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COMPARATIVE FREE RADICAL SCAVENGING CAPACITY OF THE SEED EXTRACTS OBTAINED FROM THE WHITE AND RED GRAPE BERRIES USED FOR WINE-MAKING IN TURKEY

Ayşen ÇÖLKESEN[1], Ahmet AYDIN[1], Aşkın IŞIMER[1], İlkay ORHAN[2,*], Bilge ŞENER[2]

[1]Gülhane Military Medical Academy, Department of Toxicology, 06018 Ankara, TURKEY

[2] Gazi University, Department of Pharmacognosy, Faculty of Pharmacy, 06330 Ankara, TURKEY

Abstract

Free radical scavenger capacity of the polyphenolic fractions obtained from ethyl acetate extracts of the seeds of sixteen red or white-colored samples of grape (Vitis vinifera-Vitaceae) species cultivated in Turkey has been determined in this study. On this purpose, the scavenger activity of V. vinifera extracts was evaluated via stable free radical 1,1-diphenyl-2-picryl hydrazyl ($DPPH^{\bullet}$) and superoxide radical ($O2^{-\bullet}$) generated by xanthine/xanthine oxidase (XOD) system. $DPPH^{\bullet}$ scavenger capacity of the extracts has been compared with the same concentrations of the known antioxidants such as BHT, vitamin C, gallic acid and quercetin. Vitamin C and gallic acid have been also used to compare $O2^{-\bullet}$ scavenger capacity of the extracts. Most of the extracts displayed significant $DPPH^{\bullet}$ scavenger activity.

Key words: *Free radical scavenging activity, Grape seed, Vitis vinifera, $DPPH^{\bullet}$, O_2^{-}*

Türkiye'de Şarap Yapımında Kullanılan Beyaz ve Kırmızı Üzümlerden Elde Edilen Tohum Ekstrelerinin Karşılaştırmalı Serbest Radikal Süpürücü Kapasitesi

Bu çalışmada, Türkiye'de kültürü yapılan 16 çeşit kırmızı veya beyaz üzüm (Vitis vinifera L.-Vitaceae) örneklerinin tohumlarının etilasetat ekstrelerinden elde edilen polifenolik fraksiyonların serbest radikal süpürücü etkileri tayin edilmiştir. Bu amaçla, V. vinifera ekstrelerinin süpürücü aktivitesi, stabil serbest radikal 1,1-difenil-2-pikril hidrazil ($DPPH^{\bullet}$) ve ksantin/ksantin oksidaz (XOD) sisteminin yarattığı süperoksit radikali ($O2^{-\bullet}$) aracılığı ile değerlendirilmiştir. Ekstrelerin $DPPH^{\bullet}$ süpürücü aktivitesi; BHT, vitamin C, gallik asit ve kersetin gibi bilinen antioksidanların aynı konsantrasyonları ile karşılaştırılmıştır. Vitamin C ve gallik asit, ekstrelerin ($O2^{-\bullet}$) süpürücü kapasitesini karşılaştırmak için de kullanılmıştır. Ekstrelerin çoğu dikkate değer $DPPH^{\bullet}$ süpürücü etki göstermiştir.

Anahtar Kelimeler: *Serbest Radikal Süpürme Kapasitesi,Üzüm Çekirdeği,Vitis vinifera, $DPPH^{\bullet}$, O_2^{-}*

*** Correspondence:** Tel: +90.312.2023186;
e-mail : iorhan@gazi.edu.tr,

INTRODUCTION

Reactive oxygen species are highly damaging transient chemical species formed in all cells and produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes (1). Excessive free radical production and lipid peroxidation are known to lead various disorders including cancer, atheroschlerosis, rheumatic diseases, cardiac and cerebral ischemia and degenerative diseases of the CNS such as Alzheimer's Disease (AD) (2). Although the antioxidant defense system includes both endogenously and exogenously-derived compounds, dietary antioxidants such as vitamin C and E as well as β-carotene have recently received a great attention (3,4). Phytophenolics, which are the important constituents of human diet, have been recognized as beneficial antioxidants that can scavenge harmful reactive oxygen species including $O_2^{-\bullet}$, H_2O_2, $HO^{\bullet}$, singlet O_2 and HOCl (5-7). Wine and other products derived from the grape, which is rich in procyanidins, have been demonstrated to have a high antioxidant capacity (8-10).

On the other hand, Anatolia has been a land for viticulture and wine-making since antiquity and it is one of the most cultivated fruit crops all over the world. According to the archeological ruins, wine-making and viticulture date back to B.C. 3500, due to very suitable climate conditions for vineyards in Turkey (11). Besides, Turkey ranges second after U.S.A in seedless and dried grape export. Although the Aegean region heads to the major grape production, the rest of Turkey also hosts many vineyards and wineries. Moreover, Turkey is the gene center of some types of grapes in the world.

Depending on the genetic and varietal diversity of Turkish grapes, their antioxidant capacity may show some differences. Therefore, the objective of the present study was to determine free radical scavenging activity of the ethyl acetate extracts of sixteen grape seed samples, obtained from the most popular wine-making grape cultivars of white and red colors in Turkey, against stable free radical $DPPH^{\bullet}$ and superoxide ($O_2^{-\bullet}$) radicals.

EXPERIMENTAL

Plant Materials

Sixteen types of the grape seeds, nine of which are used for wine production, and seven of which are used in table, are listed in Table 1. The seeds from Kavaklidere, Turasanlar, and Atatürk Orman Çiftliği (AOÇ) wine companies were obtained through the wastes discarded after wine-processing in these factories. Some of the materials (**GUZ**, **ABE**, **CAN**, **TAB**, and **KAK**) were collected by hand from their respective vineyards as indicated in Table 1. The **MUS** sample was purchased from Beğendik department store in Ankara, while the samples of **PAR** and **CAV** were

Table 1. Types, codes, colors, regions and collection sites of the grape seeds

Types and Codes of the Grapes Used	Colors	Regions of Cultivation in Turkey	Places Obtained
Semillon **(SEM)**	White	Tekirdağ	Kavaklidere Wine Factory
Narince **(NAR)**	White	Tokat	Kavaklidere Wine Factory
Öküzgözü **(OKU)**	Red	Elazığ	Kavaklidere Wine Factory
Carignane **(CAR)**	Red	Ceşme/Ovacık	Kavaklidere Wine Factory
Boğazkere **(BOG)**	Red	Diyarbakır	Kavaklidere Wine Factory
Papaz Karası **(PAK)**	Red	Nevşehir	Turasanlar Wine Factory
Emir **(EMI)**	White	Nevşehir	Turasanlar Wine Factory
Hasandede **(HAD)**	White	Ankara	AOÇ Wine Factory
Gül Uzümü **(GUZ)**	Pink	Ankara	A private vineyard in Ankara
Amasya Beyazı **(ABE)**	White	Amasya	A private vineyard in Ankara
Cardinal **(CAN)**	Purple	Tarsus	A private vineyard in Tarsus
Tarsus Beyazı **(TAB)**	White	Tarsus	A private vineyard in Tarsus
Müsküle **(MUS)**	White	Ankara	Beğendik Department Store
Parmak **(PAR)**	White	Nevşehir	Ankara-Local store
Çavuş **(CAV)**	White	Nevşehir	Ankara-Local store
Kalecik Karasi **(KAK)**	Red	Ankara	A private vineyard in Ankara

bought in a local store in Ankara. All of the seeds obtained were removed out of the grapes, and dried at room temperature. Prior to extraction, they were mechanically powdered.

Chemicals

Xanthine sodium, xanthine oxidase (XOD), nitroblue tetrazolium (NBT), *tert*-butyl-4-hydroxytoluene (BHT), superoxide dismutase (SOD), 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$), ethylene diamine tetraacetic acid disodium (EDTA), 3-(cyclohexylaminol)-1-propanesulfonic acid (CAPS), quercetin, sodium bisulfide, potassium dihydrogen phosphate, sodium hydroxyde, gallic acid, vitamin C, ethanol, hydrogen peroxide and hydrogen florin (Merck Co., Germany), methanol, ethyl acetate and chloroform (Carlo Erba, Italy), and Triton X-100 (Riedel de Haen, Germany) were employed in the experiments.

Preparation of polyphenolic fractions from the seeds

5 g of each type of the grape seeds was weighed separately and the extracts were prepared according to the extraction method described by Gaulejac et al. (12). Briefly, 10 mL of (1 g/L) $NaHSO_3$ solution and 10 mL of ethanol were added to the seeds and mixed on a magnetic stirrer for 2 min. After adding 20 ml of chloroform to each seed fraction, they were stirred again for a minute. Then, the mixtures were transferred into centrifuge tubes and centrifuged at 3000 rpm

for 10 min. Each mixture was divided into three portions. Hydroalcoholic solution at the top phase was separated by a pasteur pipette. Seed leftovers in the middle and chloroform phase at bottom were also separated from each other by filtration. Chloroform phase was discharged. The procedure was repeated six times for each extract. After combining the hydroalcoholic phases, they were evaporated at 30 ºC until only aqueous solution remained. The aqueous solutions were extracted with 20 mL of ethyl acetate for six times. Combined ethyl acetate extracts were evaporated at 30 ºC until dryness and the extracts obtained were stored at deep-freezer until experimental work.

Method for determination of DPPH· radical scavenger activity

1.950 mL of 0.0001 M methanol DPPH· solution and 50 µL of different concentration of each extract (20 to 400 mg/L) were added to a spectrophotometer cuvette and were shaken vigorously (Sigma, USA) (13). Decrease in absorbance at 515 nm was recorded in every 15 minute until the reaction was fixed in a spectrophotometer (Shimadzu UV-265, Japan). Remaining percentage of DPPH· was calculated by applying the standard calibration graphic, DPPH·/Methanol solutions at 10.9, 11, 12.5, 14, 16, 20, 25, 33, 50 µM concentrations. Measurements were done in triplicate for each sample and EC_{50} values were calculated.

Method for determination of superoxide radical scavenger activity

In this method, $O_2^{-\cdot}$ was produced by xanthine/xanthine oxidase (X/XOD) system in reaction medium (14). 1.7 mL of the reaction mixture containing CAPS 50 mmol/L, EDTA 0.94 mmol/L, xanthine sodium 0.05 mmol/L, NBT 0.025 mmol/L, pH 10.2 and 50 µL of different concentration of each extract (20 to 400 mg/L) were added to spectrophotometric cuvette. After adding 250 µl of xanthine oxidase (1.67 U/mL), the absorbance was measured at the beginning (A_1) and at 10^{th} minute (A_2) against air at 560 nm. Absorbance difference was calculated (A_2-A_1) for the samples, and coded as P_x. 50 µL of phosphate buffer was used in the same reaction medium in place of extract and P_1 was calculated. 50 µL of phosphate buffer in place of extract and 250 µL of distilled water in place of xanthine oxidase were used in the same reaction medium and P_0 was calculated. Unscavenged $O_2^{-\cdot}$ % was found according to the equation given below, which later the EC_{50} values were calculated:

$$\text{Unscavenged } O_2^{-\cdot} \% = (P_x - P_0) \times 100 / (P_1 - P_0)$$

Statistical analysis

Mann Whitney U test was employed in order to compare the relationship between white or red-colors of the grape seeds used and their antioxidant activity. The values were considered statistically significant if the *P* value was less than 0.05. [* $p<0.05$].

RESULTS AND DISCUSSION

Comparative antioxidant data of sixteen grape seed extracts (nine types in white color and five types in red color, one type in pink color and one type in purple color) were determined by evaluation of their DPPH$^{\cdot}$ (chemical method) and $O2^{-\cdot}$ (enzymatic method) free radical scavenger capacities (Table 2). DPPH$^{\cdot}$ scavenger activity of the extracts was compared with the known antioxidant substances such as *tert*-butyl-4-hydroxytoluene (**BHT**), vitamin C (**Vit. C**), gallic acid (**GAL**) and quercetin (**QUE**). Among the reference compounds, while the most effective one was **GAL** against both DPPH$^{\cdot}$ and $O2^{-\cdot}$ radicals, the rest of them was found to be ineffective against $O2^{-\cdot}$ radical. In DPPH$^{\cdot}$ free radical scavenger assay, the highest level of antioxidant activity was observed with **HAD** extract, while **KAK** extract had the least scavenger capacity. All of the extracts were more active than BHT. Among the extracts, **EMI**, **TAB** and **ABE** have exhibited the same activity as vitamin C in this assay. Although **KAK** was less active than vitamin C, the rest of cultivates were more active than vitamin C. When compared with **QUE**, both **SEM** and **HAD** extracts displayed a higher activity. All of the extracts have yielded lower capacity than that of **GAL**. When the $O2^{-\cdot}$ scavenger capacity of the extracts were evaluated, the seed extract of **TAB** was the most active, followed by **GUZ** and **NAR** was the least. In addition, none of the extracts investigated (except **TAB** extract) showed a remarkable antioxidant activity when assayed against the $O2^{-\cdot}$ radical *in vitro*.

Table 2. EC_{50} values of the grape seed extracts for $DPPH^{\cdot}$ and $O_2^{-\cdot}$ Radicals

Extracts	EC_{50} for $DPPH^{\cdot}$ mg/L	EC_{50} for $O_2^{-\cdot}$ mg/L
SEM	108.1±5.2	184.3±10.0
NAR	113.8±2.2	194.5±3.7
OKU	113.9±6.3	176.4±5.2
CAR	101.2±2.8	104.8±0.8
BOG	108.5±8.0	104.5±2.0
PAK	85.2±0.9*	180.0±1.5
EMI	126.7±4.9	115.0±2.6
HAD	66.6±1.7*	100.3±3.5
GUZ	105.2±1.3	74.5±7.0*
ABE	126.7±6.6	99.6±5.0
CAN	101.2±3.2	104.8±1.4
TAB	126.4±5.0	66.8±1.9*
MUS	82.2±7.8*	100.4±5.0
PAR	115.6±2.1	111.7±6.7
CAV	110.0±2.6	184.0±5.0
KAK	183.8±6.4	109.3±6.3
BHT	194.9±2.1	-
QUE	85.0±6.2	-
Vit. C	125.8±5.2	-
GAL	40.0±2.4	39.4±3.0

* $p<0.05$

Among the grape seed extracts studied herein, $O2^{-\cdot}$ scavenger activities of **HAD**, **MUS**, and **PAK** were found to be the statistically most important as compare to **GAL**, while **HAD** and **MUS** had even better EC50 values than **QUE**.

On the other hand, wine, grapes and other products derived from the grape, essential components of the Mediterranean diet, have a high antioxidant capacity, which have been mainly attributed to their polyphenolic-type components, and the byproducts of the wine-making process represent a rich source of antioxidant compounds. Grape skins are also known to be rich in the potential antioxidant compounds, namely trans-resveratrol (trans-3,5,4'-trihydroxystilbene) and piceid (3-O-β-D-glucoside) (15). Overall in the plant kingdom, polyphenols or phenolic compounds account for well over 4000 individual compounds (16). Grape seeds, as well, have been reported to be quite rich in polyphenolic compounds including proanthocyanidins, flavonoids, tannins, caffeic acid, rutin, catechin, myricetin, quercetin, epicatechin and nonflavonoids (17-19). Proanthocynanidins, polymers or oligomers of catechin units, are the major polyphenols in red wine and in grape seeds, particularly. A few studies have been performed on the chemistry of the Turkish grapes. One of them reported the total amount of phenolics in grapes of Narince type, which was also included in our study and coded as **NAR** (20). Recently, one study on antidiabetic and antioxidant activities

of the ethanolic extract of *V. vinifera* of Turkish origin reported that the extract was found to be active at 250 mg/kg dose on glutathione and malonedialdehyde levels on rat tissues (21). However, there has been no detailed study so far on the antioxidant capacity of the Turkish grape seeds.

CONCLUSION

These results from our study summarized here indicated that the apparent antioxidant potential of the extracts may possibly depend on the radical species or assay method used to determine the activity or their polyphenolic contents. Besides, our results also demonstrated that no relationship was determined between scavenger capacity and the colors of the grape seed extracts studied. The intake of unpeeled grapes could be recommended in nutritional habits as a potential source of antioxidant and anticarcinogenic phenolic compounds and the leftovers from the grapes used in wine production could be evaluated as an alternative nutraceutical for human health. To best of our knowledge, this is the first detailed report on the free radical scavenger activity of the most popular grapes used by the major wine producers in Turkey.

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