Polyphenols, Antioxidant Activity and Anti-angiogenic Potential of Red and White Grapes

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The consumption of products derived from grapes, correlates with a low incidence of cardiovascular and degenerative diseases, especially for the red varieties. The skin and seeds of grapes, rich in phenolic compounds, with high antioxidant capacities, are known to be potentially active as anti-allergics, as well as in various types of cancer by acting through different pathways, possible by modulating mast cell effects and angiogenesis. In this study we evaluated alcoholic extracts of two varieties of grapes from Serbian Banat region (Srb) and two from Romanian Banat region (Ro): red grapes (Othello-Srb, Muscat Hamburg-Ro) and white grapes (Grocanka-Srb, Chasselas Dore-Ro). The content of polyphenols and antioxidant capacity was determined spectrophotometrically. The in vivo model of the chorioallantoic membrane (CAM) assay with the aid of stereomicroscopy was applied for the assessment of the anti-angiogenic potential. The results indicated that the red grapes are the richest in polyphenols while the white grapes indicated the highest antioxidant capacity. Reduced angiogenic process was correlated with the concentration of polyphenols and antioxidant capacity, being significant for both white and red grapes from Serbia. Thus, the evaluated varieties of grapes can be considered as nutraceutic products and sources of active compounds useful in the prevention and treatment of carcinogenesis.

Keywords: red grapes, white grapes, alcoholic extract, polyphenols, angiogenesis, chorioallantoic membrane

Grapes, the fruits of *Vitis vinifera* L., are largely used as dietary products. They represent a good source of nutraceutical phytochemicals, with a high content in polyphenols, as flavonoids, proanthocyanidins, stilbenes [1]. The consumption of grape products is known to correlate with the decrease in cardiovascular disease as well as degenerative diseases [2]. Many studies indicate grape seeds are highly rich in phenolics with excellent antioxidant activities, thus having chemopreventive potential in several types of cancers [3-5]. Although rare cases of grapes and wine allergy have been reported, caused by a protein on grapes skins [6], grapes seeds were recently associated with anti-allergic effects, possible through mast cell degranulation impairment[7].

Angiogenesis is an important natural process of blood vessel formation from preexistent ones, being essential to life. However, once deregulated it becomes pathogenic and even life threatening. Angiogenesis plays a key role in tumor microenvironment, facilitating growth, survival and dissemination of cancer cells. Since its discovery many anti-angiogenic molecules were developed, some of them representing important advances introduced in clinical therapy [8]. Due to adverse reactions of these therapies, modulating the angiogenesis process through multiple pathways has become an important area of research. Mast cells and other inflammatory mediators are interesting targets and several studies indicate nutraceuticals and mainly polyphenols as potential unique modulators [9,10].

Antioxidants from grape skin and seeds are known to exert inhibitory activities on different enzymes or growth factors involved and recently were reported to impair degranulation of inflammatory mediators from mast cell, but the mechanisms are still unclear [2,7]. Although red grapes and wine are the most studied, white grapes seem interesting sources of active compounds, with low content of resveratrol or anthocyans but having similar or higher concentrations of polyphenols [11]. Therefore we aimed to investigate the content of polyphenols, the antioxidant capacity and the effect on the angiogenic process using the chorioallantoic membrane (CAM) assay of two varieties of grapes Serbian Banat region (Srb) and two from Romanian Banat region (Ro): red grapes (Othello-Srb, Muscat Hamburg-Ro) and white grapes (Grocanka-Srb, Chasselas Dore-Ro).

Experimental part

Vegetal material

The vegetal material used in this study was represented by the entire fruit of *Vitis vinifera* L. from Serbian Banat region (Srb) and two from Romanian Banat region (Ro): red grapes (Othello-Srb, Muscat Hamburg-Ro) and white grapes (Grocanka-Srb, Chasselas Dore-Ro), harvested at full ripeness, in November from local farmer vineyards from Southern Banat region in Albunara, Serbia, respectively in Recas, Romania. The fruits were washed, sorted and dried in a convection oven at 45°C for 48 h until there was no...
change in weight, then reduced to concissum (dry plant material – dpm). Samples were kept in a refrigerator at 4°C.

**Extraction**

Red grape and white grape ethanolic extracts (RGE and WGE) were prepared; 3 g of dried material were extracted with 100 mL solution of 50% (v/v) ethanol. Extraction was conducted in an ultrasonic bath. For the biological evaluation on the chorioallantoic membrane, the solvent was removed from the samples using a rotary evaporator at 30°C.

**Polyphenol content**

Total polyphenols from the grape extracts were determined using Folin-Ciocalteu reactive and an adapted method of Singleton & Rossi [12]. In short, the grape extract was pipetted into a test tube containing Folin Ciocalteu's phenol reagent previously diluted with distilled water (1:2) and after 5 min at room temperature, saturated sodium carbonate solution (Na₂CO₃ 75 g/L) was added. The content was vortexed for 15 seconds and then left to stand at room temperature for 2 h in the dark. Absorbance measurements were recorded at 760 nm using a UV-VIS Analytic Jena Spectord 205, and gallic acid (GA) was used for the construction of the standard curve. Estimation of the phenolic content was carried out in triplicate. The results were mean values and expressed as mg of gallic acid equivalents (GAE)/g of dry plant material (dpm).

**Antioxidant capacity**

The antioxidant activity of the grape extracts was determined through the FRAP (ferric reducing antioxidant power) assay. The method is based on the increase in absorbance at 593 nm due to the formation of blue colored tri-pyridyl-S-triazine complexes with Fe²⁺ (TPTZ-Fe(II)) in the presence of a reducing agent [13]. Samples were mixed with the FRAP reagent which consist of 2, 4, 6-tripyridyl triazine (TPTZ) 10 mM and FeCl₃ 20 mM in acetate buffer 0.25 M, pH 3.6 and then diluted with distilled water. After 5 min the absorbance was registered at 593 nm. The antioxidant capacity of the extracts was expressed as μM of Fe²⁺ equivalents (Fe²⁺ E) per gram of dry plant material (dpm).

**CAM assay**

The anti-angiogenic potential of grapes extracts was assessed by the chorioallantoic membrane assay, using fertilized chicken (Gallus gallus domesticus) eggs. The protocol is based on the incubation of the disinfected eggs placed in a horizontally position at 37 °C and controlled humidity. In the third day of incubation 4 mL of albumen were aspirated [14]. The next day a window was cut into the shell in order to reveal the underlying capillary net, and incubation continued until the 7th day of incubation, when 10 μL of the samples were applied inside plastic rings on top of the CAM. Dry extracts of the grapes samples were dissolved in ethanol and then diluted with distilled water (ethanol: water = 1:3) to a final concentration of 1.5 g dry extract in 100 mL solvent. The alcoholic solution was also applied as blank sample. Samples were applied each day for 7 days and the evaluation was performed in ovo daily by the aid of a stereomicroscope (Zeiss Axio V16 Stereomicroscope). Significant images were registered using Zeiss Axio Cam and Zeiss ZEN software. Morphometric analysis of the vasoproliferative response was carried out using an adapted method of Folkman and Cotran [15]. Accordingly, evaluating the number of vessels that emerge to the ring or are present inside the ring, scores on an arbitrary scale was attributed to the tested samples: 0 – reaction similar to the blank sample, 1 – slightly increased vascular density, 2/3/4 – progressively increased vascular densities, 5 – very high vascular density, hyperemic reaction.

**Results and discussions**

**Polyphenol content**

The results indicate that the highest content of polyphenols expressed as equivalents of gallic acid was detected for both white varieties from Serbia and Romania, with a very high concentration in the Serbian variety Groanka 37.2 ± 0.2 mg GAE/g dpm and 12.4 ± 0.3 mg GAE/g dpm for the Romanian variety Chasselas Dore (table 1). Both tested red varieties had similar amounts of total phenols, with slightly higher values for the Serbian variety of Othello 12.3 ± 0.2 mg GAE/g dpm compared to Muscat Hamburg variety with 11.1 ± 0.9 mg GAE/g dpm (table 1). These values are also similar to the white Romanian variety. Interestingly, with very low content of anthocyanins
or resveratrol as the red varieties, while the white grapes seem to be very rich in phenolic compounds, most probable of flavonoid type. Whereas other different studies indicated that red grapes were more concentrated in polyphenols than the white ones [16]. Other studies that evaluate a multitude of grape varieties around the world are mainly focused on seeds or skin extracts and express the values on fresh weight or liquid extract.

**Antioxidant capacity**

The grapes extracts were also investigated in what concerns their antioxidant potential, using the FRAP test. The ferric reducing antioxidant power of the samples expressed as μmol Fe²⁺/g dpm are presented in Table 1. Results show that the highest antioxidant activity is expressed by the two red grape varieties which have similar values 203.7 ± 0.5 μmol Fe²⁺/g dpm for the Hamburg variety from Romania and 202.3 ± 1.2 μmol Fe²⁺/g dpm for the Othello from Serbia. Both white tested varieties of grapes presented lower antioxidant activities. The lowest was obtained by the Romanian Chasselas dore variety with only 39.0 ± 0.8 μmol Fe²⁺/g dpm. While Serbian Grokanka variety registered around half of the red varieties antioxidant potential 113.3 ± 1.2 μmol Fe²⁺/g dpm, value that does not correlate with the polyphenol content, which had the highest value among the tested samples. With low content in compounds as anthocyanins, the white varieties have a diminished reducing power. Although, the white Grokanka variety seems an interesting source of polyphenols with diminished reducing power. Although, the white Grokanka variety seems an interesting source of polyphenols.

### Table 2

<table>
<thead>
<tr>
<th>Variety</th>
<th>Color</th>
<th>Vascular density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>n.a.</td>
<td>4.5 ± 0.50</td>
</tr>
<tr>
<td>Othello</td>
<td>red</td>
<td>2.0 ± 0.50</td>
</tr>
<tr>
<td>Grokanka</td>
<td>white</td>
<td>2.3 ± 0.4</td>
</tr>
</tbody>
</table>

Mean ± SD (n = 3)

The blue-red wine grape varieties are reported by others as highest in polyphenols, among other well-known types Cabernet Noir, the hybrid Chambourcin, and the Romanian Black Babesca, while table grapes as black Napoca from Romania presents higher content of polyphenols than Muscat of Hamburg, but also some white varieties are to be remarked as Riesling or Chardonnay. Chasselas dore white grapes from Romania were also reported by others to have low content in polyphenols compared to different table grape varieties. Usually the antioxidant capacity was in correlation with the polyphenol content, though exceptions were signaled [16-22]. Very little information could be found about the two Serbian grape varieties and none referred to polyphenol content, antioxidant effect or anti-angiogenic potential.

**Anti-angiogenic effect on CAM**

Due to their high content in polyphenols we evaluated the anti-angiogenic potential of the two varieties of grapes from Serbia. The grape extracts were applied inside a plastic ring on the 7th day of incubation in order to evaluate the effect on the rapid growing capillaries that develop inside the chorioallantoic membrane of the chick at this stage of evolution (day 7 to day 11 of incubation). The specimens were daily monitored, and the most representative images were morphometrically analyzed, on the 11th day of incubation, after 4 applications. Capillary density converging to the ring and inside the ring was observed for grape samples and blank represented only by the solvent.

After 4 days of treatment, no toxicity was signaled for neither of the tested samples, all the specimens presented a high viability. The blank sample, showed a well interconnected net of capillaries, with a high number of fine vessels formed from previous ones (fig. 1a). The white grapes sample showed a moderate reduction of the capillary density inside the application site. Though capillary sprouting could be observed, the number of vessels was lower compared to the blank specimen, possible through a delayed angiogenic process (fig. 1b). The most intense activity on the development of the capillary net during an angiogenic stage was exerted by the red grape sample. The number of new formed vessels was very low in the area of the administration, inside and outside the ring, but there was no influence on the normal development of the CAM and embryo (fig. 1c).

Morphometric analysis that was performed in triplicate expressed semiquantitative scores that correlate with the intensity of the potential anti-angiogenic effect. The values are expressed as vascular density scores and are inverse correlated with the anti-angiogenic effect.

The strongest inhibition effect on the capillary formation is expressed by the red grapes extract which obtained the lowest vascular density score (2.0 ± 0.4). White grape extract with 2.3 ± 0.50 can also be considered an active extract on the angiogenic process. Both values are considerably inferior to the score obtained for the blank specimen. Quercetin was also tested (data not shown), but the score was higher, suggesting that the complex composition of the extracts, rich in diverse phenolic structures is synergic on the impairment of the vessel formation from existing vessels. Results are correlated with the antioxidant capacity of the extracts, mainly due to the polyphenols contained, which can be explained by the fact that they block oxidative damage which leads to the activation of angiogenic genes and factors [23].

Red grapes and wine are the highly researched for their biological activity and largely consumed for their preventive properties in cardiovascular and degeneratives diseases. The complex composition could be responsible for the inhibition of mast cell degranulation, a possible mechanism for the anti-angiogenic effect. While some white grapes,
although with a low content in resveratrol and anthocyanins, are even more concentrated in polyphenols especially in the skin and seeds [11,24]. These varieties are much less investigated. Here we report new data on the polyphenol content and the potential anti-angiogenic effect of a variety of white grapes from Serbia – Grocanka, and the results are very promising. The effect induced by the white grape extract has a very similar intensity with the red Othello variety. In the literature some evaluations on red and white wines can be found, indicating that red wines are superior, but for the preparation of white wine the grapes are used without the skins [24]. Our data present the effects of ethanol extracts obtained from the whole fruit.

Conclusions
Our study revealed that both red and white grapes can represent a source of natural compounds with important content of polyphenols and antioxidant activity. The varieties of grapes tested from Banat Region presented differences concerning the content in polyphenols and antioxidant effect, Romanian red Muscat Hamburg and white Chasselas dore having less polyphenols compared to the Serbian ones. The Serbian varieties with high phenolic content were active on the angiogenic process of the CAM. The red Othello grape extracts were the most effective, but the white Grocanka grape extracts showed very similar anti-angiogenic potential. The tested grapes exert inhibitory effects on the angiogenesis process, though may be useful in pathologies with exacerbated angiogenesis like cancer.

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References

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