method [5] with modifications [6, 7]. Three ml of freshly prepared DPPH solution (2, 5 mg in 100 ml methanol) was pipetted into a spectrophotometric cuvette ($l=1$ cm) and absorbance at 515 nm is measured (A_{t_0}). Then 5 μ l of sample extract were added and after 5 min., the absorbance is measured again. (A_{t_5}). The antioxidant activity, expressed as % of inactivation, was calculated from decrease of absorbance according to formula: % inactivation = $100 - (A_{t_5}/A_{t_0} \times 100)$ [7].
- b) *ESR spectroscopy.* Nitroxide radical 2,2,6,6-tetramethyl-4-hydroxypiperidine-oxyl (Tempol) was obtained from Fluka ($p > 97\%$) and was used as oxidative agent. 1mM aqueous TEMPOL solution was added in 2 ml grape extract. Samples were measured using 20 cm length, 1 mm inside diameter quartz capillary. EPR spectra were recorded at room temperature with a PORTABLE EPR SPECTROMETER, PS 8400, Resonance Instruments Inc., operating in the X-band (~ 9.5 MHz) and equipped with a computer acquisition system. The spectrometer settings used for the experiments were as follows: modulation frequency, 100 kHz; modulation amplitude 2×10^3 ; sweep width, 100 G; sweep time, 30 s; receiver gain 5×10^2 ; number of data points, 4096. Using the same parameters, the spectra were recorded at different time intervals. The variation of the relative concentration of radical species, were obtained through double integration of the experimental spectra.

Determination of EC 50. Five successive dilutions of each extract were prepared and 5 μ l of each dilution were transferred into plastic cuvettes containing 3 ml solution DPPH. The absorption, at 515 nm was recorded at 5 (initial rate of scavenging activity) respectively 60 min (total scavenging activity). The DPPH concentration lowered by 50% was calculated from a sigmoidal curve.

Statistical analysis. All measurements, if otherwise no specified, were run in triplicate ($n=3$). One-way Anova analysis of variation, probit analysis, sigmoidal curves fit, correlations were performed with a GraphPad Prism 4 programme.

3. Results and discussion

As showed in Materials and Methods, we attempted four extraction procedures. The assessment of the solvent system, and extraction parameters to accomplish an appreciable antioxidant activity was done using a representative index, the total polyphenol content. This parameter was determined using the well established and widely used Folin-Ciocalteu method [4]. Extraction in ethanol: water mixture (50/50, v/v) under reflux was found to be more efficient in recovering the antioxidants existing inside various mechanical parts of the grapes. From the

date given in Fig. 1, with one exception (MR variety), total phenolic content decreased from seeds>skins>clusters with significant different (one-way Anova, $p \leq 0,05$). The antioxidant capacity (reducing power) of 15 extracts was performed by 2 methods: ESR spectroscopy and spectrophotometrically using DPPH radical.

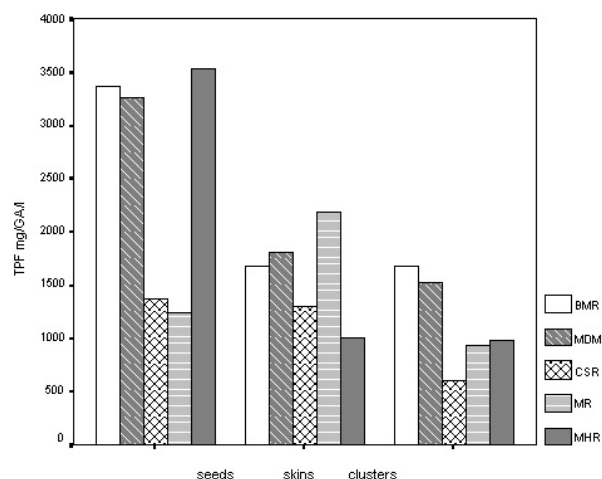


Fig. 1. Comparative diagram showed TPF contents in seeds, skins and clusters.

DPPH• molecule is a stable free radical compound that exhibits a strong absorption at 515 nm. When a reducing agent is added to DPPH the free electron is paired up and color is lost, which is measured as a decrease in absorbance [3, 6].

The antioxidant activity (AO) of the 15 hydroalcoholic extracts vs their phenolic contents (TPF) is shown in (Fig. 2).

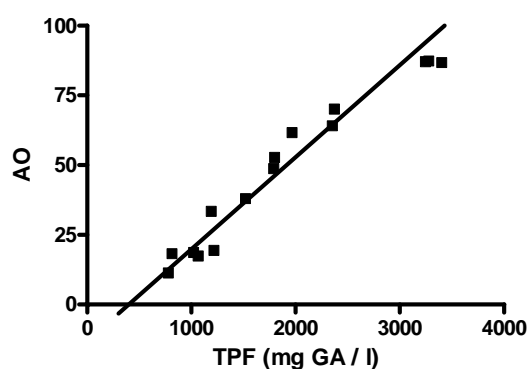


Fig. 2. Correlation between total phenolic contents (TPF) and extracts antioxidant activity (AO). (correlation coef. $R = 0.9552$, Pearson coef. $r = 0.9774$, $p < 0.0001$).

The statistical analysis showed a positive and highly significant correlation between total phenols and the antioxidant capacity. All 5 grapes varieties showed an evolution in antioxidant activity in the same order: seeds>skins>clusters, that might be explained by a decrease of activity with corresponding decrease of total

phenolic contents. This correlation suggest that, though the extracts may contain antioxidant products other than phenols, these do not contribute essentially to the antioxidant activity, and therefore, the radical scavenging activity of the extracts could be assigned mainly on the basis of their phenolic content. Similar correlations between antiradical efficiency and the polyphenolic content of red and white wines, has been reported in literature [2, 7-9].

The antioxidant activity of phenolics is due to their redox properties which allow them to act as reducing agents by donating hydrogen, by quenching singlet oxygen or by acting as metal chelators [10, 11-13]. One parameter that has been introduced quite recently for the interpretation of the results from DPPH method is the efficient concentration or EC_{50} . This is defined as the concentration of an agent that causes 50% loss of DPPH activity [2, 5]. The lower EC_{50} values correspond to higher antioxidant activities. The EC_{50} values of 3 richest extracts in phenols was thus calculated. This parameter was determined in five successive dilutions of each analyzed probe (probit analysis) and showed in Table 1.

Table 1. EC_{50} values for the hydroalcoholic extracts: BMR, MDM and MHR in DPPH test.

Extract	mg GA / l	
	EC_{50} , 5 min.	EC_{50} , 60 min.
BMR	1091	1014
MDM	1327	1260
MHR	1683	1364

The antiradical potency of extracts tested was dose-dependent since the scavenging activity of each sample diminishes once the total phenolic content decreased with dilution.

BMR-seeds extract exhibited an EC_{50} lower than the others, indicating the higher potency of its phenolic compounds.

The nitoxide radicals are relatively stable towards oxidation but they can be easily reduced to the corresponding hydroxylamines. The standard redox-potential of piperidine nitroxide derivatives ($E_0=0.2\text{eV}$ is high enough to oxidize biological compounds such as polyphenolics, ascorbic acid, semiquinones and superoxide radicals [14]. Thus, the number of paramagnetic species, decrease in time with different rates, depending on the sort of vines. Recording the EPR signal decay caused by the reaction with natural or artificial reductants or with product of metabolic reactions it is possible to draw conclusions about antioxidant capability.

In this paper we used a commonly encountered nitron spin label, nitroxidic radical Tempol (2,2,6,6-tetramethyl-4-hydroxypiperidine-oxy). The intensity of EPR signal in experimental grape extracts, was found to decrease in time and was correlated to the total content of polyphenols (Fig. 3). The rate of reaction between

polyphenolic compounds and Tempol was monitored by using double integrated residual EPR signal (Fig. 4).

Red wines are reported as rich sources of biologically active polyphenols (catechins, epicatechins, and gallic acid). They individually have been proved as having antioxidant and anticancer properties. Red wines that contains > 200 different phenolic compounds can be an important dietary source of polyphenols [15,16]. Though, less studied, the grapes must share similar patterns, so consumption of such fruits, could be associated with reduced risk of cancer especially of the gastrointestinal tract.

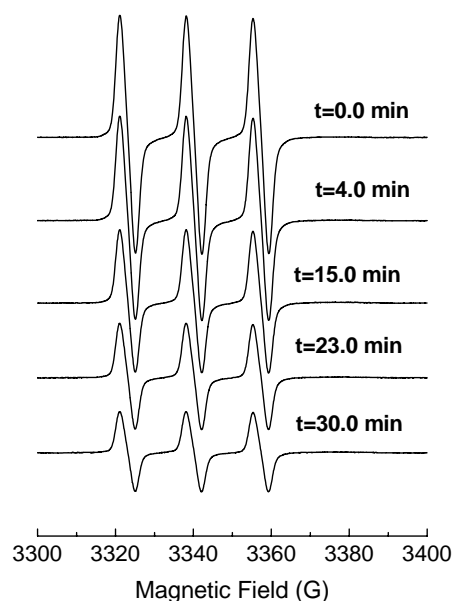


Fig. 3. The variation of the Tempol EPR signal in seeds extras (MR).

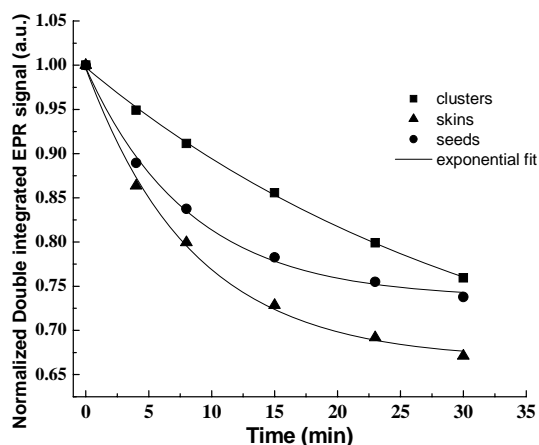


Fig. 4. Rate of decay of EPR signal of Tempol vs. extracts from parts of BMR.

4. Conclusions

The main results of our study can be summarized thus:

- an efficient polyphenols extraction from grapes might be achieved by employing, as solvent, aqueous ethanol (1:1), under reflux. The extracts with the most enhanced antioxidant capacity were from seeds.
- all extracts exhibited free radical-scavenging activity on DPPH and Tempol, in correlation with their total polyphenolic content.
- both spectrophotometry and ESR spectroscopy proved that Burgund Mare (BMR) seeds extract exhibited the highest potency of its phenolic compounds and it worth to be further investigated in biological experiments..

Currently, studies, aiming to investigate in vitro on cancer cells lines, the antioxidant, tumor-growth inhibitory activities and apoptose-inducing effect of the most active extracts are in progress in our laboratories.

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