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CHARACTERIZATION OF GRAPE SEED EXTRACTS OF NATIVE TO GEORGIA VARIETIES OF *VITIS VINIFERA* L.

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Abstract

To establish the possibility of application in cosmetology, industry of biologically active additives (BAA) or for other reasonscontent of tocopherol, total phenols, anthocyanins, proline, total proteins, soluble carbohydrates and also total antioxidant activity was studied both in unfermented and fermented seeds of two species of grape vine –*Vitis vinifera* L. (Georgian varieties Rkatsiteli, and Saperavi) and *V. labrusca* var. *izabella*, and in fermented and unfermented berry skin of Rkatsiteli, and Saperavi as well using spectrophotometrical methods. It was revealed that some amount of the studied substances remained in residues of wine making industry (both in seeds and berry skin).Thus there is a possibility of their further processing and application.

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Key words: Antioxidants, Fermented andnon-fermented grape seeds, Georgian grapevine varieties Rkatsiteli and Saperavi, *V. labrusca* var. *izabella*.

Introduction

Grapevine is one of the widely spread and valuable fruit all over the world. It is the source of a number of biologically active substances like fatty acids, vitamins, phenolic substances, etc. (Glampedaki & Dutschik, 2014). Its derivatives are used in food industry, perfumery, for medical purposes (Zhu *et al*., 2015). Grape seeds are very popular as well. Their extraction is characterized by an ability to bind free radical (Badavi *et al*., 2013). The extract is also widely used as a toxin absorbent (Irem *et al*., 2013).

Seed substances regulate the concentration of blood glucose and cholesterol, inhibit synthesis of histamine and correspondingly revealanti-allergic effect. In addition, they have a positive impact on blood flow, help in regenerating tissues and defending organism against oxidative damage (Satyam & Bairy, 2014); slow down the aging of skin, positively affect memory and vision, possess antiviral (Ali, 2002) and anti- inflammatory effects (Joshi *et al*., 2002); They have anticancer properties and impede the multiplication of tumor cells (Hemmeti *et al*., 2015).

It has been established that a number of diseases like cancer, diabetes, cardiovascular diseases, autoimmune disorders, neurodegenerative diseases and others are caused by free radicals (Doaa *et al*., 2015). Antioxidants bind free radicals and protect vital substances, like lipids, proteins, enzymes and carbohydrates from damage (Poljask *et al*., 2013). They are effective against antibiotic-caused nephrotoxicity (Lopez-Alarcona & Denicola, 2013). Grape seeds show an antioxidant activity 50 times greater than vitamins C and E (Bagchi *et al*., 2002).

Viticulture and winemaking are the ancient and significant field of Georgian agriculture. The history of grapevine cultivation is in close relation with the history of the Georgian nation. As indicated by the most recent research, Georgia is considered one of the first origins of cultivated vine (*Vitis vinifera* L.) (Ekhvaia &Akhalkatsi, 2006, Imazio *et al*., 2013).

Accordingly, viticulture and wine-making are the priority and high-profitable field in Georgia, responsible for the economic sustainability and progress of the country. In spite of the world experience, utilization of grape seeds is irrational and not fully used in Georgia. Though, it must be mentioned that this refers to residues of wine made by "Kakhetian" style. About 80% of the grape-cake (25-27% of which is made of seeds) at best may be used as food in livestock farming, or is thrown without any use. The volume of the grape-cake remained every year in Georgia after the wine making is so significant that elaboration of the effective ways of its utilization is very urgent.

Accordingly, we focused our attention on fermented seeds of grape (in Georgian "Chacha"), which remains after application of "Kakhetian" method of making wine, and may become very effective raw material for biologically active additives (BAA). Creation of new products from this type of grape waste, which may be used in livestock farming, or in medicine in form of bio-additives, seems very perspective. Detection of new sources of natural waste, for the purpose of creation of BAA, is extremely important for health care.

Grape seeds residuary after wine making by the European method represent valuable raw material for pharmaceutical industry (Jockers, 2013) but attempts to use grape seeds obtained after preparing the wine by "Kakhetian" method have not been made yet.

The purpose of this study was to investigate the content of some antioxidants in fermented grape seeds residue after making wine by "Kakhetian" method, and to establish the possibility of their further application in cosmetology, the industry of BAA or for other reasons.

Materials and Methods

Plant material: The most cultivated species in Georgia are *V. vinifera* L. and *V. labrusca* L. From these two species including three varieties such as var, Rkatsiteli (white) and var. Saperavi (red), of *V. vinifera* L. and. var. *izabella* from *V. labrusca* L. For research seven different experimental materials were chosen. All experimental material was supplied from the private vineyard of one of the authors (Maia Akhalkatsi) situated in village Shilda of Kvareli region (Georgia). Sap was squeezed out from berries to obtain unfermented seeds and berry skin of the experimental plants. Residual seeds and skin were dried at room temperature and ground for analysis. Fermented seeds and berry skin were received from the mentioned person after preparing wine by "Kakhetian" method. The content of tocopherols, proline, anthocyanins, soluble phenols, total proteins, soluble carbohydrates, and total antioxidant activity as well was studied in the experimental material.

Total proteins: Content of total proteins was studied after Lowry (1951). Bovine serum albumin served as a standard.

Anthocyanins: For determination of anthocyanins, 1g of experimental material was ground with 20 ml of ethanol and 2% HCl solution, and filtered. The extinction of the extract was measured at 529 nm by the spectrophotometer (SPEKOL 11, KARL ZEISS, Germany) (Caldwell, 1968).

Tocopherol determination: 500 mg of ground material was extracted with 20- 25ml of pure ethanol three times, at room temperature. 20 ml of 60% potassium hydroxide were added to the combined extract and it was saponified on water bath during two hours. Distilled water was used to wash the combined extract up to the point when alkaline residuals were fully removed. Later water was removed with Na2SO4, after which the received solution was evaporated on water bath, cooled and then added to the mix of alcohol-nitric acid (1 ml of concentrated HNO3: 5ml of 96° alcohol). The solution was boiled for three minutes until it turned dark red. Extinction of the extract was measured at 470nm by the spectrophotometer (SPEKOL 11, KARL ZEISS, Germany) (Fillipovich *et al*., 1982).

Proline: 0.1 g of grape seed powder was mashed in 10ml of 3% sulphosalicylic acid and filtered. 2 ml of the resulting filtrate was added to 2 ml of acid ninhydrin and 2 ml of acetic acid. After one hour exposition on water bath, 4 ml of toluene was added to the cooled extract and divided in separating funnel.The optical density of upper layer was measured on a spectrophotometer at 520 nm by the spectrophotometer (SPEKOL 11, KARL ZEISS, Germany) (Bates,1973).

Total phenols: 250 mg of grape seeds powder was boiled for 15 min in 80% ethanol. After centrifugation, the supernatant was saved, and residues of powder were mashed in 60% ethanol and boiled for 10 min. The extract was added to the primary supernatant and evaporated. The sediment was dissolved in distilled water. One ml of the obtined solution was added with the Folin-Ciocalteu reagent and optical density was measured at 765 nm by the spectrophotometer. The chlorogenic acid served as control (Badridze*et al*., 2015).

Soluble carbohydrates: Anthrone reagent was used for determining the amount of soluble carbohydrates (Turkina &Sokolova, 1971).To 100 mg of powder 96% alcohol was added for extraction (3-fold extraction). The total amount of the received extract was evaporated on the water bath and later dissolved in 5 ml of distilled water. To 0.5 ml of the tested water, the extract was added 2ml of anthrone reagent and heated in a water bath for 10 min. Further, the test-tubes were placed in the cold water bath and after 15 minutes the optical density of the solution was measured at 620 nm by the Spectrophotometer.

The total antioxidant activity: This index was measured by modified method using diphenyl-picrylhydrazyl (DPPH) (Koleva *et al*., 2002). 200mg of experimental powder was extracted two times with 96o ethanol. The extract was evaporated on a water bath and the remained sediment was dissolved in 10ml of water- alcohol mixture. 0.01 ml of the obtained solution was added with 4ml of 40μM DPPH solution and after 30 minutes of incubation in the dark, the optical density was measured at 515 nm by the spectrophotometer. The percent of inhibition was calculated.

The statistical processing of data: Means, standard deviations and minimal and maximal values were calculated as a separate data of sample quantities (Table 1). Means were compared using one-way ANOVA ($p<0.05$) post hoc range tests, to investigate the differences (Table 2). In order to determine the overall between-group differentiation, individual F-values were calculated for continuous variables of each quantitative leaf descriptor. Software package SPSS v.13.0 for Windows was used for the analysis.

Results and Discussion

Tocopherol: From the obtained results it is clear that content of tocopherols in unfermented grape seeds was higher compared with unfermented berry skin (Table 1). Unfermented seeds of Rkatsiteli were especially distinguished by this index (Table1).After fermentation content of tocopherols was decreased both in seeds and berry skin by 25-32% on average. However, the quantity of tocopherol was changed slightly in Rkatsiteli berry skin.Tocopherol is an antioxidant, which is synthesized in plants and in cyanobacteria as well. Its high concentration correlates with the intensity of different abiotic stresses (intensive light, salinity, drought, low temperature). Tocopherol synthesis was an important biochemical process in seed evolution. The fact that seeds contain it in great quantity and its synthesis prove this (Gill & Tuteja, 2010). It is established that tocopherol increases the immunity and supports to avoid cancer, cardiovascular diseases, stroke, cataract, aging. It is effective in a case of diabetes, positively affects the nervous system, wound healing, protects lungs from polluted air. In a human organism, tocopherol acts in pair with ascorbic acid. It scavenges free radicals and impairs peroxidation processes (Shao *et al*., 2008). Thus, the presence of tocopherol in waste products of wine-making, discovered in our experiments, increases their, as secondary products, practical value.

Table 1. Effects of chemical extract composition of grape seeds and berry skins of three grapevine varieties ‘Rkatsiteli’, ‘Saferavi’ and ‘Isabella’. The material data are milligrams (mg).

Characters	Data	TPH	ANC	PRN	PRT	PHS	CBH	ANO
RNBS	Mean	3,5767	2,4233	4,3767	131,4533	362,0333	7,4667	21,69
	SD	0,1079	0,353	0,1079	1,2307	10,2627	0,2517	0,3568
	Min	3,5	2,1	4,3	130,2	350,2	7,2	21,3
	Max	3,7	2,8	4,5	132,66	368,5	7,7	22,0
RWBS	Mean	3,26	1,74	1,2	36,5767	83,5667	3,0	12,45
	SD	0,2425	0,2506	0,1	0,402	1,1015	0,1	0,1609
	Min	3,0	1,5	1,1	36,2	82,5	2,9	12,27
	Max	3,48	2,0	1,3	37,0	84,7	3,1	12,58
RNGS	Mean	9,33	2,89	4,3933	99,47	567,4333	3,5033	83,3067
	SD	0,1539	0,3568	0,3523	0,3559	0,5859	0,395	0,1901
	Min	9,2	2,5	4,0	99,1	567	3,11	83,12
	Max	9,5	3,2	4,68	99,81	568,1	3,9	83,5
RWGS	Mean	6,27	2,8	1,5533	19,3567	181,1167	1,7067	24,5333
	SD	0,2524	0,3	0,0503	0,2228	0,852	0,1901	0,1155
	Min	6,0	2,5	1,5	19,1	180,3	1,52	24,4
	Max	6,5	3,1	1,6	19,5	182	1,9	24,6
SNBS	Mean	4,8967	3,7567	3,4667	124,9	135,1	2,31	91,1333
	SD	0,1762	0,2836	0,2309	0,3606	0,3606	0,2007	0,162
	Min	4,7	3,43	3,2	124,5	134,8	2,1	91,03
	Max	5,04	3,94	3,6	125,2	135,5	2,5	91,32
SWBS	Mean	3,6867	2,4033	2,0367	77,1833	68,4233	1,35	39,6733
	SD	0,1026	0,1818	0,0513	0,0981	1,157	0,15	0,0252
	Min	3,6	2,2	1,98	77,07	67,2	1,2	39,65
	Max	3,8	2,55	2,08	77,24	69,5	1,5	39,7
SNGS	Mean	7,43	8,64	4,4433	89,4167	551,31	11,6333	91,3333
	SD	0,3897	0,3816	0,1401	0,1041	0,5986	0,1528	0,1528
	Min	7,0	8,2	4,33	89,3	550,93	11,5	91,2
	Max	7,76	8,88	4,6	89,5	552,0	11,8	91,5
SWGS	Mean	5,3333	4,8033	4,2333	54,5533	282,95	2,7667	80,0233
	SD	0,3512	0,2608	0,1155	0,3281	1,0476	0,2887	0,0681
	Min	5,0	4,61	4,1	54,33	281,8	2,6	79,97
	Max	5,7	5,1	4,3	54,93	283,85	3,1	80,1
INGS	Mean	4,6067	2,72	2,6167	82,5667	451,31	4,9	44,05
	SD	0,1901	0,0854	0,1041	0,3512	0,5986	0,3606	0,4822
	Min	4,42	2,63	2,5	82,2	450,93	4,6	43,7
	Max	4,8	2,8	2,7	82,9	452	5,3	44,6
IWGS	Mean	3,1333	-	1,2133	65,5667	-	4,35	43,7333
	SD	0,1528	-	0,2203	0,3055	-	0,1803	0,4726
	Min	3	-	1	65,3	-	4,2	43,2
	Max	3,3	-	1,44	65,9	-	4,55	44,1

Abbreviations:RNBS: Rkatsiteli natural berry skin; RWBS: Rkatsiteli wine making berry skin; RNGS: Rkatsiteli natural grape seed; RWGS: Rkatsiteli winemaking grape seed;SNBS: Saperavi natural berry skin; SWBS: Saperavi wine making berry skin; SNGS: Saperavi natural grape seed;SWGS: Saperavi wine making grape seed; INGS: Isabella natural grape seed;TPH: tocopherol; ANC: anthocyanins; PRT: proteins;PHS: phenols;CBH: carbohydrates; PRN: proline;ANO: antioxidant activity.

Table 2. ANOVA indices of the investigated antioxidants of grape seeds and berry skin of three grapevine varieties 'Rkatsiteli', 'Saferavi' and 'Isabella'.

Materials		Sum of squares	df	Mean square	F	Sig
TPH	Between groups	109.722	9	12.191	228.259	0.0001
	Within groups	1.068	20	0.053		
	Total	110.790	29			
ANC	Between groups	139.701	9	15.522	210.007	0.0001
	Within groups	1.478	20	0.074		
	Total	141.179	29			
PRN	Between groups	51.711	9	5.746	193.717	0.0001
	Within groups	0.593	20	0.030		
	Total	52.304	29			
PRT	Between groups	34586.443	9	3842.938	16503.777	0.0001
	Within groups	4.657	20	0.233		
	Total	34591.100	29			
PHS	Between groups	1150421.327	9	127824.592	11527.301	0.0001
	Within groups	221.777	20	11.089		
	Total	1150643.104	29			
CBH	Between groups	264.695	9	29.411	492.255	0.0001
	Within groups	1.195	20	0.060		
	Total	265.890	29			
ANO	Between groups	25051.567	9	2783.507	39021.132	0.0001
	Within groups	1.427	20	0.071		
	Total	25052.994	29			

Anthocyanins: The highest amount of anthocyanins was found in intact seeds of Saperavi (Table 1). It was 2.6-3.7 times higher compared with other unfermented variants. Fermentation decreased the content of anthocyanins in all experimental variants. In fermented berry skins of both, Saperavi and Rkatsiteli the index was decreased by 25-27%. Fermentation caused noteworthy reduction of anthocyanins in Saperavi seeds (48%) also. Though, their content here remained higher of all unfermented variants (Table 1).Anthocyanins are flavonoid group substances, concentrated mainly in vacuoles and reveal strong antioxidant property. They possess the metal ions binding ability, which takes an active part in oxidation processes. Accumulation of anthocyanins in vacuole prevents their direct contact with the localities of active oxygen species formation. Though, the increase of anthocyanins different types of stress has been established (Badridze, *et al.*, 2015)A high content of anthocyanins is found in tea, vegetables, cocoa, crops, etc. These classes of plant substances often are named as bioflavonoids for underlining their multilateral effect on a human health. Anthocyanins are taken with a meal in the mix with other flavonoids. Their accepted daily norm is 500-1000mg. Though, if anthocyanins are received as flavonoid bio-additives (grape seed extract, Gingko biloba, etc.) their amount may be even several grams.

Total phenols: According to obtained data grape seeds contained a higher amount of phenols compared to berry skin (Table 1). Moreover, results of Rkatsiteli and Saperavi seeds were similar. The content of phenols in Isabella seeds was a bit lower compared to other seed variants. Quite high was the content of soluble phenols in Rkatsiteli berry skin as well, compared to Saperavi. Fermentation resulted in significant decrease of phenols both in seeds and berry skin (Table 1). Especially evident was an abatement of the index in tested variants of Rkatsiteli (68% and 77%, seeds and berry skin respectively. In less extent decreased the index in Saperavi (48% and 49% seeds and skin respectively was decreased.Phenols are the most active metabolites in the plant with the antioxidant activity, they exceed even ascorbic acid and tocopherols (Hernandez *et al*., 2009). Phenols neutralize the reactive oxygen species before they damage the cell (Lovdal *et al*., 2010).Phenols represent the most popular group of phyto substances, as they are widely spread among plants and in food. Their positive role in some aspects of health has been proved many times (Kroon & Williamson, 2005). The fact that scientists try to increase the content of phenols in plants, obtain derivatives with improved pharmacological properties, for their beneficial effects (Boskou, 2006).

Proline: Content of the amino acid proline in Saperavi and Rkatsiteli unfermented variants was almost equal (Table 1). Fermentation in every single tested variation was decreased except Saperavi. In Rkatsiteli berry skin it was decreased by 74%, in seeds–by 60%; in Isabella, seeds reduction was 63%. As for Saperavi, the decrease of proline was comparatively low (36%) in berry skin and remained almost the same in seeds (Table 1).

Besides taking part in proteins structure, proline is one of the widely spread competitive substances, which accumulates in plants and bacteria under unfavorable (low temperature, drought, salinity, etc. conditions). (Kuznetsov *et al*., 1999; Anjum *et al*., 2000) Moreover, it is supposed that proline takes part in flowering and development both as metabolite and as a signaling molecule. Proline takes part in cartilage and collagen formation. It keeps muscles and joints flexible and diminishes skin flabbiness and shrinking caused by ultraviolet irradiation or aging. Accordingly, proline additives may be useful in cases of osteoarthritis, chronic back pain and persistent soft tissue strains. Proline is formed from glutamic acid, and its shortage is rare in healthy individuals with a healthy diet. However, people recovering from traumatic injury, particularly skin injuries such as severe burns, may want to supplement this amino acid. People with pain caused by collagen formation or insufficient cartilage could benefit from extra proline in their diet as well. Proline may be in supplements used to promote cardiovascular health, usually in combination with vitamin C. The recommended therapeutic daily dose is between 500 to 1,000 mg, with vitamin C. Evidently, presence of proline and other above -mentioned antioxidants in fermented residues of wine-making increases the value of the by-product for its wasteless processing.

Soluble carbohydrates: Among the studied varieties of the grape vine the highest content of soluble carbohydrates was detected in unfermented seeds of Saperavi (Table 1). Since soluble carbohydrates represent the main substrate for alcoholic fermentation, it was expected to receive the significant decrease in their concentration in fermented seeds (in Saperavi seeds it reduced by 4.4 times in comparison with the unfermented variation, in other variations-by 2-2.5 times). Though, a few soluble carbohydrates were detected in fermented variants (Table 1) but it must be mentioned that content of carbohydrates was same in fermented seeds of Isabella. According to biochemical, molecular and genetic experiments soluble carbohydrates play the significant role in plant metabolism and growth, and development regulation. Accumulation of carbohydrates increases under stress conditions in different parts of the plant. Soluble carbohydrates have stress protective role (Kolupaev & Kaerec, 2010). It is established that under stress conditions carbohydrates reveal poly-functional, protective and regulative activity that means both Osmo-protective and antioxidant effect on proteins and other macromolecules. The significance of carbohydrates is demonstrated not only by participation in the synthesis of other substances but in membrane stabilization function. The are regulators of gene expression and signaling molecules as well (Pego *et al*., 2000).

Total proteins: High content of total proteins was detected in the unfermented seeds and berry skin of the studied varieties (Table 1). Though, seeds were with a bit of berry skin by this index. The significant decrease of proteins was mentioned in fermented variants of Rkatsiteli (by 72.5% and 80% respectively in skin and seeds). The index in Saperavi (by 38-39%) and Isabella (by 20%) experimental variations (Table 1) diminished to a less extent. As a result, the content of proteins appeared to be higher in fermented seeds and skin of Saperavi compared to Rkatsiteli.Proteins may serve as excellent antioxidant additives in food, because of their ability to inhibit lipid oxidation. Recently, bioactive peptides from enzymatic hydrolysis of various food proteins such as wheat gluten, soy protein, gelatin, casein, and whey protein have been shown to possess antioxidant activity (Elias *et al*., 2008).

Significant retention of proteins in the tested waste of wine-making (especially in red varieties) is one additional factor for processing of these residues for further application.

The total antioxidant activity: It can be concluded based on experimental results that unfermented seeds of Saperavi and Rkatsiteli and unfermented berry skin of Saperavi have high antioxidant activity (Table 1).

Fermentation significantly decreased this index in most experimental variants. In particular, the antioxidant activity of Rkatsiteli berry skin, which was not high in unfermented variants as well, after fermentation decreased by 1.7 times, while of seeds–by 3.4 times. Antioxidant activity of Saperavi skin diminished by 2.3 times after fermentation, while in seeds the index abated slightly (by 1.1 times). As for Isabella seeds fermentation did not affect its antioxidant activity (Table 1).Total antioxidant activity is a significant integrated index, dealing with data on summarized antioxidant activity of the tested material, without specifying the class of substances Correlation between the content of hydrophilic antioxidants and total antioxidant activity has been demonstrated in some publications (Wu *et al*., 2004). According to our data, it may be supposed that mainly the content of hydrophilic anthocyanins and total phenols is responsible for the total antioxidant activity of the tested variants.

Conclusions

By the data of chemical activity of fermented variants, it may be concluded that the residues of wine, made by “Kakhetian” method, especially fermented seeds of all tested varieties may be used as a secondary raw material for the production of bio-additives. Thus, an existence of antioxidants and other groups of organic substances in residues of wine made by “Kakhetian” method is a good reason to apply this waste in the production of bio- additives, cosmetology, or for other purposes.

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