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Nutritional constituents, phytochemical profiles, *in vitro* antioxidant and antimicrobial properties, and gas chromatography–mass spectrometry analysis of various solvent extracts from grape seeds (*Vitis vinifera* L.)

Samir Felhi[1], Noura Baccouch[2], Hichem Ben Salah[2], Slim Smaoui[3], Noureddine Allouche[2], Néji Gharsallah[1], and Adel Kadri[1,4,*]

[1]*Laboratory of Plant Biotechnology, Faculty of Science, University of Sfax, Sfax 1171, 3000, Tunisia*
[2]*Laboratory of Chemistry of Natural Substances, University of Sfax, Faculty of Sciences of Sfax, Sfax 1171, 3000, Tunisia*
[3]*Laboratory of Microorganisms and Biomolecules of the Centre of Biotechnology of Sfax, Sfax 1177, 3018, Tunisia*
[4]*College of Science and Arts in Baljurashi, Al Baha University, Al Baha 1988, Saudi Arabia*

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*Corresponding Author
Tel: +216-74-276-400
Fax: +216-74-274-437
E-mail: lukadel@yahoo.fr

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Abstract The present study revealed that the nutritive value of grape seeds (*Vitis vinifera* L.) was 383.55±0.13 Kcal/100 g, with magnesium as the most abundant mineral element (70.44±0.88 mg/L). The maximum phenolic (392.58±1.70 mg of GAE/g), flavonoid (256.16±1.60 mg of QE/g), and tannin (30.95±0.17 mg of CE/g) contents were also found in the ethanol, dichloromethane, and hexane extracts, respectively. The major phytochemical compounds in the ethyl acetate extract were identified via gas chromatography–mass spectrometry (GC–MS) analysis. The ethanol extract has the highest antioxidant activity (IC_{50}=140±1.20 μg/mL for DPPH, 145.28±0.45 mg α-tocopherol/g for total antioxidant capacity, and EC_{50}=80±1.41 μg/mL for ferric-reducing power assays). For β-carotene test, the highest antioxidant activity was obtained in the hexane extract. A satisfactory antimicrobial activity was found against a panel of microorganisms with the ethyl acetate extract as the best antimicrobial agent. Additionally, it was found that the bactericidal concentration required for the grape seed extract to kill *Listeria monocytogenes* should be less than 12.50 mg/mL (minimum inhibitory concentration=4).

Keywords: grape seed extracts, phytochemical contents, GC-MS analysis, antioxidant and antimicrobial activities

Introduction

Grape (*Vitis vinifera* L.) seeds were known as a by-product of food industry. They form a cheap source of natural antioxidant and antimicrobial agents owing to its richness in phenolic contents. Grape seed extract (GSE) is a waste product of the winery and grape juice industry (1), which contains a vast range of health benefits, such as 11% proteins, 40% fiber, 16% oil, 29.2% complex carbohydrates, 7% complex phenols, including tannins and other substances like sugars, mineral salts, and vitamins (2). The literature has confirmed the importance of GSE in pharmaceutical, food, cosmetic, and medical areas due to their effect against the deterioration and degeneration of human diseases by inhibiting the low-density lipoprotein (LDL) (3), antioxidative (4), anti-inflammatory (5), and antidiabetic (6) expressions as well as having hepatoprotective (7), renoprotective (8), and cardioprotective (9) activities. In addition, GSEs have gained considerable attention as antimicrobial compounds due to the presence of the 3,4,5-trihydroxyphenyl groups found in epigallocatechin, epigallocatechin-3-ogallate, prodelphinidin, and castalagin (10).

Therefore, this study examined the nutritional (proximate and mineral) composition and phytochemical profiles as well as gas chromatography–mass spectrometry (GC–MS) analysis of various solvent extracts to determine the percentage of phytochemical compounds in grape seeds (*V. vinifera* L.). It also aimed to assess the *in vitro* antioxidant and antimicrobial activities and justify the mode of action responsible for its antimicrobial properties against sensitive strains by kill-time analysis.

Materials and Methods

Reagents DPPH, linoleic acid, β-carotene, ascorbic acid, vitamin E, α-tocopherol, Tween 20, Tween 40, gallic acid, quercetin, Folin–

Ciocalteu, *N*,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and pyridine reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). For antimicrobial tests Mueller–Hinton Agar and Potato Dextrose Agar (PDA) were purchased from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). Spectrophotometric measurements were performed using a double-beam UV–VIS spectrophotometer.

Preparation of extracts Grapes were collected in August 2013 from Grombalia in the North East of Tunisia. The dried powdered grape seeds (200 g) were successively extracted twice (800 mL) with solvents of increasing polarity, such as hexane, dichloromethane, ethyl acetate, acetone, ethanol, and water, based on the method described by Bakari *et al*. (11).

Nutritional value and mineral composition The grape seed powder was analyzed for chemical composition (moisture, ash, fat, protein, and carbohydrates) using the AOAC procedures (12). The protein concentration was determined using the Kjeldahl method. The crude protein content was estimated by multiplying the total nitrogen content by the factor of 6.25. The fat was gravimetrically determined after the Soxhlet extraction of the powdered sample with hexane. Carbohydrate levels were calculated by subtracting the total sum of protein, fat, ash, and moisture from 100% dry weight sample. Energy was calculated according to the following equation: Energy value (kcal)=4×(g protein)+4×(g carbohydrate)+9×(g fat).

Two grams of dry powder was placed in a porcelain crucible and ashed at 550°C for 8 h. Subsequently, the ash was dissolved in 1 mL of concentrated hydrochloric acid and 100 mL of distilled water. Next, it was cooled and filtered through Whatman filter paper. From this ash, we performed the assay for determining metals/minerals, such as Mg, Ca, Fe, Zn, Ni, Cu, Pb, Hg, and Cd, via atomic absorption spectroscopy (AAnalyst 200; PerkinElmer, Waltham, MA, USA).

Determination of total phenolic, total flavonoid, and tannin contents The phytochemical contents of GSE were determined according to the method described by Bakari *et al*. (11). The tannin contents in extracts were analyzed using the modified vanillin assay described by Sun *et al*. (13). Each test was performed thrice.

GC–MS identification of phytochemical constituents

***Sample preparation and derivatization*:** The method described by Zuo *et al*. (14) was applied for the silylation of the phenolic extract and standards. For the silylation procedure, a mixture of BSTFA (200 μL), 200 μL extract of grape seeds in acetonitrile, and pyridine (50 μL) were mixed and mechanically shaken for 2 min at room temperature and consecutively placed in a water bath at 80°C for 60 min. At this point the samples were ready to be injected into the gas chromatography–mass spectrometer.

***GC analysis*:** The trimethylsilyl derivatives of phytochemical compounds were identified using the GC–MS, as cited by Bakari *et al*. (11). The GC column was a 30 m (0.25 mm i.d., film thickness 0.25 μm) HP-5MS (5% phenyl) methyl siloxane capillary column. The injector temperature was 250°C. The oven temperature was maintained at 100°C for 1 min, increased to 260°C at a rate of 4°C/min, and subsequently maintained at 260°C for 10 min. The detector temperature was 250°C. Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The sample volume in the direct injection mode was 1 μL. Electron impact ionization (EI) required an ion energy of 70 eV and a scanned mass range of 50–550 m/z. The identification of compounds was achieved by comparing the retention times with those of the authentic compounds and the spectral data obtained from the Wiley and NIST libraries.

Antioxidant activities

***DPPH-free radical scavenging, β-carotene–linoleic acid bleaching assay, and ferric-reducing power assay (FRAP)*:** All these tests were determined by spectrophotometric method based on the method described by Kadri *et al*. (15).

Total antioxidant capacity (TAC) (phosphomolybdenum method) The TAC of GSE was evaluated by the method described by Prieto *et al*. (16). It was expressed as mg of α-tocopherol equivalent per g dry weight. The experiment was also conducted using α-tocopherol (concentration: 0.0–0.1 mg/mL and correlation coefficient (R^2)=0.991) as a reference antioxidant.

Antimicrobial activity The bacteria and fungi used in this study included the following Gram-positive bacteria: *Bacillus cereus* JN 934390, *Bacillus subtilis* JN 934392, *Enterococcus faecalis*, *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes*, and *Micrococcus luteus*. It also included the following Gram-negative bacteria: *Salmonella enteric serotype* Enteritidis ATCC 43972, *Salmonella enteric serotype* Typhimurium, *Escherichia coli* ATCC 25922, and *Klebsiella pneumonia*. Additionally, fungal strains, such as *Aspergillus niger*, *Fusarium phyllophilum* AB587006, and *Penicillium* sp. were tested. The determination of the inhibition zone diameter (IZ), minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) were based on the same protocol as described by Daoud *et al*. (17).

***Kill-time analysis*:** To characterize the antibacterial activity of an extract over time, kill-time analysis is frequently applied using a slightly modified version of the method proposed by Ben Hsouna *et al*. (18). Each test was performed twice.

Statistical analysis The experimental results concerning this study were expressed as a mean±standard deviation (SD) of the 3 parallel measurements. A correlation and regression analysis was conducted using the Microsoft Excel program packaged in Microsoft Office 2010 (Microsoft Corporation, Redmond, WA, USA), and the values (p<0.05) were considered to be statistically significant with a probability of 5% to be wrong.

Results and Discussion

Nutritional value and mineral composition The results of the nutritional value and the mineral composition of the grape seed powder are shown in Table 1. The moisture content was the most frequently used parameter in food storage and preservation with a value of 3.20±0.05 g/100 g, which is nearly close to that found by Sousa *et al*. (19); however, it was lower than that obtained by Tangolar *et al*. (20). The lowest value of the moisture indicates the low sensitivity of grape seed to microbial growth and explains its great nutritional density. The obtained ash level of 4.50±0.05 g/100 g indicates the richness of grape seed in mineral elements. The amount of fat of grape seed observed in this study was 2.87±0.02 g/100 g, which is very similar to that obtained by Bampi *et al*. (21) in the residues of grape (2.56 g/100 g) and lower than that reported by Sousa *et al*. (19) (8.16 g/100 g). The low level of fat shows that grape seed is not a source of accumulation of lipids that cause arteriosclerosis and therefore improves the usage of the grape seed extract for individuals suffering from cardiovascular disease (22). The amount of protein in grape seed is 6.93±0.01 g/100 g, which is lower than that obtained by Sousa *et al*. (19). The carbohydrate content was higher (82.50±0.00 g/100 g) than that reported by Bampi *et al*. (21) and Sousa *et al*. (19) in the grape pomace flour. The observed differences can be attributed to the variability of the studied cultivars and climatic conditions. The high amount of carbohydrates in the grape seed extract indicates that it can be classified as a food rich in carbohydrates when compared to the other levels of nutrients. Based on protein, fat, and carbohydrate content, the grape seed energy content was 383.55±0.13 kcal/100 g, justifying its uses as a rich nutrient source. Sousa *et al*. (19) reported a lower value for grape seed (224 Kcal/100 g).

The contents of nutrient elements are listed in Table 1 with the predominance of grape seed in Mg (70.44±0.88 mg/100 g), followed by Ca (44.69±0.85 mg/100 g), Fe (5.05±0.36 mg/100 g), and Zn (2.05 ±0.35 mg/100 g); however, Ni, Cu, Pb, and Cd were less than 1 mg/ 100 g.

The richness of grape seed in Ca is essential for blood clotting. It is also helpful to maintain a healthy blood pressure for the normal functioning of the brain. Mg is known for its role as a source of the energy production process. Its presence is crucial in preventing fat absorption and producing proteins, enzymes, and antioxidants. Further, Fe has several vital functions in the body due to its role as the oxygen transporter of the protein hemoglobin, which is required for the energy production process and the enzymatic synthesis of RNA and DNA.

Table 1. Nutrient composition of grape seeds

Nutrients	Content
Proximate composition (per 100 g)	
Moisture (g)	3.20±0.05
Ash (g)	4.50±0.05
Fat (g)	2.87±0.02
Protein (g)	6.93±0.01
Total carbohydrate (g)	82.50±0.00
Energy (Kcal)	383.55±0.13
Minerals (mg/100 g)	
Magnesium (Mg)	70.44±0.88
Calcium (Ca)	44.69±0.85
Iron (Fe)	5.05±0.36
Zinc (Zn)	2.05±0.35
Nickel (Ni)	0.93±0.42
Copper (Cu)	0.54±0.10
Lead (Pb)	0.14±0.02
Mercury (Hg)	0.03±0.00
Cadmium (Cd)	0.00±0.00

Values are the means of triplicate experiments±SD.

Extraction yields The results of the preliminary investigation on the extraction yields of GSEs in various solvents are listed in Table 2. The above results of extraction yields indicated that the water extract produced the maximum phytochemical yield (3.90%), followed by the ethyl acetate (2.97%), ethanol (2.78%), hexane (2.77%), acetone (2.67%), and dichloromethane (2.24%) extracts. The difference in the extraction of the yields among the various GSEs was statistically significant (p<0.05).

The changes in the extraction yields appear to be related to the difference in the polarity of extracts of the components and to the

Table 2. Extract yield, total phenolic content (TPC), total flavonoid content (TFC), and tannin content (TC) in different solvents of grape seed extracts (GSEs)

Solvent extract	Extract yield % (w/w)	TPC (mg GAE/g)	TFC (mg QE/g)	TC (mg CE/g)
Hexane	2.77±0.10^{b}	120.37±1.50^{d}	131.82±1.20^{b}	30.95±0.17^{a}
Dichloromethane	2.24±0.22^{b}	106.82±1.80^{e}	256.16±1.60^{a}	28.37±0.76^{b}
Ethyl acetate	2.97±0.36^{b,a}	335.64±3.12^{b}	103.4±2.40^{c}	24.48±1.05^{c}
Acetone	2.67±0.28^{b,c}	278.71±2.50^{c}	50.59±3.50^{d}	17.06±1.12^{d}
Ethanol	2.78±0.28^{b}	392.58±1.70^{a}	44.55±1.45^{e}	27.40±1.08^{b}
Water	3.90±0.64^{a}	23.96±1.40^{f}	11.53±1.90^{f}	ND

Values are averages±SD of triplicate analysis.
ND, not detected.
For each extraction solvent, values in the same column followed by a different letter (a, b, c, d, e, and f) are significantly different (p<0.05).
Results are ranked in ascending order: a>b>c>d>e>f.

solvents used, which play a crucial role in increasing the solubility of phytochemical compounds. The previously obtained higher yield from the water extract may be related to the richness of GSE in pigments, enzymes, and bioactive components that are soluble in water (12).

Total phenolic, flavonoid, and tannin content The results of phytochemical analysis of the total phenolic content (TPC), total flavonoid content (TFC), and tannin content (TC) in various extracts are listed in Table 2. As shown in Table 2, the ethanol extract exhibited the highest TPC (392.58±1.70 mg GAE/g), followed by ethyl acetate (335.64±3.12 mg GAE/g), acetone (278.71±2.50 mg GAE/g), hexane (120.37±1.50 mg GAE/g), dichloromethane (106.82± 1.80 mg GAE/g), and water (23.96±1.40 mg GAE/g) extracts, with a significant difference between them (p<0.05). Additionally, the TFC varied from 11.53±1.90 to 256.16±1.60 mg QE/g. Among all extracts, the highest level of the TFC recorded was for dichloromethane (256.16±1.60 mg QE/g), followed by the hexane (131.82±1.20 mg QE/g), ethyl acetate (103.4±2.40 mg QE/g), acetone (50.59±3.50 mg QE/g), ethanol (44.55±1.45 mg QE/g), and water (11.53±1.90 mg QE/g) extracts. Indeed, it was observed that the hexane extract accounted for the highest amount of tannin (30.95±0.17 mg CE/g), followed by dichloromethane (28.37±0.76 mg CE/g), ethanol (27.40±1.08 mg CE/g), ethyl acetate (24.48±1.05 mg CE/g), and acetone (17.06±1.12 mg CE/g); however, the water extract was not found in tannin.

The results of this study suggest that the solvent extract considerably influenced the extraction capacity of the phenolic compounds. The recovery of polyphenols from plant is influenced by the solubility of the phenolic compounds in the solvent used for the extraction process (23). In the current research, 6 solvents with different polarities were used. The results indicated that ethanol had the highest level of phenolic content. This observation is in agreement with the previous studies, indicating that ethanol is the best solvent to extract a large range of phenolic compounds from plants (24,25). It has been pointed out that the phytochemical constituents exhibit a greater antioxidative action in biological systems, acting as scavengers of singlet oxygen, free radicals, hydrogen donors, and reducing agents owing to their high redox potentials (11).

GC–MS identification of phytochemical compounds The analysis of the phyto-constituents of plant extracts via GC–MS provides the most sensitive detection of bioactive compounds (26). Phenolic compounds in the ethyl acetate extract were analyzed using GC–MS. The GC–MS analysis of the extract facilitated the identification of 12 different compounds representing approximately 93.00% of the total extract. Table 3 lists the phytochemical constituents identified in the extract along with their retention times, molecular formula, and peak area. The major phytochemical compounds were linoleic acid (32.36%), pimaric acid (12.16%), caffeic acid (10.49%), p-hydroxyphenylacetic acid (8.78%), and gallic acid (7.22%). These results suggest that the seeds of *Vitis vinifera* L. exhibited a high content of the total phytochemical compounds. Kumar and Vijayalakshmi (27) have reported that linoleic acid is a major constituent in the GSE.

These major phytochemical compounds present in the extract contain certain important medicinal activities for the future drug discovery system, such as linoleic acid having the antioxidant and antimicrobial activities (28). On the other hand, caffeic and gallic acids had the antimicrobial, anti-inflammatory, and antioxidant activities (29,30). The studied seed extract may have great potential for use in food industries as a source of bioactive molecules, such as phenolic compounds for dietary supplements or functional foods. Therefore, the GSEs were found to exhibit a therapeutic effect and could be used as food sources by the free radicals, which affect the organoleptic properties and edibility of foods (31,32).

Antioxidant activities

***DPPH radical scavenging activity*:** DPPH is a stable nitrogen-centered free radical that provides information about the potential of an antioxidant to inhibit oxidative cell damages by preventing reactive radical species from attacking key biomolecules such as lipoproteins

Table 3. Molecular mass (MM) and important ions present in the mass spectra of silylated phytochemical compounds* in grape seed ethyl acetate extract by gas chromatography–mass spectrometry (GC–MS)

Peak No.	Retention time (min)	Name of the compound	MM (silylated compounds)	Peak area (%)	Identified ions (m/z)
1	7.71	Benzoic acid	194	2.73	77, 105, 135, 179, 194
2	8.07	Gallic acid	458	7.22	147, 179, 281, 458
3	14.67	p-Hydroxybenzoic acid	282	2.49	193, 223, 267, 282
4	16.15	p-hydroxyphenylacetic acid	296	8.78	149, 164, 179, 296
5	20.40	p-Coumaric acid	308	3.46	219, 249, 293, 308
6	20.64	Palmitic acid	328	0.63	73, 117, 145, 313, 328
7	27.65	Ferulic acid	338	2.80	219, 249, 293, 279, 308, 323, 338
8	29.46	Caffeic acid	396	10.49	179, 191, 219, 396
9	30.51	Linoleic acid	352	32.36	73, 95, 129, 337, 352
10	34.28	Oleic acid	354	4.46	73, 117, 145, 339, 354
11	42.68	Stearic acid	356	5.42	73, 117, 341, 356
12	50.19	Pimaric acid	374	12.16	73, 121, 359, 374

* Identified as TMS derivatives.

and polyunsaturated fatty acids in biological and food systems. The antioxidant activity is expressed as IC_{50} (i.e., the concentration of the antioxidant required to scavenge 50% of the initial DPPH radicals) (Table 4), and the potential of the GSE ranged significantly (p<0.05) as follows: the vitamin E (IC_{50}=17±0.11 μg/mL)>ascorbic acid (IC_{50}= 26±0.16 μg/mL)>ethanol (IC_{50}=140±1.20 μg/mL)>dichloromethane (IC_{50}=300±3.10 μg/mL)>hexane (IC_{50}=400±2.60 μg/mL)>acetone (IC_{50}= 450±3.34 μg/mL)>ethyl acetate extracts (IC_{50}=550±5.30 μg/mL). The higher radical scavenging ability of the ethanol GSE can be attributed to its higher level in TPC.

***β-carotene–linoleic acid bleaching assay*:** This test is based on the discoloration or bleaching of β-carotene caused by radicals released upon the oxidation of the linoleic acid in the emulsion by measuring the ability of an antioxidant to inhibit lipid peroxidation. As shown in Table 4 (results were expressed in terms of IC_{50}), the GSE displayed various degrees of scavenging with the strongest effect observed for hexane (IC_{50}=87±1.92 μg/mL), which is similar to that of the standard ascorbic acid (IC_{50}=87±2.82 μg/mL), followed by ethyl acetate (243± 4.41 μg/mL), ethanol (282±2.82 μg/mL), water (300±2.14 μg/mL), and acetone (550±4.70 μg/mL). The correlation coefficient between the TPC and TFC and the IC_{50} values of the β-carotene bleaching assay was significant (R^2=0.50 and R^2=0.78, respectively). In addition, our data indicate that the GSE phenolics and flavonoids were observed as major sources of natural antioxidants.

***FRAP*:** The FRAP assay approach is based on a single-electron transfer involving the monitoring of the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). The reducing power was expressed as EC_{50} (effective concentration at which the absorbance is 0.5). The results summarized in Table 4 revealed that the ethanol extract exhibited the highest activity (EC_{50}=80±1.41 μg/mL), followed by ethyl acetate (EC_{50}=160± 3.70 μg/mL), acetone (EC_{50}=200±4.00 μμg/mL), and water (EC_{50}=200 ±2.49 μg/mL); however, the dichloromethane and hexane extracts exhibited considerably lower activity (p<0.05) with EC_{50}=640±3.82 and 720±5.70 μg/mL, respectively. In this study, high positive correlations were also observed between the TPC and ferric-reducing antioxidant power of the grape seeds (R^2=0.593). Additionally, these correlations were found between the TFC of the GSE and their antioxidant activity assessed by the FRAP method (R^2=0.76). The reducing ability of the grape seed, as determined by the FRAP assay, was attributed to the magnitude conjugation of the phenolic compounds as well as phenolics and flavonoids.

***TAC*:** This assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH, which is measured at 695 nm. The results listed in Table 4 show that the antioxidant capacity of the GSEs decreased in the following order: the ethanol (145.28±0.45 mg α-tocopherol/g extract)>hexane (138.99±2.41 mg α-tocopherol/g extract)>acetone (108.38±4.13 mg α-tocopherol/g extract)>ethyl acetate (60.34±2.91 mg α-tocopherol/g extract)>dichloromethane (54.48±2.69 mg α-tocopherol/g extract)>water extracts (30.01±2.01 mg α-tocopherol/g extract). This excellent antioxidant activity might be attributed to the richness of polyphenols in these extracts. We noted the positive correlation with the TPCs (R^2=0.51); however, with flavonoid contents, this correlation was significantly less (R^2=0.13).

Antimicrobial activity In the present study, the antimicrobial activity of the different GSEs in different solvents was investigated against 13 microorganisms, including 10 bacteria (6 Gram-positive and 4 Gram-negative) and 3 fungi, which were compared to the controls (chloramphenicol for bacteria and cyclodextrine for fungi), and assessed quantitatively by IZ, MIC, MBC, and MFC values. The results depicted in Table 5A and 5B show that the ethyl acetate extract possesses the strongest antibacterial activity against *Listeria monocytogenes* (42.5±1.5 mm) and *Micrococcus luteus* (38.5±1.5 mm), which were about 2 and 3 times higher than the positive control, respectively. On the other hand, the ethanol extract displayed a considerably better antibacterial activity (27.0±2.0 mm) than chloramphenicol (12.0±0.0 mm); however, the water extract exhibited the lowest activity. Among all extracts, only ethanol and water are sensitive to fungal strains; however, the others were

Table 4. DPPH-free radical scavenging activity, β-carotene–linoleic acid assay, ferric-reducing power assay (FRAP), and total antioxidant capacity (TAC) of grape seed extracts (GSEs)

Solvent extract	IC_{50} (μg/mL)		EC_{50} (μg/mL)	TAC
	DPPH	β-carotene	FRAP	(mg of α-tocopherol/g extract)
Hexane	400±2.60^{c}	87±1.92^{e}	720±5.70^{a}	138.99±2.41^{a}
Dichloromethane	300±3.10^{d}	ND	640±3.82^{b}	54.48±2.96^{e}
Ethyl acetate	550±5.30^{a}	243±4.41^{d}	160±3.70^{d}	60.34±2.91^{d}
Acetone	450±3.34^{b}	550±4.70^{a}	200±4.00^{c}	108.38±4.13^{b}
Ethanol	140±1.20^{e}	282±2.82^{c}	80±1.41^{e}	145.28±0.45^{c}
Water	ND	300±2.14^{b}	200±2.49^{c}	30.01±2.01^{f}
Vitamin E	17±0.11	–	–	–
Ascorbic acid	26±0.16	87±2.82	79±1.41	–

Values are averages±standard deviation of triplicate analysis.
ND, not detected.
–, not tested.
For each extraction solvent, values in the same column followed by a different letter (a, b, c, d, e, and f) are significantly different (p<0.05).
Results are ranked in ascending order: a>b>c>d>e>f.

remained unaffected, except for the ethyl acetate extract, which was active only against *Fusarium phyllophilum* (13.0±1.0 mm).

The results of the MIC, MBC, and MFC values were found to vary with extraction solvent and were generally in accordance with those recorded for IZ (Table 5A). The MIC values ranged between 1.56 and 25.00 mg/mL, whereas the MBC values were similar or twofold higher than the MIC values. However, in some cases, the MBC values were recorded with a higher value than the strongest MIC and MBC values. In agreement with the report of Gatsing *et al*. (33), an extract is bactericidal when MBC/MIC4 and bacteriostatic if MBC/MIC>4. The data listed in Table 5B indicate that many MBC/MIC ratios for the crude extracts were below 4, suggesting their bactericidal effects on several Gram-positive and Gram-negative bacteria, excluding the bacteriostatic effects of ethanol and ethyl acetate for *Listeria monocytogenes* and *Salmonella* Enteritidis, respectively. However, for the fungicidal degree of extracts, as described by the MFC/MIC

Table 5A. Antimicrobial activities of GSEs against bacterial and fungal strains

Strains	Inhibition zones diameter (mm)[1)]						
Bacterial	Hexane	Dichloromethane	Ethyl acetate	Acetone	Ethanol	Water	Chloram.[3)]
Gram-positive bacteria							
Bacillus cereus	–[2)]	–	11.0±1.0	–	10.0±0.0	10.0±0.0	26.0±1.0
Bacillus subtilis	12.5±0.5	12.5±1.0	15.0±1.0	11.0±1.0	11.5±0.5	10.5±0.5	24.0±0.0
Enterococcus faecalis	–	–	12.0±0.0	–	–	–	12.0±1.0
Staphylococcus aureus	–	14.0±2.0	12.5±1.0	16.5±1.5	17.0±1.0	10.0±1.0	16.5±0.5
Listeria monocytogenes	15.0±1.0	11.5±0.5	42.5±1.5	12.0±0.0	18.5±0.5	11.0±1.0	20.0±2.0
Micrococcus luteus	13.5±0.5	12.0±1.0	38.5±1.5	11.0±0.0	27.0±2.0	11.0±0.0	12.0±0.0
Gram-negative bacteria							
Salmonella Enteritidis	10.0±1.0	13.5±0.5	20.0±0.0	–	14.0±1.0	11.0±0.0	16.0±0.0
Salmonella Typhimurium	10.0±0.0	12.0±0.0	14.5±0.5	11.5±0.5	13.5±0.5	12.0±1.0	17.0±1.0
Escherichia coli	10.5±0.5	12.0±1.0	13.0±1.0	14.5±0.5	11.0±0.0	15.5±1.5	23.5±0.5
Klebsiella pneumoniae	–	–	12.5±0.5	–	–	–	22.0±1.0
Fungal strains							Cyclo.[4)]
Aspergillus niger	–	–	–	–	12.0±0.5	20.0±2.0	17.0±1.0
Fusarium phyllophilum	–	–	13.0±1.0	–	15.0±1.0	25.0±2.0	14.5±0.5
Penicillium sp.	–	–	–	–	14.0±2.0	21.0±1.0	14.0±1.0

Values are mean ± standard deviation of 3 separate experiments.
[1)]Diameter of the inhibition zones of extract, including diameter of well 6 mm.
[2)]no inhibition.
[3)]Chloramphenicol was used as a standard antibiotic at a concentration of 15 μg/well.
[4)]Cycloheximide was used as a standard antibiotic at a concentration of 20 μg/well.

Table 5B. Determination of the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) (mg/mL) of GSEs

Solvents	Hexane			Dichloromethane			Ethyl acetate			Acetone			Ethanol			Water		
Strains	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
Gram-positive bacteria																		
Bacillus cereus							12.5	25.0	2				6.25	12.5	2	12.5	25.0	2
Bacillus subtilis	12.5	12.5	1	25.0	12.5	0.5	25.0	12.5	0.5	12.5	25.0	2	6.25	12.5	2	12.5	25.0	2
Enterococcus faecalis							12.5	25.0	2									
Staphylococcus aureus				12.5	12.5	1	12.5	25.0	2	12.5	25.0	2	6.25	12.5	2	12.5	25.0	2
Listeria monocytogenes	3.12	6.25	2	3.12	12.5	4	3.12	6.25	2	6.25	12.5	2	1.56	12.5	8	12.5	25.0	2
Micrococcus luteus	3.12	6.25	2	3.12	6.25	2	3.12	12.5	4	6.25	12.5	2	3.12	12.5	4	12.5	25.0	2
Gram-negative bacteria																		
Salmonella Enteritidis	12.5	25.0	2	12.5	25.0	2	1.56	12.5	8				6.25	12.5	2	12.5	25.0	2
Salmonella Typhimurium	12.5	25.0	2	6.25	25.0	4	3.12	12.5	4	6.25	25.0	4	3.12	6.25	2	6.25	12.5	4
Escherichia coli	6.25	25.0	4	12.5	25.0	2	12.5	25.0	2	12.5	25.0	2	12.5	25.0	2	12.5	25.0	2
Klebsiella pneumoniae							12.5	25.0	2									
Fungal strains	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC
Aspergillus niger													6.25	25.0	4	12.5	25.0	2
Fusarium phyllophilum							12.5	25.0	2				12.5	25.0	2	6.25	25.0	4
Penicillium sp.													6.25	25.0	4	6.25	25.0	4

ratios, the results showed that the ethyl acetate extract possess fungicidal degree for *Fusarium phyllophilum*, and the same effect was determined with ethanol and water extracts for *Aspergillus niger*, *Fusarium phyllophilum* and *Penicillium* sp. with MFC/MIC4.

In the present study, it is worth noting that the sensitivity of the Gram-positive and Gram-negative bacteria is due to the morphological differences among the cell membranes of tested microorganisms, particularly for the Gram-negative bacteria as their outer phospholipidic membrane carries the structural lipopolysaccharide components and makes their surfaces highly hydrophilic (17). On the other hand, the higher susceptibility of the Gram-positive bacteria can be explained by the fact that they possess only a peptidoglycan layer that facilitates the infiltration of hydrophobic compounds. Thus, in agreement with other reports, the antibacterial activity of the extract may be due to the diversity of the phytoconstituents present in the GSEs and to the fact that different antibacterial compounds are present in the plant extracts, which may differently and synergistically act to affect the antimicrobial properties. Both ratios of MBC/MIC for bacteria and those of MFC/MIC for fungi were evaluated, and the results revealed different behaviors depending on the powerful extract with the effectiveness of ethanol and ethyl acetate for *Listeria monocytogenes* and *Salmonella* Enteritidis as a bacteriostatic agent and ethanol and water for *Aspergillus niger*, *Fusarium phyllophilum*, and *Penicillium sp* as a fungicidal agent.

***Kill-time analysis: Effect of ethyl acetate extract on viable counts of Listeria monocytogenes*:** kill-time kinetics is an assay used to elucidate a new antimicrobial agents for a better understanding on the pharmacodynamics of antimicrobial agents. Therefore, the effect of grape seed ethyl acetate extract on viable counts of selected bacteria, such as *Listeria monocytogenes*, was investigated to confirm its effect and clarify its action mechanism. The kinetics was performed using different concentrations of the ethyl acetate extract (MIC, 2 MIC, 4 MIC, and 8 MIC). The results summarized in Fig. 1 in terms of the log10 CFU/mL demonstrate the effect of the ethyl acetate extract on the viable count of *Listeria monocytogenes* at the MIC (3.12 mg/mL) and 2 MIC (6.25 mg/mL) values. As shown in Fig. 1, the number of viable cells decreases over the 60-min period of the test and reaches 2 and 1 log CFU/mL for MIC and 2 MIC, respectively. At higher concentrations of 4 MIC (12.50 mg/mL) and 8 MIC (25.00 mg/mL), no viable cells were observed after 20 min. Consequently, the bactericidal concentration required for the GSE to kill *Listeria monocytogenes* should be less than 12.50 mg/mL (4 MIC). The MBC results have confirmed that the biocidal effect of the extract may be applied on the examined organisms, which encouraged the isolation and development of new antimicrobial agents that were exhibited by the kill-time assessment of the extract (34).

In conclusion, grape seeds contain high levels of important nutrients, including minerals, protein, lipid and carbohydrates, as well as antioxidant and antimicrobial activities. The results demonstrate that the extraction solvents affect the contents of the phenolic, flavonoid, and tannin compounds and their antioxidant and antimicrobial properties in various degrees. The highest content of phenolic, flavonoid, and tannin were obtained with the ethanol, dichloromethane, and hexane extracts, respectively. Considering the antioxidant activities evaluated via DPPH-free radical scavenging activity, FRAP, and TAC, the best activity was demonstrated by the ethanol and hexane extracts for β-carotene–linoleic acid bleaching assay. The high correlation between the total phenolic and antioxidant activities indicates that the total phenolics are the main contributors to antioxidant activities. Furthermore, our results revealed the potential effect of the ethyl acetate extract from the grape seeds as a source for natural health products or natural food preservatives owing its high antibacterial and antifungal activities against a panel of food pathogenic bacteria and fungus. A particular attention has been focused on the characterization by GC–MS of the phytochemical composition of the ethyl acetate extract, which has potential bioactivities and high content of phenolic compounds. Our results are agree well with the findings that demonstrate the use of grape seeds as a promising candidate for medicinal health functions and various antimicrobial and antioxidant applications in food, cosmetics, and other fields.

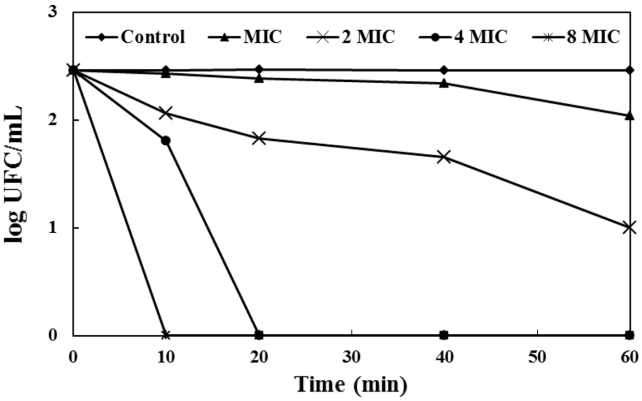

Fig. 1. The bactericidal effect of grape seed ethyl acetate extract on *Listeria monocytogenes* strain. Samples were collected at different incubation times, and viability was determined by the plate colony count procedure [colony forming unit (CFU)].

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