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CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES OF SEEDS FROM FOUR VITIS VINIFERA TUNI SIAN VARIETIES

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A B S T R A C T

Thousands of polyphenolic compounds have been identified in various plants. Recently, a number of studies showed that beneficial effects of grapes are related to the presence of polyphenols, with multiple biological activities. The aim of this study was to compare the polyphenol profiles as well as biological activities of the seeds of four Tunisia cultivated grape cultivars, syrat, merlot, cabernet sauvignon and carignan. The total phenolic content of seed extracts varied between varieties and seemed to be correlated to the scavenging activity. Preliminary antibacterial results showed a good growth inhibitory activity of syrat cultivar against *Staphylococcus epidermidis*. To our knowledge, this is the first of such study being performed on this bacterium, known as a leading cause of Tunisian hospital-acquired infections. Gas Chromatography Mass Spectrometry analysis identified 20 polyphenol components, flavonoids being the most abundant in all extracts, followed by phenolic acids, resveratrol, tyrosol, and syringaldehyde. We also noticed a deficiency of three phenolic acid compounds (salicylic acid methyl ester, ferulic acid, Sinapic acid) as well as a flavonoid one (myricetin) in merlot cultivar, which could be responsible for its low antioxidant activity. These results will help in the selective exploitation of the seeds obtained from winemaking wastes, as well as in further pharmacological and/or *in vivo* investigations.

Keywords: Grape, polyphenols, antibacterial, antioxidant.

Abbreviations

HCl:	Hydrochloric acid
BSTFA:	N,O-Bis(trimethylsilyl)trifluoroacetamide
TMCS:	Trimethylchlorosilane
GCMS:	Gas chromatography mass spectrometry
E.COLI:	Escherichia coli
B.cereus:	Bacillus cereus
MIC:	Minimal Inhibitory Concentration
S. epidermidis:	Staph epidermidis
TMS:	Trimethylsilyl
GSE:	Grape Seed Extract

INTRODUCTION

More than 10,000 polyphenolic compounds have been identified in various plants such as vegetables, tea, cereals, medical plants, microalgae, and fruits (Li *et al*., 2012; Deng *et al*., 2013; Li *et al*., 2014). A wide variety of polyphenolic compounds have been characterized in grapes which are one of the oldest and most consumed fruit of the world. Among *Vitis* species, *Vitis vinifera* is the most cultivated around the world, not only for its use in wine production, but also for its consumption as fresh fruit or as juice, especially in Mediterranean diet (Carton *et al*., 2007). A number of studies have shown that beneficial effects of grape, particularly those of the seed, are related to the presence of polyphenols, mainly flavonoids and phenolic acids, with antioxidant, anti-inflammatory, antimicrobial, antiviral, antiulcer, antimutagenic and cancer preventive properties (Tyagi *et al*., 2003; Shahidi and Yeo, 2018). Otherwise, for valorization objectives, Saïdani-Tounsi *et al.* (2009) proposed three Tunisian grape varieties (muscat, syrah and carignan) seed extracts as natural antioxidant agents,

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and confirm that these extracts represent a significant source of phenolic compounds. Moreover, Charradi *et al*. (2011) demonstrated that grape seed and skin extract alleviates high-fat diet-induced obesity and heart dysfunction by preventing cardiac siderosis. Furthermore, Kadri *et al*. (2015) determined the effect of short term (seven-days) administration grape seed and skin extract on ischemia/reperfusion (I/R) injury in a rat model of global ischemia. They noticed that almost all I/R-induced disturbances were prevented by GSSE pretreatment as oxidative stress; transition metals associated enzyme activities, brain damage size and histology.

In the same context, and in order to gain more knowledge towards the biological potential of grape seed extract (GSE), we performed a comparative analysis of four Tunisia cultivated cultivars: 'carignan', 'syrat', 'merlot' and 'cabernet sauvignon'. We compared their polyphenolic content and their antioxidant effect using the DPPH assay. We also investigated their antimicrobial potential against six bacterial strains and *Candida albicans*. To our knowledge, although many works valorized the antibacterial effect of GSE, it is the first study that presents the growth inhibitory effect of Tunisian GSE on *Staphylococcus epidermidis* which is a leading cause of Tunisian hospital-acquired infections (Mekni *et al*., 2012). Interesting results were obtained suggesting the potential of GSE as a natural antibacterial compound. A comparative analysis of the four cultivars' polyphenol composition, obtained by gas chromatography-mass spectrometry (GC-MS), was also conducted and discussed in relation with their biological activities.

MATERIALS AND METHODS

Plant material: The examined cultivars: ''merlot'', ''carignan'', ''syrat'' and ''cabernet sauvignon'' of *Vitis vinifera* were harvested from Grombalia vineyards (North-Eastern Tunisia) in 2014. Winemaking waste was collected from Tardi cooperative winery (Ain Ghelal). Seeds were manually selected, air dried and grounded with a coffee grinder (Brandt MX5SLIMV) until a fine powder was obtained.

Polyphenols' extraction: Polyphenols' extraction was carried out according to the method of Mau *et al*. (2001) with little modifications. Grape seeds powder (10 g) was stirred with 100 ml of methanol at room temperature at 150 rpm for 24 h and filtered through Whatman filter paper. The methanolic extract was then rotary evaporated at 40°C to dryness. The dried extract thus obtained was dissolved in methanol to a concentration of 10 mg/ml and stored at 4°C for further use.

Determination of total phenolics: Total phenolic contents were assayed using the Folin–Ciocalteu reagent, according to the method of Li *et al*. (2007). Briefly, 200 µl of the obtained extract was added to 1:10 diluted Folin–Ciocalteu reagent (1 ml). After 4 min, 800 µl of saturated sodium carbonate solution (about 75 g/l,) was added. The absorbance of the reaction mixture was measured at 760 nm with Shimadzu UV-160 spectrophotometer after incubation for 2 h at room temperature. Gallic acid was used as a reference standard, and the results were expressed as milligram gallic acid equivalent (mg GAE) per gram of dry weight. The estimation of phenolic compounds in the extracts was carried out in triplicate.

Measurement of the radical-scavenging activity: The potential antioxidant activity of plant extracts was determined on the basis of the scavenging activity of the stable DPPH free radical. The antiradical capacity of the samples was estimated according to the procedure reported by Brand-Williams and Cavelier (1995) and slightly modified. The methanol DPPH solution (1.95 ml, 6×10^{-5} mol/l) was added to 0.05 ml of different concentrations of GSE (5, 10, 20, 30, 50, and 100 µl/ml). After 30 min in room temperature, the solution absorption was measured at 517 nm with a Shimadzu UV-160 spectrophotometer versus methanol as a blank. DPPH scavenging effect was calculated by the following ratio: (Acontrol- Asample/ Acontrol) × 100, where A control is the absorption of the DPPH solution and A sample is the absorption of the DPPH solution after the addition of the sample. The antiradical activity was expressed as IC50 ($mgml^{-1}$), the concentration required to cause a 50% DPPH inhibition.

Antimicrobial assay: The antibacterial and anti-candida activities of grape seed methanolic extracts were conducted using agar disc diffusion method according to Celiktas *et al*. (2007) and Naeini *et al*. (2009), respectively. Briefly, microbial suspension (10^6 cells/ml) was seeded in Mueller Hinton agar plates. Filter paper discs were impregnated with 10 to 20 µl of the extract (equivalent to 3 mg/ml). Plates were incubated at 37°C for 24 hours and the size of the inhibitory zones (including disk diameter) was measured. The Minimum inhibitory concentration (MIC) was determined against *S. epidermidis* showing the higher diameter zone (12.7 mm in response to 'syrat' extract). The MIC value was

calculated as recommended by Taye *et al*. (2011). Bacterial growth was determined by measuring the OD600 nm using a microplate reader (Bioteck, ELx 800). MIC was defined as the lowest concentration inhibiting bacterial growth.

Phenolic compounds composition

Extraction of phenolic compounds: The extraction of phenolic compounds from grape seeds was carried out as follows. Plant material (10 g) was reduced to a fine powder and macerated in a shaker apparatus (60 °C for 24 h) with 200 ml of 80% aqueous ethanol (v/v) acidified to pH 2 with some drops of concentrated HCl. Extract was filtered using Whatman paper number 4. After ethanol evaporation in a rotary evaporator, the resulting aqueous solution was extracted three times with 30 ml of ethyl acetate in a separatory funnel and the organic solutions were combined and evaporated to dryness.

Derivatization procedure: For GC–MS analysis of phenolic compounds, grape seed extracts were derivatized in order to introduce silyl groups into molecules. The dried extracts were treated by adding BSTFA+TMCS 1% anhydrous pyridine (0.2 mL, 1:1). The mixture was heated at 60 °C for 30 min and derivatized sample submitted to GC–MS analysis.

GC-MS analysis: GC/MS analysis was performed on an Agilent GC system 7890A coupled with a mass spectrometer Agilent 5975C inert XL MSD with electron impact ionization (70 eV). An HP-5 ms capillary column (30 m x 0,25 mm coated with 5% phenyl methyl silicone, 95% dimethyl polysiloxane, 0,25 µm film thickness) was used. Oven temperature was programmed at 180°C for 1 min, then heated to 280 °C at a rate of 10 °C/min, it was kept constant at 280°C for 5 min, finally raised to 300°C at a rate of 15 °C/min and held for 10 min, transfer line temperature was 280°C. The carrier gas was He with a flow of 1 ml/min and a split ratio of 20/1. Scan time and mass range were 1 s and 50-1050 m/z, respectively. The identification of phenolics was assigned by matching their recorded mass spectra with those stored in the Wiley 09 NIST 2011 mass spectral library of the GC/MS data system and those found in recent publications (Table 1).

Statistics: Data were expressed as mean ± SE of at least three replicates, and the analysis of variance (two-way analysis of variance) was performed on Microsoft Excel 2007. Differences were considered statistically significant at $p < 0.05$.

Table 1. GC/MS analysis of phenolic compounds.

Compound	Molecular weight	TMS groups	TMS derivatized molecular weight	m/z (relative intensity)
Tyrosol	138,068	2	282,147	179 (100), 73 (54), 282 (18), 180 (15)
Hydroxybenzoicacid	138,032	2	282,111	73 (100), 267 (32), 45 (13), 91 (94)
Salicylicacidmethyl ester	152,047	0	152,047	120 (100), 92 (72), 152 (48), 65 (22)
Vanillicacid	168,042	2	312,121	73 (100), 267 (48), 297 (41), 282 (35), 312 (32)
Protocatechuicacid	154,027	3	370,145	73 (100), 193 (84), 370 (37), 45 (21), 355 (21)
Syringicacid	198,053	2	342,132	327 (100), 312 (78), 297 (69), 73 (54), 253 (48)
p-Coumaricacid	164,047	2	308,126	73 (100), 219 (55), 293 (47), 308 (38), 249 (24)
Gallicacidethyl ester	198,053	3	414,171	73 (100), 281 (60), 414 (37), 45 (22), 282 (14)
Gallicacid	170,022	4	458,180	73 (100), 281 (26), 458 (25), 45 (17), 459 (11)
Ferulicacid	194,058	2	338,137	338 (100), 73 (82), 323 (41), 249 (40), 308 (40)
Caffeicacid	180,042	3	396,161	396 (100), 219 (82), 73 (69), 397 (40), 381 (28)
Ellagicacid	302,006	4	521,126	73 (100), 217 (52), 521(29), 129 (29), 75 (25)
Sinapicacid	224,068	2	368,148	368 (100), 338 (83), 73(50), 353 (43), 369(29)
Syringaldehyde	182,058	1	254,097	224 (100), 73 (42), 239 (38), 254 (28), 223 (21)
Resveratrol	228,243	3	444,793	73 (100), 444 (84), 445 (31), 443 (11), 135(7)
Epicatechin	290,079	5	650,277	368 (100), 73 (72), 355 (35), 369 (34), 370 (16)
Catechin	290,079	5	650,277	368 (100), 73 (72), 355 (35), 369 (34), 370 (16)
Catechin gallate	442,376	8	946,371	560 (100), 78 (73), 369 (43), 281(31), 147(20)
Quercetin	302,043	5	662,240	575 (100), 576 (48), 73 (43), 577(27), 647(17)
Myricetin	318,038	6	750,275	73 (100), 735 (45), 736 (28), 737 (18), 647 (14)

RESULTS AND DISCUSSION

Total phenolic content: As indicated in Figure 1, the total phenolic content (TPC) of grape seed extracts varied between varieties and ranged from 158.45 ± 2.33 mg GAE/g DW in 'carignan' to 312.56 ± 3.9 mg GAE/g DW in 'cabernet sauvignon'. 'syrat' and 'merlot' exhibited a TPC mean of 295.11 mg GAE/g DW and 221.34 mg GAE/g DW, respectively.

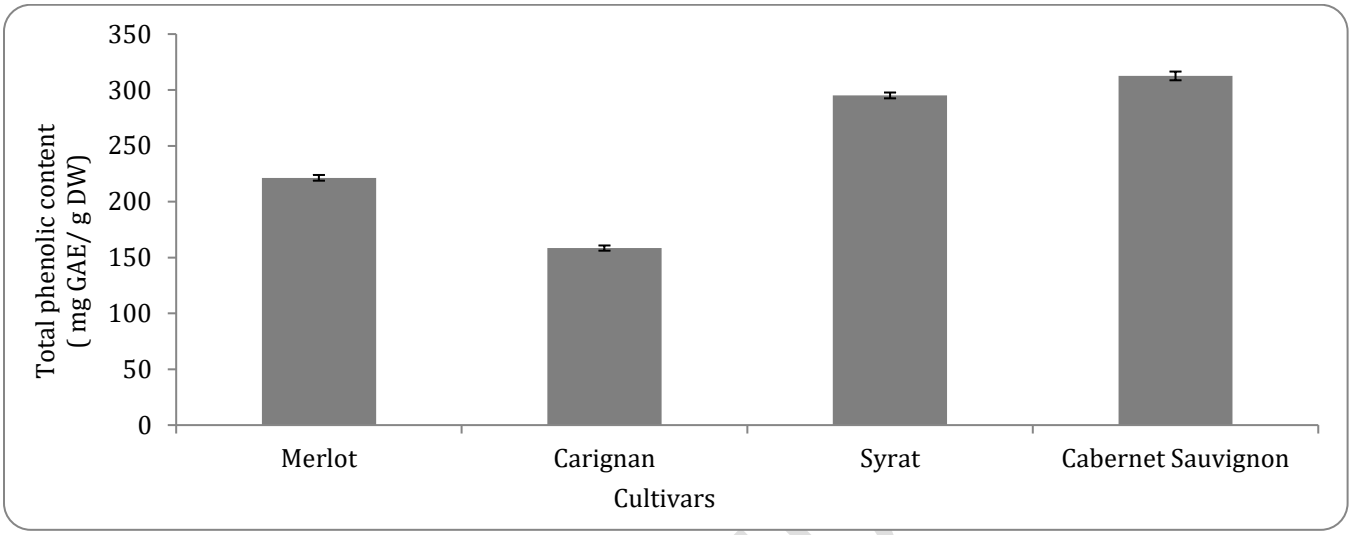

Figure 1. Total phenolic content in 'merlo', 'carignan', 'syrat' and 'cabernet sauvignon'.
mg GAE/g DW: milligrams gallic acid equivalent/ gram dry weight. Data are presented as mean ± standard deviation for at least three replicates.

DPPH radical scavenging activity: As it is known, the lower the IC50 value the higher the antioxidant activity of the extracts. As indicated in Figure 2, we noticed that all the seed extracts exhibited a higher IC50 value than that of the control (BHT: 17.34 µg/ml). It seems that there is a correlation between scavenging activity of GSE and total phenolic content. In fact, 'cabernet sauvignon' and 'syrat' had the highest antioxidant activities with IC50s of 20±0.36 and 20.3±0.38 µg/ml, respectively, whereas the IC50s of 'carignan' and 'merlot' were 21.49±0.32 µg/ml and 25.15±1.3 µg/ml, respectively.

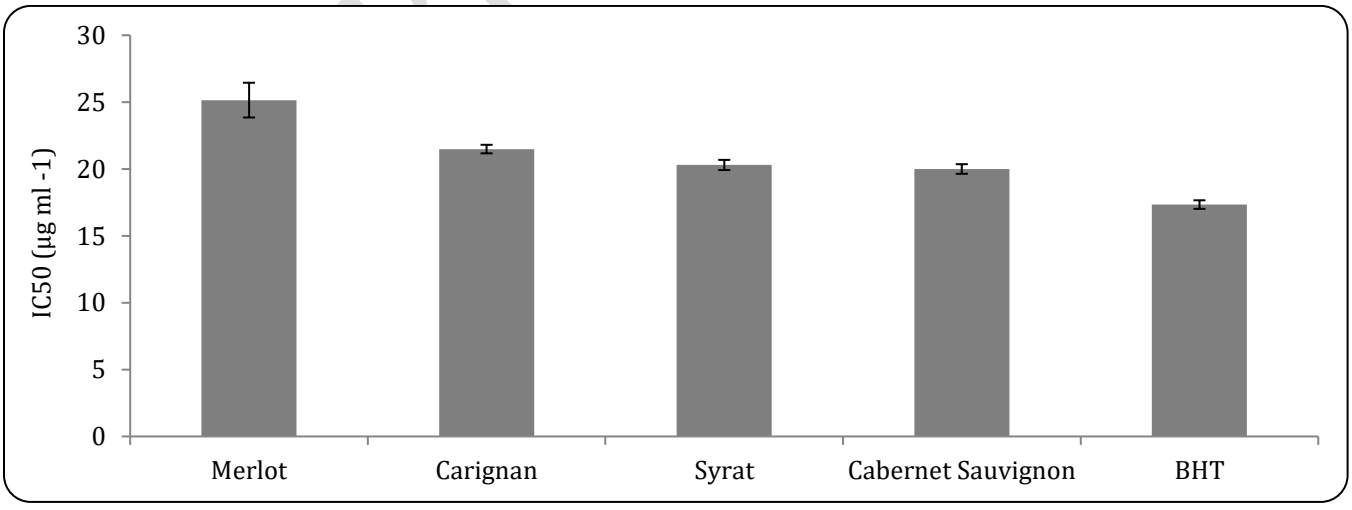

Figure 2. DPPH radical scavenging activities of 'merlot', 'carignan', 'syrat' and 'cabernet sauvignon'. Data are presented as mean ± standard deviation for at least three replicates.

Antimicrobial activity: As illustrated in Figure 3, all the GSEs exhibited a potent activity against the tested microorganisms except *E. coli*, *Erwinia* and *Candidaalbicans*. These findings confirm the reports that GSE is most effective against gram-positive bacteria than gram negative ones (Jayaprakasha *et al*., 2003; Filocamo *et al*., 2015). The pathogenic capability of gram-negative bacteria is usually associated with the presence of lipopolysaccharide layer, which might be involved in reducing the sensitivity of these bacteria against natural extracts (Delgado-Adámez *et al*., 2012). Interestingly, *S. epidermidis* showed the highest sensitivity, with zone inhibition diameters values equal to 12.7, 10, 9.7 and 7.7 mm in response to 'syrat', 'merlot', 'cabernet sauvignon' and 'carignan', respectively. To better elucidate the antibacterial effect of the four GSEs on *S. epidermidis,* MIC test was performed. 'Syrat' was the most effective extract against *S. epidermidis* with a MIC value of 200 µg/ml followed by 'merlot', 'cabernet sauvignon' and 'carignan' with MIC values of 375, 500 and 587.5 µg/ml, respectively. The antibacterial potential of grape extracts was generally attributed to their phenolic composition (Jayaprakasha *et al*., 2003; Baydar *et al*., 2004; Delgado-Adámez *et al*., 2012).

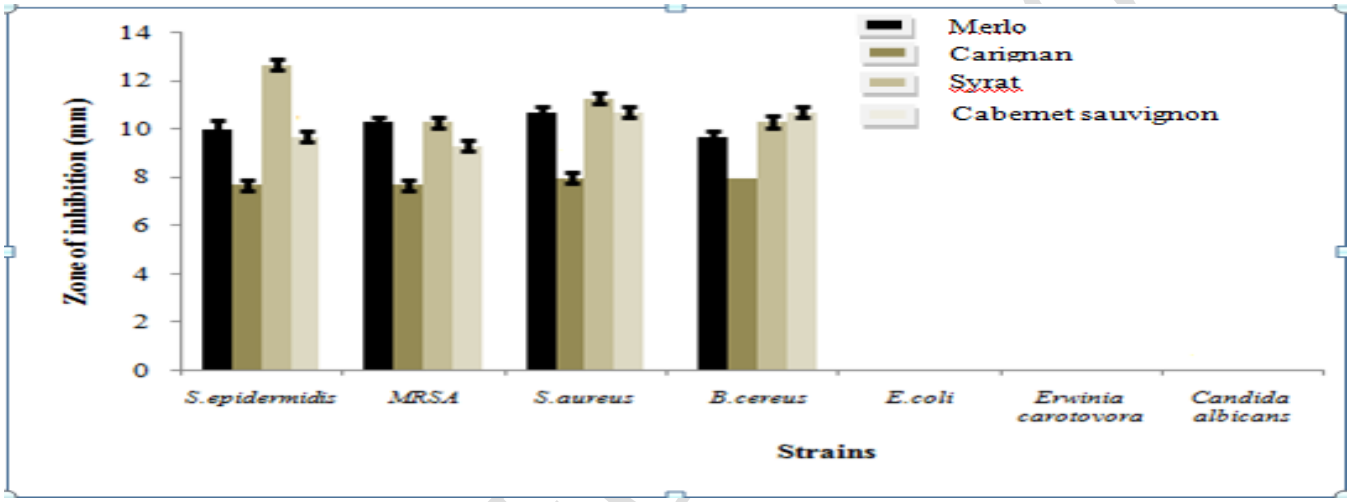

Figure 3. Effect of grape seed extracts on growth of bacteria strains and on *Candida albicans*. *S. epidermidis*: *Staphylococcus epidermidis*; MRSA: methicillin-resistant *Staphylococcus aureus*; S. aureus: *Staphylococcus aureus*; B. cereus: *Bacillus cereus*. No growth inhibitory effect of GSEs on *Escherichia coli*, *Erwinia carotovora* and *Candida albicans*. Data are presented as mean ± standard deviation for at least three replicates.

Determination of individual phenolic compounds in the GSE: Table 2 shows the individual compounds of GSE identified by GCMS. Phenolic compounds mainly consisted of flavonoids, but also of phenolic acids, resveratrol, tyrosol and finally syringaldehyde.

In agreement with previous works (Revilla and Ryan, 2000; Silván *et al*., 2013), flavonoids were the most abundant phenolic compounds in all extracts. They consisted of four flavonol compounds, mainly catechins and epicatechins, but also low levels of quercetin and myricetin. Moreover, only 'cabernet sauvignon' exhibited a low rate of catechin galloate (0.12%). Flavonoids have been known to possess numerous medicinal actions and properties (Lacopini *et al*., 2008). The amount of non-flavonoid content is dependent on the grape varieties. Resveratrol (or 3,5,4′-tri-hydroxy stilbene), syringaldehyde (or 3,5–dimethoxy–4–hydroxybenzaldehyde) and tyrosol were most abundant in 'cabernet sauvignon'. Resveratrol is a phytoalexin, it is the most widely studied stilbene found in small fruit such as grape, berries, peanuts, and some medicinal plants (Burns *et al*. 2002; Orallo, 2008; shetty, 2011). It possesses many biochemical and physiological properties including estrogenic, antiplatelet, and anti-inflammatory actions (Lekli *et al*., 2010). Important biological activities and cardioprotective effect were also demonstrated for tyrosol (Gris *et al*., 2011). On the other hand, syringaldehyde showed bioactive properties such as antioxidant, antimicrobial and anti-oncogenic (Ibrahim *et al*., 2012). Another non-flavonoid class corresponded to phenolic acids, average composition consisted of hydroxybenzoic acid, salicylic acid methyl ester vanillic

acid, protocatechuic acid, p-coumaric acid, gallic acid ethyl ester, ferulic acid, caffeic acid, ellagic acid, sinapic acid and finally gallic acid. As expected, for all analysed cultivars, the major phenolic acid compound corresponded to gallic acid (3,4,5-trihydroxybenzoic acid) which was reported to present numerous biological activities (Marino *et al*., 2014).

Table 2. Gas chromatography mass spectrometry (GC-MS) identification of phenolic compounds. RT: retention time.

Cultivar		'carignan'	'syrat'	'merlot'	'cabernet sauvignon'
Compound name	RT		Area %		
Tyrosol	5.031	0,87	0,77	1,45	1,9
PHENOLIC ACIDS					
Hydroxybenzoic acid	5.179	0,10	0,09	0,25	0,84
Salicylic acid methyl ester	5.305	0,39	0,37	0	0,78
Vanillic acid	7.36	0,25	0,29	0,72	0,78
Protocatechuic acid	8.13	0,40	0,43	0,85	2,47
Syringic acid	9.16	0,013	0,20	0,21	1,61
p-Coumaric acid	9.61	0,095	0,11	0,50	1,94
Gallic acid ethyl ester	9.74	0,16	0,16	1,13	1,50
Gallic acid	10.007	7,89	7,15	8,17	7,83
Ferulic acid	11.605	0,005	0,04	0	0,22
Caffeic acid	12.32	0,13	0,11	1,18	0,94
Ellagic acid	24.37	0,11	0,1	2,78	0,13
Sinapic acid	14.813	0	0,02	0	0,05
PHENOLIC ALDEHYDES					
Syringaldehyde	7.278	0,310	0,28	0,17	0,5
STYLBENES					
Resveratrol	20.69	0,12	0,10	1,24	1,5
FLAVONOIDS					
Epicatechin	21.764	45,42	47,00	36,81	36,13
Catechin	21.92	43,28	42,33	41,34	37,91
Catechin galloate	27.783	0	0	0	0,12
Quercetin	24.172	0,16	0,13	3,04	2,94
Myricetin	29.534	0,26	0,23	0	0,29

CONCLUSION

The present study compared the phenolic composition as well as the antioxidant and antibacterial potentials of four Tunisian grape seed extracts. Phenolic composition analysis showed varietal differences and this result would contribute to the selective exploitation of the seeds obtained from winemaking wastes. Our findings demonstrate that the four extracts presented considerable antioxidant and antibacterial activities, and can be proposed as nutritional supplement as well as natural preservatives. We emphasize that syrat displayed the most important radical scavenging activity and growth inhibitory effect, particularly against *staph epidermidis*. Thus, this cultivar could be exploited in future pharmacological and *in vivo* investigations.

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