

Antioxidant Activity of Phenolic Compounds from Different Grape Wastes

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Abstract

Winery pomace (from red and white grapes) was extracted under various conditions using different solvents. The antioxidant activity of solvent extracts was investigated by DPPH radical scavenging method. Ethanol extract exhibited the highest antioxidant activity compared to the other solvent (BuOH, EtOAc, Me₂Cl₂ and pet.ether). There was a correlation between antioxidant activity and total phenol content. HPLC analysis of the extracts showed that gallic and cinnamic acid was the major phenolic compounds in winery pomace. Various phenolic compounds such as catechin, rutin, rosmarinic, chlorogenic, caffeic, vanillic, coumaric acids were also identified.

Keywords: Grape pomace; Phenolic compounds; HPLC; DPPH

Introduction

There is an increased evidence for the participation of free radicals in the etiology of various diseases like cancer, diabetes, cardiovascular diseases, autoimmune disorders, neurodegenerative diseases, aging, etc [1].

Antioxidants are agents which scavenge the free radicals and prevent the damage caused by them. They can greatly reduce the damage by neutralizing the free radicals before they can attack the cells and prevent damage to lipids, proteins, enzymes, carbohydrates and DNA [2]. Antioxidants can be classified into two major classes i.e., enzymatic and non-enzymatic. The enzymatic antioxidants are produced endogenously and include superoxide dismutase, catalase, and glutathione peroxidase. The non-enzymatic antioxidants include tocopherols, carotenoids, ascorbic acid, flavonoids and tannins which are obtained from natural plant sources [3]. A wide range of antioxidants from both natural and synthetic origin has been proposed for use in the treatment of various human diseases [4]. There are some synthetic antioxidant compounds such as butylated hydroxytoluene, butylated hydroxyanisole and tertiary butyl hydroquinone, commonly used in processed foods. However, it has been suggested that these compounds have shown toxic effects like liver damage and mutagenesis [5]. Flavonoids and other phenolic compounds of plant origin have been reported as scavengers of free radicals [6].

In the last few years, an increased attention has been focused on the industrial pomaces, especially those containing phenols from residual plant raw materials. There has been an upsurge in the exploitation of the pomace materials generated by the wine industry. Wine pomace is characterized by the presence of natural antioxidants and is characterized by high-phenolic contents due to poor extraction during winemaking, and thus making their utilization worthwhile [7]. In recent years, the use of grape seed extracts (GSE) has gained ground as a nutritional supplement in view of its antioxidant activity. The by-products obtained after winery exploitation, either seeds or pomaces, constitute a very cheap source for the extraction of antioxidant flavanols, which can be used as dietary supplements, or in the production of phytochemicals, providing an important economic advantage [8] and additive value will be added to the residue. It is hoped that information on the total phenolic compounds and antioxidant activities of plant extracts and their individual phenolic compounds can be used as criteria to retard or prevent lipid oxidation in a variety of food products. The aim of this work is to investigate the effect of extracting solvents on the phenol content of winery pomace extracts and evaluate the antioxidant activity

of winery pomace extracts in order to develop an effective procedure for the recovery of phenolic compounds from winery pomace with special consideration to their utilization as antioxidants for foods.

Materials and Methods

Methanol (MeOH), ethanol (EtOH), acetone (Me₂CO), butanol (BuOH), ethyl acetate (EtOAc), methylene chloride (Me₂Cl₂) and pet. ether used were in an analytical reagent grade and purchased from Merck (Darmstadt, Germany). Folin-Ciocalteu phenol reagent, Phenolic standards have from 98–99% purity and free radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH) were purchased from Sigma Chemical Co. (Sigma-Aldrich Company Ltd., Great Britain).

Grape pomace samples

Four grape varieties were selected for this study, and their wastes were produced and obtained as follows:

- Grenache waste obtained from El kroom company, consist of skin and seeds (red berries).
- Thompson seedless waste obtained from El kroom company, which consist of skin only as the species is seedless one (white berries).
- Red Romy consists of skin and seeds, obtained from local market and pomace was produced in the medicinal and aromatic plants Dept. NRC (red berries).
- Crimson grape obtained from local market and pomace produced in the lab (red berries).
- Each sample was hand divided into their parts; skin, seeds and pomace.

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Received December 18, 2013; **Accepted** January 25, 2014; **Published** February 05, 2014

Citation: Gengaihi SEI, Ella FMA, Emad MH, Shalaby E, Doha H (2014) Antioxidant Activity of Phenolic Compounds from Different Grape Wastes. J Food Process Technol 5: 296. doi:10.4172/2157-7110.1000296

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Seed obtained were air dried and weighted, pomace was oven dried at 50°C, then ground to a fine powder. The powdered samples were then kept at -4°C until used.

Extraction of total phenolic and total flavonoid compounds

One gram of air dried pomace powdered pomace were extracted at room temperature with petroleum ether (40-60°C) till complete extraction to remove lipid, waxes, pigments, sterols and non phenolic compounds. Then the mark was extracted with (acidified EtOH (0.1% HCl), 80% EtOH, 50% EtOH and Me₂CO) several times (10 ml x 4) till complete extraction. The combined extract was transferred to measuring flask 100 ml. and completed to 100 ml.

Estimation of total flavonoid

The total flavonoids content was determined using the method adopted by Meda et al. [9]. Three ml of 2% aluminium trichloride (AlCl₃) in methanol (Fluka Chemie, Switerland) was mixed with the same volume of the extract. Absorption was read at 415 nm (UV.VIS, 2041 spectrophotometer) after 30 min against a blank sample consisting of a 3 ml AlCl₃ with 3 ml methanol. The total flavonoids content was determined using a standard curve of rutin (Sigma–Aldrich Chemie, Steinheim, Germany) as standard. The mean of three readings was used and expressed as mg of rutin equivalents /100 g of air dried pomace sample.

Estimation of total phenolic content

Folin–Ciocalteu method was used to determine total phenolic content as chlorogenic acid (sigma) according to the method described by Meda et al. [9]. Concentration of the total phenol was plotted from the chlorogenic acid calibration curve. The mean of three readings was used and the total phenolic content was expressed in mg of chlorogenic acid equivalents /100 g of air dried pomace sample).

Chromatographic procedure for flavonoid and phenolic acids

Powdered grape wastes were extracted with EtOH (80%) by soaking at room temperature. The combined methanol extracts were concentrated under reduced pressure at 45°C. The crude residue was dissolved in hot water, left overnight, filtered and was successively partitioned with methylene chloride (Me₂Cl₂), ethyl acetate (EtOAc) and n-butanol (BuOH). The HPLC system was a HP 1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped

with an auto-sampler, quaternary pump and a diode array detector. The measurements were integrated by Chemstation chromatographic software interfaced to a personal computer.

The analytical column was ZORBAX Eclipse XDB C18 column (15 cm x 4.6 mm ID, 5 μm, USA). Operative conditions were: mobile phase A, methanol; mobile phase B, 2% acetic acid; flow rate, 1mL min; fixed wavelength, 280 and 360 nm; injected quantity, 20 μL; elution program, A (%) / B (%): 0 min 5/95; 10 min 25/75; 20 min 50/50; 30 min 100/0; 40 min 5/95. Identification of phenolic compounds was performed by comparison with the retention times of standard substances [10].

DPPH radical-scavenging activity

As described by Mahakunakorn et al. [11], scavenging capacity was measured spectrophotometrically at 517 nm. Inhibition (%) was plotted against the sample concentration (100ug/ml in MeOH) in the reaction system; The mixture was shaken vigorously (2,500 rpm) for 1min then left to stand for 60 min in the dark. ascorbic acid and BHT (100 ug/ml in MeOH) were used as a positive control, the percentage inhibition of the DPPH radical was calculated according to the following formula:

$$\% \text{ Inhibition} = ((A \text{ control} - A \text{ sample}) / \text{control}) \times 100$$

Where A is absorbance

Statistical analysis

Data were expressed by two-way analysis of variance (ANOVA), COSTAT Software Computer Program. Significance values between groups were at P < 0.01 [12].

Results and Discussion

Chemical characterization of winery pomace had prior necessity to evaluate its potential, to determine the extraction yield and to be controlled qualitatively.

Total phenolic content

Phenolic compounds react with Folin Ciocalteu's Reagent (FCR) only under basic conditions (adjusted by aqueous sodium carbonate). Dissociation of a phenolic proton in basic medium leads to a phenolate anion, which is capable of reducing FCR in which the molybdate in testing system is reduced forming a blue colored molybdenum oxide with maximum absorption near 765 nm. The intensity of blue coloration produced is proportional to the total quantity of phenolic compounds present in the testing samples. Data of polyphenolic contents of different pomace samples are presented in Table 1. Results in Table 1 reveal that the total phenols in skin extracts were lower than in seed one; the highest concentration of total phenolic was found in Grenache noir seeds, skin and Red romy seeds. The extractive capacity of phenolic components from pomace material is considerably depend on the type of solvents. The best extraction efficiency was achieved by ethanol: 0.1 % HCl (acidified alcohol) and 80 % EtOH whereas pure ethanol resulted in poor phenolic contents. The technique of phenolic isolation from a plant material, including the methods and type of extracting solvent, depends generally on the type of phenolic compound and the solvents [13]. Results of previous studies showed that the extraction yield of phenolic and flavonoid content is greatly expressed in acidified alcohol [14], and this holds with our results.

Total flavonoids

The yields obtained by using various extractants (solvents) and their composition of total flavonoids are shown in Table 2. The highest

Wastes (W)	Solvents(S)				Mean
	EtOH : HCl	50% EtOH	80% EtOH	Me ₂ CO	
Red Romy Skin	14.61 ± 0.3	1.81 ± 0.07	1.95 ± 0.1	1.54 ± 0.2	4.98 ^d
Red Romy seed	15.54 ± 0.6	14.64 ± 0.37	14.19 ± 0.9	13.21 ± 0.5	14.40 ^b
Crimson Skin	13.78 ± 0.7	1.80 ± 0.07	1.74 ± 0.3	1.48 ± 0.0	4.70 ^d
Thompson Seedless Skin	1.13 ± 0.3	3.83 ± 0.46	6.47 ± 0.9	2.37 ± 0.1	3.45 ^e
Grenache Noir skin	20.63 ± 0.6 ^b	8.53 ± 0.21	11.87 ± 2.9	7.30 ± 0.3	12.08 ^c
Grenache Noir seed	28.50 ± 0.9 ^a	11.37 ± 0.25	14.90 ± 0.5	16.33 ± 0.8 ^c	17.78 ^a
Mean	15.70 ^a	7.00 ^c	8.52 ^b	7.04 ^c	-
LSD _{0.01}	W=1.14		S=0.93	W*S interaction=1.72	

Data are represented as mean ± S.E.

Statistical analysis is carried out by two way analysis of variance using COSTAT program.

Unshared letters between brackets are significant value between groups.

Table 1: Total phenolic content (mg/g) in different grape pomaces using different extracting solvents.

Wastes (W)	Solvents (S)				Mean
	EtOH : HCl	50% EtOH	80% EtOH	Me ₂ CO	
Red romy Skin	0.71 ±0.00	0.31 ±0.00	0.30 ±0.00	0.29 ±0.01	0.40 ^d
Red romy seed	1.79 ±0.01 b	0.65 ±0.00	0.66 ±0.00	0.57 ±0.00	0.92 ^c
Crimson Skin	0.66 ±0.00	0.39 ±0.00	0.37 ±0.00	0.32 ±0.00	0.43 ^d
Thompson seedless skin	1.01 ±0.01	0.93 ±0.00	2.13 ± 0.02 ^a	1.54 ±0.01 ^c	1.41 ^a
Grenache noir skin	1.42 ±0.02	0.69 ±0.00	0.94 ±0.01	1.02 ±0.01	1.02 ^b
Grenache noir seeds	1.34 ±0.01	0.64 ±0.00	0.74 ±0.01	0.58 ±0.00	0.83 ^c
Mean	1.16 ^a	0.60 ^d	0.86 ^b	0.72 ^c	
LSD _{0.01}	W= 0.10 S = 0.08 W*S interaction = 0.15				

Data are represented as mean ± S.E.

Statistical analysis is carried out by two way analysis of variance using COSTAT program

Unshared letters between brackets are significant value between groups

Table 2: Total flavonoid content (mg/g) in different grape pomaces using different extracting solvents.

Wastes	Extract	Concentration mg/100g									
		Catechin	Gallic	Caffeic	Vanillic	Cumaric	Ferulic	Cinnamic	Chlorogenic	Rosmarinic	Rutin
Red romy skin	EtOAc	151	3.84	-	-	-	-	138	20.7	-	20.8
	BuOH	0.91	7.09	-	-	-	2.85	-	2.44	-	-
	EtOH	2.78	30.6	5.49	6.21	0.85	3.88	37.7	8.21	187	52.2
Red romy seeds	EtOAc	6.81	16.4	-	9.55	-	5.25	7.47	6.56	-	104
	BuOH	20.4	366	-	171	4.48	235	2690	49.1	218	482
	EtOH	-	212	-	94.5	114	216	1146	370	7.98	17.2
Crimson skin	EtOAc	-	6.34	6.8	1.61	-	-	1949	59.1	16.4	129
	BuOH	4.31	85	-	26.2	46.3	-	425	-	79.2	996
	EtOH	1.62	23.9	5.56	-	15.6	4.68	82.4	-	2.71	207
Thompson seedless skin	EtOAc	2.68	82.5	29.6	0.51	-	-	4.81	16.3	51.2	9.63
	BuOH	5.93	15.3	26	6.76	2.39	-	518	-	55.6	866
	EtOH	7.53	1058	231	4.34	90	36.6	457	-	453	749
Grenache noir skin	EtOAc	0.57	3.99	17.7	-	-	12.8	-	20.2	-	-
	BuOH	15.3	48.8	22.3	11.6	1.53	88	42	30.2	-	75.3
	EtOH	-	22.6	29.8	2.1	46.1	91.8	187	-	116	284
Grenache noir seeds	EtOAc	-	3.48	6.04	-	-	-	-	-	-	-
	BuOH	7.93	67.8	153	11.8	1.72	-	105	24.1	-	5.54
	EtOH	-	77.1	15.6	26.6	11.7	12.1	56.4	61.3	-	43

Table 3: concentration of phenolic acids (mg/100g) in the investigated samples by HPLC.

flavonoid content was found in Thompson pomace and the lowest one were in red romy skin and crimson skin.

High Performance Liquid Chromatography (HPLC)

It is obvious that the total phenolic content measured by the Folin-Ciocalteu procedure does not give a full picture of the quality or quantity of the phenolic constituents in the extracts as reported in literature [15,16]. PLC is the preferred technique for both separation and quantification of phenolic compounds [17]. Various factors affect HPLC analysis of phenolics, including sample purification, mobile phase, column types and detectors [18]. In general, purified phenolics are applied to an HPLC instrument utilizing a reversed phase C18 column (RP-C18), photo diode array detector (PDA) and polar acidified organic solvents [19]. The HPLC analysis of the phenolic compounds in different extracts (EtOAc, BuOH and 80 % EtOH) were employed using the previous condition and were compiled in Table 3. HPLC analysis of different extracts of grape pomace showed a complex mixture of phenolics that was difficult to resolve. Several phenolic compounds, which are representative of the diverse structural types, were identified. Table 3 reveals that the alcoholic extract of romy seeds had the highest amounts of ferulic, coumaric, vanillic, chlorogenic (216, 114, 94, and 370 ug/100g respectively). While Thompson seedless pomace had the highest amount of gallic, caffeic and rosmarinic acid (1058, 231 and 453 ug/100g respectively). Butanolic extract of Crimson skin had the

highest rutin amount (996 ug/100g); while the butanolic extract of romy seeds had the highest cinnamic acid amount (2690ug/100g).

These finding are in agreement with Kallithraka et al. [20] they indicated that the ethanol extraction of grape seed had high content of catechin, Rodtjer et al. [21] showed that the extraction yield of phenolic compounds is greatly depending on the solvent polarity [22]. According to Yilmaz and Toledo [23] they concluded that aqueous solutions of ethanol, methanol was better than a pure compound solvent system for the extraction of phenolics compound from Muscadine seed. Also, other studies have established that the phenolics and flavonoids content of extracts are strongly depend on the type of the solvent as well as on the different concentrations used.

Wang and Helliwell [24] reported that aqueous ethanol was better than aqueous methanol for extraction of tea flavonoids. In extracting phenolic compounds from peanut skin, ethanol and methanol were more effective than water, with ethanol being the most efficient extraction solvent [25]. Meanwhile, the methanol was the solvent with best results for phenols from pine sawdust, while in almond hulls ethanol was the best extraction solvent [26]. Jung et al. [27] compared the influence of different solvents and they found out that the ethanol extracts contained higher amounts of total phenolics and flavonoids than water and methanol extracts from wild ginseng leaves.

Wastes	Inhibition % (100ul/ml)					Mean
	EtOH	EtOAc	BuOH	Me ₂ Cl ₂	Pet.ether	
Romy Skin	81.94 ±2.69	24.25 ± 0.4	25.15 ± 0.5	25.8 ± 1.8	23.6 ± 0.0	36.1
Romy seeds	93.31 ±1.27 ^a	59.9 ±1.3	64.1 ± 3.2	26.6 ± 0.8	27.3 ± 1.8	54.2 ^b
Crimson skin	74.33 ±2.44	26.1 ± 1.4	27.3 ± 0.9	23.6 ± 0.0	27.3 ± 0.9	35.7
Thompson seedless skin	89.05 ±2.04	54.05 ± 0.5	33.15 ± 0.4	29.1 ± 0.9	27.1 ± 0.9	46.5
Grenache noir	91.47 ±2.55	72.25 ± 2.3	47.3 ± 0.4	26.35 ± 1.8	20.6 ± 1.8	51.6
Grenache noir seeds	90.23 ±1.02	100 ± 0.5	79.2 ± 0.7	27.8 ± 0.09	23.8 ± .6	64.2 ^a
Mean	86.7 ^a	56.1	46	26.5	25	
Ascorbic			90.15 ±2.5			
BHT			94.1 ±2.03			

Data are represented as mean ± S.E.

Statistical analysis is carried out by two way analysis of variance using COSTAT program

Unshared letters between brackets are significant value between groups

Table 4: In vitro DPPH radical scavenging activity of different grape pomaces extracted with different solvents.

Antioxidant activity

The most common methods to determine antioxidant activity in a practical, rapid and sensitive manner are those that involve a radical chromophore, simulating the reactive oxygen species, and the free radical DPPH, of purple coloration that absorbs at 515 nm, is one of the most widely used for *in vitro* evaluation of plant extracts and fractions. Dealing with antioxidant activity of different grape pomaces extracted by different solvent, Table 4 reveals that ethanol extract of different pomaces produced the higher radical scavenging activity when compared with other solvents i.e. EtOAc, BuOH, Me₂Cl₂ and Pet. ether solvents. Alcoholic extract of Red romy seeds gave the highest antioxidant activity than ascorbic and other grape pomaces; which may be attributed to its major components of phenolic acids like ferulic, coumaric, vanillic, gallic, chlorogenic and cinnamic. The different antioxidant activities of phenolic extracts can be attributed to different extracting solvent as the antioxidant activity depends on the type and polarity of the extracting solvent, the isolation procedures, the purity of active compounds, as well as the test system [28].

EtOAc, BuOH, Pet.ether extracts exhibited lower antioxidant activity than EtOH, The high antioxidant activity of ethanol extract can be also attributed to its major components, i.e. gallic acid and catechin. There is one exception that EtOAc of Grenache noir exhibited the highest antioxidant activity.

A positive correlation was well established with the EtOH of romy seeds and Grenache noir seeds where the highest values of polyphenols (14.2 and 14.9 mg/g respectively) in this fractions relate to the good DPPH radical inhibition results (93.31 and 90.23% respectively). This correlation between phenolics compounds and the antioxidant capacity (DPPH method) is in good agreement with the results of Mustafa et al. [29], Surveswaran et al. [30] and Janovik et al. [31].

Some studies suggest that it is not always possible to correlate the total phenolics and antioxidant capacity. This can be explained by several factors, including the presence of different active compounds in the plant that can modify the antioxidant capacity, the synergistic effects of different compounds, the experimental conditions, and the mechanisms of the methods used for antioxidant reactions. Structural factors include the nature of the phenolic groups and the changes caused by glycosylation [32]. There are also compounds that react strongly with the DPPH, and others that have a slower reaction rate [33].

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