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Total phenolic content, antioxidant capacity and phytochemical profiling of grape and pomegranate wines

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The object of this study was to determine the phenolic profile, the total phenolic content and antioxidant capacity of pomegranate wine and compare to multi-varietal red wine using different spectrophotometric and spectrometric techniques. Total phenolic content was determined by the Folin-Ciocalteu assay. The antioxidant capacity was measured by the DPPH and the ABTS radical scavenging assays. The radical-scavenging capacity was higher for pomegranate wine (statistically significant difference was observed for the DPPH assay) in agreement with its higher total phenolic content (383.19±18.22 and 296.57±25.23 mg gallic acid equivalents/100 mL for pomegranate and grape wine respectively). Customized HPLC–PDA–ESI–MS^n and GC–MS methods were applied for the identification and chemical characterization of the phenolic compounds for both wines. Identification using LC-MS was based on their λ_{max} (nm) and the characteristic fragments which derived from the sequential fragmentation in MS while GC-MS was based on commercial libraries and mass spectra of authentic standards. Eighty one different phenolic compounds were characterised by LC-MS and one hundred eight compounds by GC–MS after different chemical hydrolysis regimes. The study signifies the prior treatment with alkaline hydrolysis which had a considerable effect on the detection of phenolic compounds. The results showed that the combination of LC-MS and GC–MS methods allowed the detection of different compounds while results from both techniques are complementary and may confirm each other. Phytochemicals with proven biological activities including antimicrobial, antiviral and chemoprotective, have been identified mainly in pomegranate wines. Furthermore, a significant diversity between pomegranate and grape wines was observed, in terms of their phenolic content and antioxidant profiles indicating the-nutritive and health-promoting effects of pomegranate wine.

Introduction

The pomegranate fruit has been used extensively in the folk medicine of many cultures. Nowadays, pomegranate is regarded as a dietary source of bioactive compounds which possess several health effects like maintenance of redox balance, protection from cardiovascular diseases, diabetes, and neurodegenerative diseases as Alzheimer's and cancer.[1]
The consumer's perception about pomegranate is increasingly positive since it is related to numerous health benefits. Towards this direction, pomegranate has been characterized as "superfruit with healing power", "the new fat buster", "the antioxidant bomb", etc. It is worthy that in a single year, 194 studies have been published, more than any other fruit.
Edible parts of pomegranate fruit constitute the 50% of total fruit weight and contain 80% juice and 20% seeds. Fresh juice contains 85% water, 10% total sugars, and 1.5% pectin, ascorbic acid, and polyphenolic flavonoids. Pomegranate seeds are a rich source of crude fibers, pectin, and sugars.[2]
Apart from the edible part of the fruit, several other types of products have been presented including fermented ones. The elaboration of pomegranate wine has been recently pointed out as a novel means of exploitation of pomegranates. Pomegranate wines have already received increased attention and have been examined for their increased health benefits. Significant compositional alterations take part during pomegranate winemaking processes, resulting in wines with a promising phytochemical profile.[3]
On the other hand, grape wine, which is the most important fermented fruit juice, is oftentimes documented for its favourable properties, arising in connection with its high content in phenolic compounds. Grape wine contains more than 500 compounds, originating either from the grapes or from the metabolic pathways as by-products of yeast activity during fermentation.[4] These compounds, especially polyphenols, have been reported to have considerable anticarcinogenic, cardioprotective, antiinflammatory and antibacterial properties as well as high antioxidant and antiradical activity.[5] Furthermore, it is well known that phenolic compounds play an important role in red wine color, bitterness and astringency, as well as a range of other tactile

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or "mouth feel" organoleptic characteristics.[6] The consumption of natural antioxidants from wines has been reported to be easier and surely more agreeable than the consumption of free radical scavengers from vegetables.

Numerous studies have reported the phenolic composition and antioxidant activity of grape wines from different varieties or regions.[5,7] However, to our knowledge, there are only few reports on evaluated pomegranate wine with respect to its phenolic profile as well as to its antioxidant capacity.[1,8-10] To this extend, the main goal of the current study is to assess comparatively the total phenolic content, the antiradical activity and the most noticeable phenolic compounds using LC-MS^n and GC-MS analyses, in pomegranate and grape red wine samples. Moreover another target is to elucidate the phenolic compounds identification using the combination of a GC-MS analysis after different hydrolysis methods with LC-MS^n analysis.

Results and discussion

Total phenolic content and antiradical activity of pomegranate and grape wines

Total phenolic content and antiradical activity of pomegranate and grape wines are presented in Table 1. Total phenolic content (expressed as mg gallic acid equivalents/100 mL of wine) of pomegranate wine was found significantly ($P<0.05$) higher than the corresponding of the grape wine.

Scientific research has shown that total phenolic content and profile present high qualitative and quantitative variability, depending on a number of factors as geographical origin, fruit type and variety, method of vinification, wine maturation, wine storing and their in between interactions.[11-13] Specifically, the phenolic content of Cabernet red wine was found 200.5 mg GAE / 100 mL, of elder grape wine 175.3 mg GAE/100 mL, of blueberry wine 167.6 mg GAE/100 mL, of gooseberry wine 150.9 mg GAE/100 mL, of cherry wine 99.1 mg GAE/100 mL, of raspberry wine 97.7 mg GAE/100 mL, of cranberry wine 97.1 mg GAE/100 mL, of plum wine 55.5 mg GAE/100 mL, of apple wine 45.1 mg GAE/100 mL, of peach wine 41.8 mg GAE/100 mL, of Chardonnay white wine 28.7 mg GAE/100 mL, of pear wine 31.0 mg GAE/100 mL and of wine from grapes Riesling 25.0 mg GAE/100 mL.[12] Yoo *et al.*[14] reported that the phenolic content of Cabernet and Shiraz red wines from Australia ranged from 141.71 to 358.86 mg GAE / 100 mL Moreover, Rastija and Srečnik[13] reported that the phenolic content of grape wines from Croatia ranged from 19.1 to 65.2 mg GAE/100 mL for white wines, while for red wines from 115.6 to 261.9 mg GAE/100 mL. It is reported that the phenolic content of white wines from Argentina and Italy ranged from 21.6 to 85.4 mg GAE/100 mL, of an Italian rosé wine was 130.4 mg GAE/100 mL and of red wines from Brazil, Chile, Portugal and Italy ranged from 161.5 to 417.7 mg GAE/100 mL.[7] Furthermore, Zhuang *et al.*[8] reported that the total phenolic content of pomegranate wine was found 491.14±4.81 mg GAE/100 mL. According to the literature data, red wines are particularly richer in phenolic constituents than white and rosé wines. The average value for total phenolic content of pomegranate wine in this study was higher to those reported for red grape wines.

Antiradical activity (expressed as ascorbic acid and trolox equivalents per 100 mL of wine) from the examined wines was found to vary in the same manner as with phenolic content (Table 1). If it is assumed that the wines from different fruits have different profile of phenolic compounds, the higher antiradical capacity of pomegranate wine as compared with grape wine is probably related not only to the higher polyphenol content but also to the presence of different phenolic compounds in them. Since pomegranate wine was found to have higher antiradical capacity combined with higher phenolic content than red wine, as well as the examined wines were produced by the same winemaking conditions, it is estimated that the pomegranate wine could be an interesting proposal for economic exploitation of pomegranates.

Table 1: Total phenolic content and antiradical activity of pomegranate and grape wines

Parameters	Pomegranate wine	Grape wine
Total phenolic content (mg gallic acid equivalents/100 mL)	383.19±18.22a	296.57±25.23b
DPPH scavenging capacity (mg AA equivalents /100 mL)	82.65±0.59a	78.18±0.66b
ABTS (mg Trolox equivalents/100 mL)	90.82±1.96a	87.23±2.01a

Results represent means ± SD (n = 10 separate samples); Means in the same row bearing different letters differ significantly (P < 0.05).

LC-MS analysis of pomegranate and grape wines

HPLC–PDA–ESI–MS^n analysis was performed to study the composition of phenolic compounds in pomegranate and grape wines. For each compound identifying, the λmax (nm), the ion from the positive (ESI+) or the negative (ESI–) ionization modes as well as the characteristic fragments which derived from the sequential fragmentation in MS, are provided (Table 2). For the identification of each phenolic compound the fragments (m/z) obtained from MS^1, MS^2, MS^3 and MS^4 spectra were comparatively studied with those from respective literature data.[10,15,16, 39.]

According to Table 2, eight and twenty one anthocyanidinic compounds were identified in the pomegranate and grape wine respectively. Anthocyanins attribute wine color and quality and their profile is used to classify the grape cultivars and to inspect the wine authenticity. All anthocyanin 3-monoglycosides and 3.5-diglycosides showed a common fragmentation pathway by the loss of one and two glucose units (m/z = 162) respectively, from the protonated molecule $[MH^+]$ (Table 2). Therefore, MS/MS approach permits anthocyanin aglycone and sugar moiety characterisation. The main anthocyanins found in grape wine were glycosides of delphinidin, cyanidin, pelargonidin, petunidin, peonidin and malvidin, while in pomegranate wine only glycosides of delphinidin, cyanidin and pelargonidin were found. Therefore, the profile of anthocyanins can successfully be used for authentication and adulteration issues in pomegranate wines. In accordance to the findings of the present study, Gómez-Caravaca *et al.*[17] reported that 3-monoglycosides and 3.5-diglycosides of cyanidin, delphinidin and pelargonidin are responsible for the red color of the pomegranate products.

Furthermore, in the same samples, twenty six and thirty non-anthocyanidinic phenolic compounds as well as two and four organic acids were determined respectively (Table 2). Pomegranate wine was found to contain ellagic acid, gallagic acid punicalagin, pedunculagin, punicalin and their derivatives, whereas red grape wine contained only ellagic acid-dihexoside. The above compounds exhibit high antioxidant and antiproliferative activities.[18] Gallagic acid is an analogue of ellagic acid containing four gallic acid residues. Pedunculagin, which is an ellagitannin, provide protection against inflammation, cancer, virus and bacteria, suppress lipid peroxidation, reduce blood urea nitrogen and improve mental condition.[19] Another interesting finding was the detection of brevifolin carboxylic acid (BCA) via the fragment ion at m/z 247, which is attributed to brevifolin[20] and of valoneic acid bilactone via the characteristic pseudomeolecular ion at m/z 469 and the fragment ion at m/z 425,[21] in pomegranate wines. The above compounds were also found in the juice, peel and mesocarp of *P. granatum*.[15] Brevifolin carboxylic acid has been shown to inhibit hepatitis B virus (HBV) replication and tumor growth.[22] According to Fracassetti *et al.*[21] and in agreement to the above findings, valoneic acid bilactone is also detected in plants containing ellagitannins. Flavonols (laricitrin, quercetin, syringetin and myricetin) and their glycosides were also identified in grape wine, which are the most common wine flavonoid. The identification of such compounds is important since they allow a better characterization of grape varieties.[23] Furthermore, grape wine was found to contain chlorogenic, *cis*-cinnamic and caftaric acid (caffeic acid conjugated with tartaric acid).

GC-MS analysis of phytochemical constituents of pomegranate and grape wines

The pomegranate and grape wines were also studied after chemical hydrolysis in comparison to non-hydrolyzed ones by GC-MS analysis. The main advantage of GC-MS vs LC-MS is the volatility of phenolic compounds via derivatization and their release from the glycoside and ester bonds via alkaline and acidic hydrolysis to volatile derivatives.[24] The results of GC-MS analysis (Table 3) were used in order, a) to assess the phytochemical constituents profile in pomegranate and grape wines, including phenolic acids, phenolic compounds and other compounds and b) to compare and confirm LC-MS analysis findings. The compound structure identification was based on the retention time (Rt) of respective standards and their mass spectra characteristic fragments. Specifically, eleven compounds were identified by comparison with reference standards. The mass spectra of the unknown components were compared with those of the known components in the MS libraries' spectra (NIST05, NIST05s, NIST08, NIST08s, NIST21, NIST107, WILEY7, PMW_TOX2, SZTERP). According to the results (Table 3) of non-hydrolyzed wine samples, only few phenolic acids and phenolic compounds were identified. Therefore, it seems that the wine samples contained mostly bound phenolics as glycosides, complexes and/or polymers. According to the results of Table 3, it seemed that the type of hydrolysis plays an important role in determining the quality of phenolic and other compounds via GC-MS analysis. Furthermore, alkaline hydrolysis resulted in a greater number of detected phenolic and other compounds compared to acidic and post alkaline acidic hydrolyses. Further observations derived from the GC-MS analysis are discussed below.

Regarding phenolic acids, in pomegranate wine, gallic acid was detected in non-hydrolyzed samples and after acidic and post alkaline acidic hydrolyses, whereas in grape wine only after alkaline hydrolysis. After post alkaline acidic hydrolysis, salicylic and 4-hydroxybenzoic acids were detected in pomegranate and grape wines, respectively. Moreover in both wine samples, vanillic acid was detected after alkaline and post alkaline acidic hydrolyses, whereas isovanillic and protocatechuic acids after post alkaline acidic hydrolysis. The presence of the above mentioned free hydroxybenzoic acids in wines resulted from the hydrolysis of flavonoids as anthocyanidins and flavan-3-ols and of ellagitannins, which were detected by LC-MS analysis (Table 2). In accordance to the above findings, Ross *et al.*[25] reported that gallic and protocatechuic acids can be obtained from the acid hydrolysis of hydrolysable tannins. Furthermore alkaline hydrolysis seems to release gallic acid from ethyl-gallate, which could be formed during vinification and was detected by LC-MS analysis (Table 2). Caffeic acid, which is the most characteristic isomer of hydroxycinnamic acids in wines,[26] was detected in all studied samples of pomegranate and grape wines. By relating the results of GC-MS and LC-MS analysis (Tables 2 and 3), the presence of caffeic acid in wines, both in the free form, as well as in the form of caffeic acid- hexosides and as ethyl-caffeate was confirmed. Furthermore, p-coumaric and ferulic acids were detected only after alkaline hydrolysis. This result is in accordance to Kim *et al.*[27] who reported that acidic hydrolysis may degrade cinnamic acid derivatives, as p-coumaric and ferulic acids. Interestingly, baccharin was detected in pomegranate wine after alkaline hydrolysis. This finding is of high value, since baccharin is a natural phenolic compound derived from p-coumaric acid which is reported to have high chemoprotective activity against genomic and chromosomal damages.[28] Another interesting outcome was the detection of β-phenyllactic acid in non-hydrolyzed pomegranate and grape wines as well as after post alkaline acidic hydrolysis of pomegranate wines and of 4-hydroxyphenyllactic and mandelic acids after acidic hydrolysis, in pomegranate wine. It is reported that aromatic amino acids produce secondary metabolites such as phenyllactic acid from phenylalanine and hydroxyphenyl lactic acid from tyrosine via shikimate pathway.[29] Phenyllactic acid is a relatively new antimicrobial agent and inhibitor of *L. monocytogenes*, *Gram*-positive bacteria, *Gram*-negative bacteria and fungi.[30,31] Mandelic (or 2-hydroxy-2-phenylacetic) acid, which is an isomer of cresotinic and oxymethylbenzoic acid, has been also found to possess antibacterial and antibiotic properties. Mandelic and

Table 2: Phenolic compounds detected in pomegranate and grape wine samples using HPLC–PDA–ESI–MS^n analysis

Anthocyanidinic Compounds	λ_{max} (nm)	M^+ (m/z)	MS^2 (m/z)	MS^3 / MS^4 (m/z)	Pomegranate Wine	Grape Wine
Cyanidin	510	287	269, 241, 213, 177, 113			√
Cyanidin-3-O-monoglucoside	515, 280	449	287		√	
Cyanidin-3-O-(6-acetyl-glucoside)	522	491	449, 287			√
Cyanidin-3-O-(6-coumaroyl-pentoside)	528	565	419, 287			√
Cyanidin-3-O-(6- coumaroyl-glucoside)	522, 308	595	449, 287			√
Cyanidin-3-O-mono pentoside	512	419	287			√
Cyanidin-3-rutinoside (antirrhinin)	503, 274	595	449, 287			√
Cyanidin 3-O-(6-caffeoyl-glucoside)	513, 276	611	449, 287		√	
(Epi)catechin-cyanidin-3,5-diglucoside	520, 280	899	**737**, 575	**575** / 449, 423, 329, 287	√	
(Epi)gallocatechin-cyanidin-3,5- diglucoside	520, 280	915	**753**, 591	**591** / 573, 465, 423, 329, 287	√	
Delphinidin-3-O-monoglucoside (myrtillin)	522, 277	465	303		√	√
Delphinidin 3-O-(6-caffeoyl- glucoside)	521	627	465, 303		√	√
(Epi)gallocatechin-delphinidin-3,5- diglucoside	520	931	**769**, 607	**607** / 589, 481, 439, 345, 303	√	
Malvidin-3-O-monoglucoside	526	493	331			√
Malvidin-3,5-O-diglucoside	524	655	493, 331			√
Malvidin-3-O-(6-acetyl-glucoside)-5-O-glucoside	530	697	655, 493, 331			√
Malvidin-3-O-(6-caffeoyl-glucoside)-5-O-glucoside	528	817	655, 493, 331			√
Pelargonidin-3-O-monoglucoside	503, 274	433	271		√	
Pelargonidin-3,5-O-diglucoside	510	595	433, 271			√
Pelargonidin-3-O-(6-caffeoyl-glucoside)	511	595	433, 271			√
Pelargonidin-3-O-(6- coumaroyl-glucoside)	511	579	433, 271			√
Peonidin-3-O-monoglucoside	516	463	301			√
Peonidin-3-O-(6-coumaroyl-glucoside)-5-O-glucoside	532, 520	771	625, 463, 301			√
Peonidin-3-O-(6-caffeoyl-glucoside)-5-O-glucoside	532, 520	787	625, 463, 301			√
Peonidin-3-O-monoglucoside-pyruvic acid	520	531	369			√
Petunidin-3-O-monoglucoside	524	479	317			√
Petunidin-3-O-(6-caffeoyl-glucoside)-5-O-glucoside	522	803	641, 479, 317			√
Non-Anthocyanidinic Phenolic Compounds	**λ_{max} (nm)**	**$[M–H]^-$ (m/z)**	**MS^2 (m/z)**	**MS^3 (m/z)**	**Pomegranate Wine**	**Grape Wine**
Apigenin-rhamnoside (detected as formic acid adduct)	272, 334	461	415	269, 161	√	√
Brevifolin carboxylic acid	277, 354	291	247	203	√	
Caffeic acid (3,4-Dihydroxycinnamic acid)	240, 326	179	161, 135		√	√
Caffeic acid-hexoside	232, 330	341	**179**, 161, 135	135	√	
cis-Caftaric acid	244, 326	311	179, 149			√
(+)-Catechin	276	289	261, **245**, 205, 203, 179	203, 227, 187, 161, 217		√
Chlorogenic acid (5-Caffeoylquinic acid)	238, 325	353	217, 191			

cis-Cinnamic acid	267	147	129			√
Coumaric acid-hexoside	234, 312	325	**187**, 163, 145, 119	119		√
Digalloyl-HHDP-hexoside	236, 256	785	**633**, 615, 483, 301	301, 257, 229	√	
Dihydrokaempferol-3-O-rhamnoside	267, 344	433	269, 179, 151			√
Dihydroquercetin-O-hexoside	258, 352	465	447, 339, 151			√
Dimeric procyanidin	280	577	425			√
Dimers of tergallagic-O-hexoside	376	631	**933**, 961, 451, 301, 299, 271	257, 229	√	
Ellagic acid	254, 368	301	**301**, 257, 229, 185	301, 284, 257, 229	√	
ellagic acid deoxyhexoside	250, 372	447	301	257, 229, 185	√	
Ellagic acid-dihexoside	254, 362	625	**463**, 301	301, 257, 191	√	√
Ellagitannin II	232, 253	643	481	355, 319, 301, 257, 193, 175		√
Ellagitannin III	232, 254	643	**481**, 463, 355, 301, 283	301, 300, 283		√
Ellagitannin VII	232, 254	951	907	889, 783, 605, 481, 301, 271	√	
Ellagitannin VIII	232, 253	953	**935**, 463, 301	891, 463, 343, 301	√	
Epicatechin	238, 278	289	261, **245**, 205, 203, 179	203, 227, 187, 161, 217		√
Ethyl caffeate	248, 328	207	179, 135		√	√
Ethyl gallate	280	197	169, 125		√	√
Gallagic acid	260, 377	601	**299**, 271, 243	271	√	
Gallagyl ester I	260, 379	1083	**1065**, 1021, 807, 721, 601, 575	763, 601, 575, 549, 425, 301, 299	√	
Gallagyl ester II	260, 379	1083	**1065**, 1021, 959, 807, 601, 575	301, 299	√	
Gallic acid (3,4,5-Trihydroxybenzoic acid)	232, 272	169	125	125	√	√
Galloyl-HHDP-glucose	286, 233	633	**463**, **301**, 275, 249	301, 257, 229, 185	√	
Glucoside ester of coumaric acid	234, 310	325	163, 145, 119			√
Galloyl-HHDP-DHHDP-hexoside (Granatin B)	365, 274	951	**933**, 631, 613, 301	631, 613, 301 299	√	
Laricitrin-3-O-glucoside	354	493	331	316, 193, 179		√
Myricetin-3-O-galactoside	262, 352	479	317			√
Myricetin-3-O-glucoside	266, 352	479	317, 179			√
Pedunculagin isomer (bis-HHDP- glucose)	276, 377	783	**765**, 481, **301**, 275	746, 301, 299, 301, 275, 229	√	
Pedunculagin isomer (bis-HHDP- hexoside)	268	783	**631**, 451, 425, 301	433	√	
Pedunculagin derivative	276	951	907	783, 481, 301	√	
Punicalagin isomer	258, 378	1083	**781**, 721, **601**, 575	721, 601, 299, 299, 271	√	
Punicalin derivative	258, 378	1101	1057, **781**, 721, 601	721, 601		
Punicalin a or b	258, 378	781	721, **601**	299, 271	√	
Quercetin-3-O-glucuronide	254, 352	477	301		√	√
Quercetin-3-O-rhamnoside	256, 352	447	301			√
Quercetin-3-O-xyloside	254, 356	433	301			√
Resveratrol (cis or trans)	284	227	159, 143			√
Syringetin-3-O-glucoside	248, 324	507	345			√
Syringetin-hexoside	274	507	**345**, **327**, 315	327, 315, 312, 296, 283, 268		√
Syringic acid	274	197	182, 167, 123			√
trans-Cinnamic acid	320	147	103			√
trans-coumaryltartaric acid	310	295	163			√
Valoneic acid bilactone	257, 365	469	425	407, 300	√	
Organic Acids		**$[M–H]^-$ (m/z)**	**MS^2 (m/z)**	**MS^3 (m/z)**	**Pomegranate Wine**	**Grape Wine**
Amber acid (Succinic acid)		117			√	√
Citric acid		191	**173**, 111	111, 67	√	√
L-malic acid		133	**115**, 87	71		√

Tartaric acid		149				√

Ions with relative abundance greater than 10% are shown; [M]+: molecular mass under positive ionization conditions; [M–H]–: molecular mass under negative ionization conditions; HHDP: hexahydroxydiphenic acid; Each successive MSn analysis applies on the ion shown in bold in the preceding column.

Table 3. Compounds identified in pomegranate and grape wines based on standards and GC-MS spectra libraries

A/A	Compounds	Pomegranate wine				Grape wine			
		non-hydrolyzed	hydrolyzed			non-hydrolyzed	hydrolyzed		
			alkaline	*post alkaline acidic*	*acidic*		*alkaline*	*post alkaline acidic*	*acidic*
1	BHT		√	√	√		√	√	√
2	benzoic acid	√	√	√	√	√	√	√	√
3	gallic acid	√		√	√		√		
4	4-hydroxybenzoic acid							√	
5	salicylic acid			√					
6	protocatechuic acid			√				√	
7	vanillic acid		√	√			√	√	
8	isovanillic acid			√				√	
9	homovanillic acid				√				
10	cinnamic acid		√	√			√	√	
11	hydrocinnamic acid	√	√	√		√	√	√	
12	caffeic acid	√	√	√	√	√	√	√	√
13	ferulic acid		√				√		
14	p-coumaric acid		√				√		
15	baccharin		√						
16	β-phenyllactic acid	√		√		√			
17	4-hydroxyphenyllactic acid				√				
18	mandelic acid				√				
19	2,3,4-trimethoxymandelic acid			√					
20	3-hydroxyphenylacetic acid					√	√		√
21	4-hydroxyphenylacetic acid							√	√
22	3-(4-hydroxy-3-methoxyphenyl)propanoic acid		√						
23	tyrosol	√			√				
24	phenylpyruvic acid	√	√	√	√	√	√	√	√
25	Pyruvic acid					√	√	√	√
26	1,2-benzenedicarboxylic acid		√				√	√	
27	1,2-benzenedicarboxylic acid, diisooctyl ester		√						
28	1,3-benzenedicarboxylic acid		√						
29	1,4-benzenedicarboxylic acid		√			√	√		
30	1,2,4-benzenetricarboxylic acid			√				√	
31	benzenehexacarboxylic acid							√	
32	4-hydroxyphenylpropionic acid				√				
33	1,3-dihydroxy-12H-benzo[b]xanthen-12-one		√						
34	malonic acid		√		√	√	√	√	
35	(4-hydroxy-2,5-dimethylphenyl)maleic acid							√	
36	Mannoonic acid		√						
37	vanillin						√		
38	(±)-catechin						√		
39	catechol		√				√		
40	catecholpyruvate	√				√			
41	luteolin		√						
42	quercetin		√	√			√	√	
43	quercetin-3-O-glucuronide		√				√		
44	quercetin-3-O-glucoside		√				√		
45	isorhamnetin		√						
46	4-acetyl-3-methoxyisocoumarin							√	
47	aesculetin (6,7-dihydroxycoumarin)	√							
47	4-Butyldihydrocoumarin			√					
49	vitamin B6–pyridoxine				√				
50	3-vanilpropanol				√				

51	2-(4-hydroxyphenyl)ethanol			√				√	√
52	succinic acid	√			√	√			√
53	Isopropenyl succinate		√				√		
54	Ethyl succinate								√
55	2-hydroxyglutaric acid	√							
56	2-oxo-pentanoic acid	√							
57	2-hydroxy-pentanoic acid	√							√
58	Hexadecanoic acid	√	√	√		√	√	√	
59	Heptadecanoic acid								
60	Octadecanoic acid	√	√	√		√	√	√	
61	11-Eicosanoic acid	√	√						
62	Pyrotartaric acid	√			√	√			√
63	Itatonic acid	√							
64	Methyl-maleic acid	√				√			
65	Fumaric acid	√							
66	Succinoic acid	√							
67	Citric acid	√							
68	Isocitric acid			√				√	√
69	2-Ethylcaproic acid		√						
70	Succinic dialdehyde			√					
71	2,2,3,4-Tetramethylpentane		√						
72	Octane, 6-ethyl-2-methyl-		√						
73	Acetaldehyde ethyl amyl acetal		√						
74	2,4-Dimethylhexane		√						
75	3-Pentanethiol		√						
76	1-Hexadecanol		√				√	√	
77	1-Octadecanol			√				√	
78	Oxalic acid, isobutyl pentyl ester		√	√			√	√	
79	2-Oxo-n-valeric acid		√	√					
80	valeric acid			√					
81	2-hydroxy-valeric acid					√			
82	3-hydroxy-valeric acid			√					
83	Phloroglucitol		√						
84	2,2-Dimethylpentanol		√				√		
85	n-Undecane		√				√	√	
86	n-1-Undecene		√				√		
87	3,4-Dimethyl decane						√		
88	3,3,4-Trimethyldecane			√					
89	Ethyl-boronic acid		√						
90	Erythronic acid-gamma-lactone		√						
91	3,7-Dimethyl-1-octanol		√						
92	2,2-Dimethyl-3-hexanone		√						
93	2-Allyl-1,4-dimethoxy-6-methylbenzene		√						
94	1-Hexadecene		√						
95	Neopentyl benzoate		√						
96	Oxalic acid, isobutyl propyl ester		√	√			√	√	
97	Decanoic acid						√		
98	2-Decenoic acid		√						
99	1-Dodecene		√				√		
100	n-Dodecanol		√				√	√	
101	n-Dodecanal		√				√		
102	3-Furoic acid			√				√	
103	2-Ketoisocaproic acid								√
104	3-Methyl-2-hydroxy-butanoic acid		√	√			√	√	√
105	1-Tetradecanol			√	√				√
106	n-Tetradecanoic acid			√					
107	Tartaric acid						√		√
108	Octane, 2,3,3-trimethyl						√		

phenyllactic acids were also identified in heather honey.[32] Phenylpyruvic acid was detected in all studied wines, whereas pyruvic acid only in grape wines. Pyruvic acid is a flavoring agent and yeast metabolite formed during fermentation.[33] Phenylpyruvic acid is a keto-acid that is an intermediate of phenylalanine metabolism to phenyllactic acid.[34] Tyrosol and hydroxyphenylacetic acid isomers were also detected in pomegranate and wine samples, respectively. The presence of such compounds suggesting that they might be microbial metabolites produced during fermentation and derived from the shikimic acid pathway via phenylpyruvic acid.[35] Coumarin derivatives were also detected by GC-MS analysis. Coumarin is a naturally occurring secondary plant product with pleasant flavor. The biosynthesis of coumarin in plants is via hydroxylation, glycolysis, and cyclization of cinnamic acid.[36] The presence of quercetin in wines after alkaline and post alkaline acidic hydrolyses confirm the detection of quercetin glycosides by LC-MS analysis (Table 2). Isorhamnetin (3-methylquercetin), which was detected after alkaline hydrolysis in pomegranate wine, possess *in vitro* anti-inflammatory activity and prevents endothelial cell injuries.[37]

The identification of acids such as tartaric (from grapes) and succinic acids (from grapes and pomegranates) along with oxalic, fumaric, isocitric and citric acid (from fermentation process) influences the pH of wines. Succinic acid is the main dicarboxylic acid produced by wine yeast during fermentation and its production is stimulated by the presence of glutamate or from sugars.[38]

The synthetic additive BHT was detected in wine samples, both after basic and acidic hydrolysis, and is probably added during vinification for wine preservation. BHT is widely used in food industry as a preservative.

Experimental

Chemicals, standards and solvents

All reagents, standards and solvents were used as previously described by Lantzouraki *et al.*[39]

Sampling and sample preparation

Pomegranate and grape red wines used in this study were produced in Armenia from Armavir region in 2013 and purchased from a wine store in Athens. A total of ten pomegranate semi dry red wine samples from Wonderful variety, aging for 1 year in oak barrels, with 11.5%(v/v) of alcoholic content, were assayed.

Concerning grape wine, ten dry red wine samples from Areni and Nerkeni varieties, aging for 2 years in oak barrels, with 12.5%(v/v) of alcoholic content, were evaluated. The same classical vilification process in steel tanks was applied for both types of wine. Grape and pomegranate wine bottles were stored in the dark and analyzed immediately after opening.

Determination of total phenolic content (TPC)

The total phenolic content (TPC) of each sample was determined applying a micromethod of Folin–Ciocalteu's colorimetric assay as described by Lantzouraki *et al.*[39]

Methods for determining the antiradical and antioxidant activity.

a) Scavenging Activity on 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$).

The antiradical activity of wine samples was evaluated by using the stable 2,2-diphenyl-1-picryl-hydrazyl radical ($DPPH^{\bullet}$) as described by Lantzouraki *et al.*[39]

b) Scavenging Activity on 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet +}$).

The antiradical activity of wine samples was determined according to the method described by Lantzouraki *et al.*[39]

Chemical hydrolysis of wines.

In order to identify the grape and pomegranate wines' phenolic compounds by GC–MS analysis, mild alkaline and acidic hydrolysis of the studied samples were performed using the method described by Lantzouraki *et al.*[39] During the hydrolysis, the glycosidic bonds of glycosylated phenolic compounds are cleaved and the hydrolyzed products are analyzed after silylation.

a) Mild alkaline hydrolysis. Briefly, 1.5 mL of wine was treated with 1.5 mL of a solution consisting of NaOH 4 M - ascorbic acid 2% (w/v) - EDTA 14 mM. The solution was vortexed for 5 min and remained at room temperature in dark for 16 h. Phenolics were extracted with 1.5 mL of diethyl ether-ethyl acetate solution (DE/EA, 1:1, v/v). The mixture was vortexed for 60 s and cooled for 10 min. After phase equilibration, phenolic compounds from alkaline hydrolysis, are transferred to the upper DE/EA organic layer.

b) Post alkaline acidic hydrolysis. The bottom aqueous layer resulting from alkaline hydrolysis was treated with 1.5 mL of a solution consisting of HCl 3 M – ascorbic acid 1% (w/v) – EDTA 5 mM. The solution was vortexed for 5 min and incubated in a water bath at 85 oC for 60 min. Phenolics were extracted with 2.0 mL of diethyl ether-ethyl acetate solution (DE/EA, 1:1, v/v). The mixture was vortexed for 10 min and cooled for 10 min. After phase equilibration, phenolic compounds from acidic hydrolysis, are transferred to the upper DE/EA organic layer.

c) Acidic hydrolysis. In 1.5 mL of wine, 1.0 mL of a solution, consisting of HCl 3 M – ascorbic acid 1% (w/v) – EDTA 5 mM, was added. The further experimental procedure followed the protocol described above.

Silylation of the phenolic compounds.

Silylation procedure was performed according to the method described by Lantzouraki *et al.*[39]

Gas chromatography/mass spectrometry analysis of phenolic compounds.

Qualitative analysis was performed on a mass spectrometer QP2010 Series (Shimadzu USA MANUFACTURING, Inc., Kyoto, Japan) as described by Lantzouraki *et al*.[39] Electron impact (EI) ionization was produced by accelerating electrons from a filament through a difference of 70 eV. A non-polar column was used (DB-5 MS, 30 m, 0.25 mm i.d. and 0.25 µm film thickness; Agilent, USA).

Liquid Chromatography – Tandem Mass Spectrometry (LC–MS^n).

a) Instrumentation. Phenolics separation was carried out using a Thermo Scientific Surveyor Plus HPLC–PDA–ESI–MSn system (San José, CA, USA). The platform comprised of a Thermo Scientific Surveyor HPLC Pump Plus, a Thermo Scientific Surveyor Autosampler Plus Lite, a Thermo Scientific Accela PDA Detector and a LCQ FLEET mass spectrometer with electrospray ionization (ESI). The data were processed using the Xcalibur software program (version 2.1).

b) Chromatographic conditions and mass spectrometry. The separation of phenolics was carried out using a Finnigan Surveyor system and a Hypersil Gold Column (3 µm, 2.1 × 100 mm, Thermo, Palo Alto, CA) protected with a security guard cartridge (Hypersil Gold, 3 µm, 10 × 2.1 mm i.d.) as described by Lantzouraki *et al*.[39]

c) Mass spectrometry analysis. Separate injections were run for analysis of the sample in both positive and negative electrospray ionization (ESI) modes as well as for different collision energies for MS^n analysis. According to the method described by Setandreu et al.,[30] positive and negative modes were applied for anthocyanidinic and non-anthocyanidinic compounds' determination, respectively.

The mass spectrometer parameters for positive ion mode were: source voltage, 3.5 kV; capillary voltage, 9 V; capillary temperature, 300 °C; sheath gas flow, 50 (arbitrary units); sweep gas flow, 20 (arbitrary units); full max ion time, 300 ms; and full micro scans, 3.

The mass spectrometer parameters for negative ion mode were: source voltage, 4.0 kV; capillary voltage, –18 V; capillary temperature, 300 °C; sheath gas flow, 50 (arbitrary units); sweep gas flow, 20 (arbitrary units); full max ion time, 300 ms; and full micro scans, 3.

Data dependent scan MS^n analyses for positive ions were carried out with the following conditions: collision energies 15, 17, 25, 30, 35 (arbitrary units); width, 1.00; repeat count, 2; repeat duration, 0.5 min; exclusion size list, 25; exclusion duration, 1.00 min; exclusion mass width, 3.00; scanned mass range (m/z), 260–1000.

Data dependent scan MS^n analyses for negative ions were carried out with the following conditions: collision energies 15, 25, 30, 35 (arbitrary units); width, 1.00; repeat count, 2; repeat duration, 0.5 min; exclusion size list, 25; exclusion duration, 1.00 min; exclusion mass width, 3.00; scanned mass range (m/z), 100–1600.

Statistical Analysis

All determinations (N = 10 samples per wine type) were carried out in triplicate. Values were averaged and reported along with the standard deviation (S.D.). All data were analyzed with One-Way ANOVA Post Hoc Tests and pairwise multiple comparisons were conducted with the Tukey's honestly significant difference test. Possibilities less than 0.05 were considered as statistically significant ($P<0.05$). All statistical calculations were performed with the SPSS package (IBM SPSS Statistics, version 19.0, Chicago, IL, USA) statistical software for Windows.

Conclusions

The present comparative study between pomegranate and grape wine products has highlighted the value of pomegranate wine in terms of total phenolic content and scavenging activity. Furthermore, LC and GC-MS based analyses were implemented to identify phytochemicals and more specifically phenolic compounds, putative antioxidant metabolites. Alkaline, acidic and post alkaline acidic hydrolysis, liquid-liquid extraction (LLE), and trimethylsilyl (TMS) derivatization procedures were implemented for GC-MS analysis and have resulted the detection of greater number of phenolic and other compounds, compared to LC-MS analysis without sample pretreatment. In general, alkaline hydrolysis has produced the highest number of compounds followed by post alkaline acidic, as detected by the GC-MS compared to other hydrolyses or no treatment conditions. Between studied substrates, 54 different compounds have been detected and identified in pomegranate wines compared to 38 compounds which have been detected in grape wines after alkaline hydrolysis. Between the identified metabolites, baccharin, a natural phenolic chemoprotective compound, phenyllactic and mandelic acids with significant antimicrobial properties, and brevifolin carboxylic acid, a hepatitis B virus (HBV) replication and tumor growth inhibitor have been identified. Overall, results showed a significant diversity between pomegranate and grape wines indicating that the higher antiradical capacity combined with the higher phenolic content of the first may be a promising basis of its exploitation.

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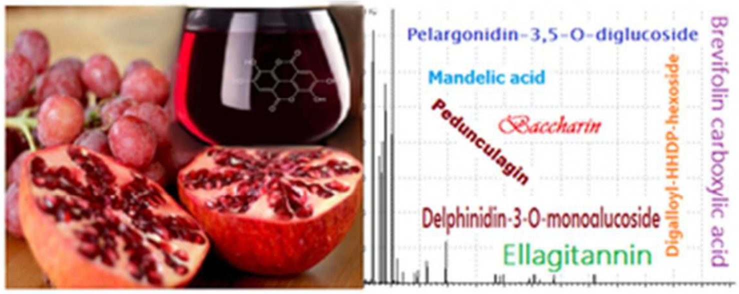

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