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Antilisterial Activities of Polyphenol-Rich Extracts of Grapes and Vinification Byproducts

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The quantitation of the main polyphenols and the assessment of the total polyphenolic content (TPC) in polyphenol-rich extracts of grape berries and vinification byproducts, obtained from *Vitis vinifera* cultivars of the Greek islands, are presented. The results indicated that seed extracts contain high concentrations of flavan-3-ols and their derivatives, whereas pomace and stem extracts consist of significant amounts of flavonoids, stilbenes, and phenolic acids. In particular, stems—a scarcely studied class of grape byproduct—were also characterized by high *trans*-resveratrol and ε-viniferin content. The evaluation of their in vitro antilisterial activities revealed as most potent the seed and stem extracts of the red variety Mandilaria. Their minimum inhibitory concentrations (MICs) were assessed using a Malthus apparatus by two methods, a plate count technique and an automated technique that combines the conductance measurements with the common dilution method. The results revealed the usefulness of the conductance method as an alternative rapid means for the MIC estimation, whereas the respective values (0.26 and 0.34 for seeds and stems) indicated that both extracts represent an inexpensive source of potent natural antilisterial mixtures, which may be incorporated in food systems to prevent the growth of *Listeria monocytogenes*.

KEYWORDS: Grape berries; grape pomace; grape seeds; grape stems; total polyphenolic content (TPC); antilisterial activity; food preservation

INTRODUCTION

Preservative agents are required to ensure that manufactured foods remain safe and unspoiled. The increasing number and severity of food-poisoning outbreaks worldwide have stimulated public awareness about the safety of foods in general. The annual health care cost traced to a few selected foodborne pathogens such as *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* spp. was estimated at 5−6 billion euros per year (*1*). On the other hand, the excessive use of preservatives, many of which are suspect because of their potential carcinogenic and teratogenic attributes or residual toxicity (*2, 3*), has created mistrust by the European consumer that is threatening to become a long-term problem. The increasing resistance of some pathogens associated with food-borne illness is also of great concern. To remedy this, the stakeholders (e.g., Food Industry and European authorities) have to show increased vigilance with regard to all safety and quality issues (*4, 5*). Because consumers need to feel reassured that the foods are safe, there is increasing pressure on food manufacturers and authorities (i) to completely remove synthetic additives/preservatives from their food products, (ii) to search for and develop new types of effective, nontoxic harmless compounds, or (iii) to adopt more "natural" alternatives for the safety or maintenance or extension of a product's shelf life. Among these "alternative routes", extracts from spices, herbs, and edible plants are the subject of considerable research activity. In this regard, the inhibitory activity of grapes and seed extracts against a variety of microorganisms is well established (*6–9*). Their activity has been attributed to the presence of polyphenols, a group of secondary plant metabolites with diverse chemical structures and functions that are produced as responses to various forms of physiological stress. Grapes are particularly rich in bioactive polyphenols, especially flavonoids, stilbenes, and phenolic acids. Some polyphenols are extracted into wine, but their majority remains in the vinification waste (pomace, stems, and seeds), which accounts for about 13% of the processed grape weight (*10*). The vinification byproducts are traditionally used either as animal food or as source of various products such as ethanol, alcoholic beverages, tartaric and citric acid, grape seed oil, and dietary fiber (*11*), which constitute processes of limited economical interest. Thus, their exploitation as a source of high added value polyphenols (or their enriched extracts) is particularly attractive because this combines the profitable venture with a significant advance in environmental protection around the wine-producing zones.

Among the various grape polyphenols, flavonoids are the most studied and known to possess antibacterial activities, which have

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Table 1. Quantitation of Polyphenolic Content of Extracts (Milligrams per Gram) from Grape Berries and Vinification Byproducts Assayed

	grape berries				seeds				pomace				stems			
	Voidomato	Mandilaria	Asyrtiko	Aidani	Voidomato	Mandilaria	Asyrtiko	Aidani	Voidomato	Mandilaria	Asyrtiko	Aidani	Voidomato	Mandilaria	Asyrtiko	Aidani
gallic acid	1.98	2.24	1.03	3.11	6.46	2.61	4.98	1.78	20.44	13.64	10.78	5.71	11.49	12.84	41.44	2.27
(+)-catechin	102.20	95.92	151.67	43.69	117.75	135.72	173.75	105.14	10.96	9.32	105.57	14.91	46.74	85.81	95.39	68.29
(−)-epicatechin	55.14	40.49	31.35	17.97	79.40	33.38	38.20	20.46	15.04	8.72	37.39	3.34	11.14	0.43	4.34	*b*
procyanidin B3	21.01	20.35	24.51	9.34	18.00	18.24	27.38	19.56	4.12	3.61	9.96	2.01	20.54	31.55	26.01	25.79
procyanidin B2	16.14	17.40	10.63	23.25	29.46	25.61	19.38	15.30	7.83	3.22	24.01	3.58	*b*	*b*	5.15	8.55
epicatechin gallate	60.57	30.57	40.39	5.83	73.71	1.79	1.99	1.78	1.47	0.53	2.93	0.58	5.61	7.78	3.14	4.46
trans-caftaric acid	0.25	1.05	4.31	0.12	*b*	*b*	*b*	*b*	0.12	6.40	*b*	*b*	16.11	1.62	4.87	12.25
trans-resveratrol	0.07	*b*	*b*	*b*	*b*	*b*	*b*	*b*	0.24	2.00	0.33	1.93	5.47	17.56	12.62	11.42
ε-viniferin	0.33	0.57	0.17	*b*	*b*	*b*	*b*	*b*	2.00	7.55	0.83	6.02	12.79	31.42	20.15	14.53
quercetin 3-*O*-galactoside	3.32	2.73	3.49	6.26	*bb*	*b*	*b*	*b*	3.96	4.63	1.56	1.91	12.06	6.63	8.20	19.22
quercetin 3-*O*-glucoside	1.74	1.08	1.12	6.54	*b*	*b*	*b*	*b*	2.68	2.62	7.29	3.03	3.86	4.61	2.06	7.16
quercetin 3-*O*-rhamnoside	0.92	*b*	4.73	3.85	*b*	*b*	*b*	*b*	0.87	1.78	3.34	0.89	0.90	0.46	0.34	1.53
quercetin	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	3.83	3.26	4.75	2.77	0.80	0.60	3.40	0.32
kaempferol	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	1.53	0.06	0.54	0.29	*b*	0.20	0.32	0.04
caffeic acid	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	0.38	0.05	*b*	0.51	*b*	0.34	*b*
syrigic acid	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	0.95	4.16	0.17	0.44	0.14	0.07	0.46	*b*
p-coumaric acid	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	0.47	0.25	0.12	0.12	0.08	0.04	0.01
ferulic acid	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	0.06	*b*	0.03	*b*	0.03	0.04	*b*
TPC[a]	472.4	467.4	492.7	333.2	811.95	620.00	428.2	325.37	376,71	207.79	465.3	107.12	494.2	536.8	484.3	367.1

[a] Total polyphenols; milligrams of gallic acid per gram of extract. [b] Below the detection limit.

been attributed to their interaction with extracellular soluble proteins and/or bacterial cell walls (*12*). In particular, catechins are known to inhibit in vitro the action of many bacteria, such as *Vibrio cholerae*, *Streptococcus mutans*, and *Shigella* (*12*), whereas (−)-epigallocatechin gallate is a potent Gram-positive bactericidal that acts by damaging the respective bacterial membranes (*13*).

The main goal of the present study is the detailed assessment of Greek vinification byproduct (pomace, stems, and seeds) content in bioactive polyphenols and the investigation of their activities against *L. monocytogenes* Scott A. This organism is of great importance because human infections from *L. monocytogenes* often affect the pregnant uterus, the central nervous system, or the bloodstream (*14, 15*). Symptoms vary, ranging from mild flu-like symptoms and diarrhea to life-threatening infections characterized by septicemia and meningoencephalitis (*15*). In pregnant women, the infection affects fetuses, which will be either born severely ill or die in uterus (*14*).

Finally, the minimum inhibitory concentration (MIC) of the most potent products was determined using either the traditional plate count technique or an automated technique that combines the conductance measurements with the dilution method. Both methods were applied on a Malthus apparatus, whereas the determinations accuracy was ensured via the mathematical processing of data.

MATERIALS AND METHODS

Grapes and Vinification Byproduct. The samples studied were grape berries, seeds, pomace, and stems obtained from the most representative red (Mandilaria and Voidomato) and white (Asyrtiko and Aidani) varieties of *Vitis vinifera* species that are cultivated in the Greek islands. All samples were obtained from Santorini island during the 2006 harvest and vinification campaign. Grape berries were manually destemmed and unseeded, weighed, freeze-dried, mill-powdered, and stored in a freezer. Stems and seeds were directly obtained by manual separation during the destemming and deseeding processes, while the whole pomace samples were obtained after the respective vinification process. These samples were air-dried, mill-powdered, and stored at room temperature.

Reagents and Standards. Calibration curves were constructed for the following polyhenols: gallic acid, (+)-catechin, (−)-epicatechin, procyanidin B2, epicatechin gallate, *p*-coumaric acid, ferulic acid, 3,4-dihydroxybenzoic acid, caffeic acid, syringic acid, vanillic acid, kaempferol, quercetin, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-rhamnoside, and *trans*-resveratrol (all obtained from Sigma-Aldrich) and procyanidin B3, *trans*-caftaric acid, and *trans*-ε-viniferin [isolated by preparative high-performance liquid chromatography (HPLC)].

The Folin−Ciocalteu reagent, used for the determination of the total polyphenolic content, was purchased from Fluka. All remaining chemicals were obtained from Sigma-Aldrich.

All solvents were purchased from J. T. Baker as analytical (polyphenol extraction) or HPLC (chromatographic analyses) grades. For the chromatographic analyses HPLC-grade water was prepared using a Milli-Q system, whereas all HPLC solvents were filtered prior to use through cellulose acetate membranes of 0.45 μm pore size.

Extracts Preparation. Ten grams of dried sample (powdered grape berries, skins, seeds, pomace, or stems) were poured into a 50 mL mixture of MeOH/H_2O/1.0 N HCl (90:9.5:0.5 v/v) and sonicated in an ultrasonic bath for 10 min. The solvent was separated by filtration, and the remaining solid was re-extracted three additional times, using the same solvent system and procedure. The combined extracts were evaporated under vacuum, resulting in a slurry, which was dissolved in 30 mL of MeOH/H_2O (1:1) and centrifuged for 10 min (7000 rpm). The supernatant liquid was extracted with petroleum ether (3 × 30 mL) to remove the lipids and concentrated under vacuum. The remaining residue was poured into 30 mL of brine and extracted repetitively with ethyl acetate (EtOAc, 4 × 30 mL). Thus, all sugars remained in the aqueous layer. Then, the combined organic layers were dried over anhydrous $MgSO_4$ and evaporated under vacuum. The remaining solid was weighed and dissolved in methanol (MeOH) to 1 mg/mL, membrane filtered (0.45 μm), and subjected to HPLC analysis.

To avoid polyphenol degradation, all of the aforementioned activities were performed in the absence of direct sunlight and at temperatures below 35 °C.

HPLC Analyses. All HPLC analyses were carried out on a Thermo Finnigan 3000 system equipped with a quaternary pump, an autosampler, a degasser, and a diode array detector (DAD). The column used was a Lichrosphere C_{18} column (250 mm × 4.1 mm, particle size = 5 μm) with a guard column of the same material (8 × 4 mm). Injection was by means of a Rheodyne injection valve (model 7725I) with a 20 μL fixed loop. Chromatographic data were acquired and processed using ChromQuest version 4.1 software.

The HPLC method used is a modified version of the method developed by Tsao and Yang (*16*). More specifically, the analysis was carried out at 30 °C (maintained by a column thermostat) using samples of 20 μL, which were directly injected by means of a Rheodyne

Table 2. Growth of *Listeria monocytogenes* Scott A in BH Broth and the Effect of Extracts on Detection Time, Maximum Conductance, and Final Viable Count

extract[a]	concn (%, w/v)	detection time (h)	max conductance (μS)	final viable count,[b] log (CFU/mL)
Grapes				
Voidomato	1	—	—	<1
	0.40	19.9 ± 0.7	375 ± 15	8.54
	0.10	9.7 ± 0.1	410 ± 10	8.78
Mandilaria	1	—	—	<1
	0.40	18.4 ± 0.9	390 ± 5	8.57
	0.10	9.7 ± 0.1	415 ± 0	8.85
Asyrtiko	1	—	—	<1
	0.40	38.9 ± 1.7	115 ± 20	6.58
	0.10	9.4 ± 0.3	410 ± 10	8.94
	1	—	—	<1
Aidani	0.40	20.0 ± 1.9	335 ± 15	7.38
	0.10	9.4 ± 0.1	410 ± 5	8.21
Seeds				
Voidomato	1	—	—	<1
	0.40	—	—	3.22
	0.10	10.4 ± 0.3	405 ± 10	8.81
Mandilaria	1	—	—	<1
	0.40	20.7 ± 1.5	345 ± 15	7.89
	0.10	9.3 ± 0.4	400 ± 10	8.85
Asyrtiko	1	—	—	<1
	0.40	31.3 ± 2.1	135 ± 15	7.22
	0.10	9.8 ± 0.2	415 ± 5	8.75
	1	43.7 ± 2.3	100 ± 15	6.25
Aidani	0.40	11.4 ± 0.9	400 ± 10	8.87
	0.10	9.5 ± 0.0	420 ± 0	8.84
Pomace				
Voidomato	1	—	—	<1
	0.40	18.4 ± 0.9	385 ± 5	8.76
	0.10	9.7 ± 0.1	420 ± 0	8.88
Mandilaria	1	—	—	<1
	0.40	38.3 ± 1.9	180 ± 20	7.35
	0.10	9.3 ± 0.2	410 ± 10	8.92
Asyrtiko	1	—	—	<1
	0.40	16.3 ± 0.4	390 ± 10	8.85
	0.10	9.8 ± 0.2	415 ± 10	8.78
	1	36.7 ± 1.9	115 ± 20	6.74
Aidani	0.40	15.1 ± 0.8	395 ± 15	8.47
	0.10	9.8 ± 0.1	420 ± 5	8.7
Stems				
Voidomato	1	39.3 ± 1.9	120 ± 20	7.28
	0.40	12.8 ± 0.9	370 ± 10	8.75
	0.10	9.6 ± 0.1	425 ± 5	8.89
Mandilaria	1	—	—	<1
	0.40	—	—	5.16
	0.10	10.5 ± 0.1	410 ± 10	8.8
Asyrtiko	1	—	—	<1
	0.40	43.3 ± 2.3	105 ± 15	6.55
	0.10	9.8 ± 0.2	415 ± 5	8.89
	1	—	—	<1
Aidani	0.40	16.3 ± 0.5	285 ± 15	8.75
	0.10	9.9 ± 0.1	415 ± 5	8.86
positive control (benzoic acid)	1	—	—	<1
	0.40	10.9 ± 0.3	415 ± 10	8.65
	0.10	9.3 ± 0.1	415 ± 5	8.79
negative control (BH broth)		9.3 ± 0.0	420 ± 5	8.85

[a] The experiments were carried out in triplicate for 48 h, and the standard deviation is given. [b] Level of inoculation = 10^3 CFU/mL.

injection valve (model 7725I). The gradient eluted consisted of solvent A [obtained by the addition of 3% acetic acid (AcOH) in 2 mM sodium acetate (AcONa) aqueous solution] and solvent B (acetonitrile, CH_3CN). Run time was set at 70 min with a constant flow rate at 1.0 mL/min, in accordance with the following gradient time table: at zero time, 95% A and 5% B; after 45 min, the pumps were adjusted to 85% A and 15% B; at 60 min, 65% A and 35% B; at 65 min, 50% A and 50% B; and finally at 70 min, 100% B. This routine was followed by a 30 min equilibration period with the zero time mixture prior to injection of the next sample. The analysis was monitored at 280, 320, and 360 nm simultaneously. Three replicate experiments were carried out for each sample examined. Peaks were identified by comparing their retention

Table 3. Growth of *Listeria monocytogenes* Scott A in BH Broth and the Effect of Antibacterial Compounds on Detection Time, Maximum Conductance, and Final Viable Count

compound[a]	concn (%, w/v)	detection time (h)	max conductance (μS)	final viable count,[b] log (CFU/mL)
gallic acid	1	—	—	<1
	0.40	14.9 ± 0.3	385 ± 20	8.45
	0.10	9.5 ± 0.1	410 ± 15	8.75
(+)-catechin	1	17.5 ± 0.9	365 ± 20	7.95
	0.40	9.3 ± 0.2	400 ± 15	8.78
	0.10	9.4 ± 0.1	405 ± 10	8.87
(−)-epicatechin	1	15.2 ± 0.8	395 ± 15	8.28
	0.40	9.7 ± 0.2	410 ± 15	8.58
	0.10	9.3 ± 0.1	420 ± 10	8.72
	1	11.7 ± 0.6	410 ± 15	8.55
procyanidin B2	0.40	9.4 ± 0.1	415 ± 10	8.78
	0.10	9.3 ± 0.2	420 ± 10	8.49
epicatechin gallate	1	20.5 ± 1.5	375 ± 15	7.85
	0.40	9.9 ± 0.3	400 ± 20	8.82
	0.10	9.3 ± 0.2	410 ± 15	8.75
quercetin	1	—	—	<1
	0.40	12.1 ± 0.7	405 ± 15	8.80
	0.10	9.2 ± 0.3	410 ± 5	8.75
kaempferol	1	14.7 ± 0.8	405 ± 15	8.45
	0.40	9.3 ± 0.2	415 ± 10	8.72
	0.10	9.4 ± 0.1	405 ± 0	8.57
	1	—	—	3.45
caffeic acid	0.40	9.2 ± 0.2	410 ± 5	8.75
	0.10	9.3 ± 0.2	420 ± 5	8.72
syringic acid	1	35.7 ± 2.4	255 ± 20	7.35
	0.40	10.1 ± 0.3	405 ± 15	8.82
	0.10	9.2 ± 0.4	410 ± 10	8.72
p-coumaric acid	1	—	—	<1
	0.40	12.3 ± 0.4	400 ± 15	8.52
	0.10	9.4 ± 0.4	420 ± 5	8.85
ferulic acid	1	—	—	<1
	0.40	11.1 ± 0.5	410 ± 15	8.75
	0.10	9.5 ± 0.3	405 ± 10	8.68
positive control (benzoic acid)	1	—	—	<1
	0.40	10.9 ± 0.3	415 ± 10	8.65
	0.10	9.3 ± 0.1	415 ± 5	8.79
negative control (BH broth)		9.3 ± 0.0	420 ± 5	8.85

[a] The experiments were carried out in triplicate for 48 h, and the standard deviation is given. [b] Level of inoculation = 10^3 CFU/mL.

time and UV−vis spectra with the reference compounds, and data were quantitated using the corresponding curves of the reference compounds as standards. All standards were dissolved in methanol.

Identification of Pure Compounds. All isolated compounds were unambiguously characterized on the basis of their physical and spectral data. Their structural elucidation was achieved by means of the following spectroscopic data analyses: ^{1}H NMR spectra measured on a Bruker DRX400 spectrometer (400 MHz) and ^{13}C NMR on a Bruker AC200 spectrometer (50 MHz). The corresponding signals were unambiguously assigned using the following 2D NMR techniques: COSY, COSY-LR, HMQC, and HMBC. All of the aforementioned 2D experiments were performed using standard Bruker programs. The optical rotations were measured with a Perkin-Elmer 341 polarimeter. Optical rotations were measured on a Perkin-Elmer 341 polarimeter.

Procyanidin B3. ^{1}H NMR and ^{13}C NMR data are consistent with those previously reported (*17*); ES-MS *m/z* 601 [M + Na].

trans-Caftaric Acid. ^{1}H NMR and ^{13}C NMR data are consistent with those previously reported (*18*); ES-MS *m/z* 335 [M + Na]; mp 123−125 °C.

trans-ε-Viniferin. ^{1}H NMR and ^{13}C NMR data are consistent with those previously reported (*19, 20*); ES-MS *m/z* 505 [M + Na]; $[\alpha]^{22}_D$ +64.4 (c 0.5, MeOH).

Assessment of the Total Phenolic Content (TPC). The TPC of the extracts was determined in accordance with a modified version of the Folin−Ciocalteu method (*21*), applied in 96-well microplates. Stock solutions (10 mg/mL) of the sample extracts were prepared in MeOH, and further dilutions were performed with CH_2O/EtOH (85:15). Fifty

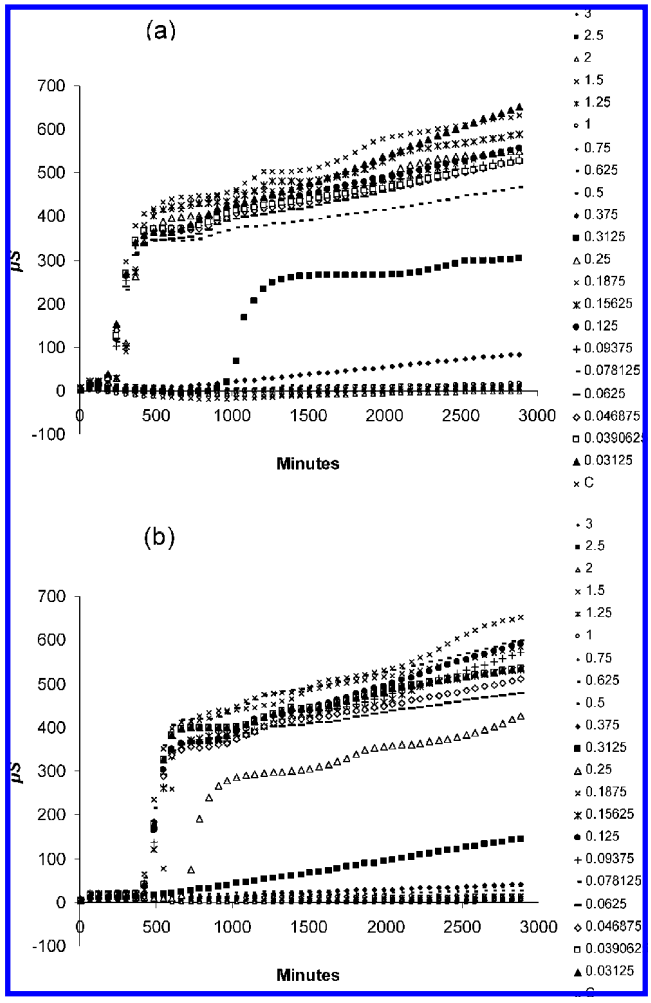

Figure 1. Typical graphs for *Listeria monocytogenes* at 10^3 CFU/mL level of inoculation as observed by conductance measurements at increased concentrations (% w/v) of Voidomato seeds extract (**a**) and Mandilaria stems extract (**b**) in BH broth (C = control).

microliters of the diluted solution was pipetted in the wells in triplicate. One hundred microliters of 20% (w/v) sodium carbonate (Na_2CO_3) was added in each well, followed by 50 μL of Folin−Ciocalteu 2-fold diluted, and the plates were placed in the dark for 1 h at room temperature. Absorbance was measured at 650 nm, and the results were expressed as milligrams of gallic acid per gram of extract.

Bacterial Cultures. *L. monocytogenes* Scott A (kindly provided by Dr. Eddy Smid ATO-DLO) was grown overnight in flasks containing brain heart (BH) broth (Merck catalog no 1.100493), with shaking at 30 °C. The cells were harvested by centrifugation at 3000*g* for 15 min at 4 °C (model ALC, high-speed centrifuge 4239R). Then, they were washed twice and resuspended in a Ringer solution (LabM, Bury, U.K.) to provide a bacterial concentration ranging between 10^6 and 10^7 colony-forming units (CFU)/mL.

Antilisterial Assays. The antibacterial activity against *L. monocytogenes* Scott A was determined by a conductance method that monitors the bacterial growth using the Malthus system (Radiometer International, Copenhagen, Denmark). A typical Malthus conductivity cell contains platinum electrodes that allow the detection of the conductance changes as a response to the bacterial metabolism in the growth medium. Thus, it is feasible to monitor the conductance changes at defined intervals and record the corresponding data. The detected changes are expressed in microsiemens (μS), which can be recorded graphically as conductance curves. The detection time signal appeared when there were three consecutive measurements of 6 μS minimum. Maximum conductance is expressed in microsiemens (μS) and indicates the conductance of the conductance/time curve at the early stationary phase. In the present study, the growth medium (BH broth) was dispensed to 2.0 mL final volume into sterile closed glass reaction cells (121 °C/15

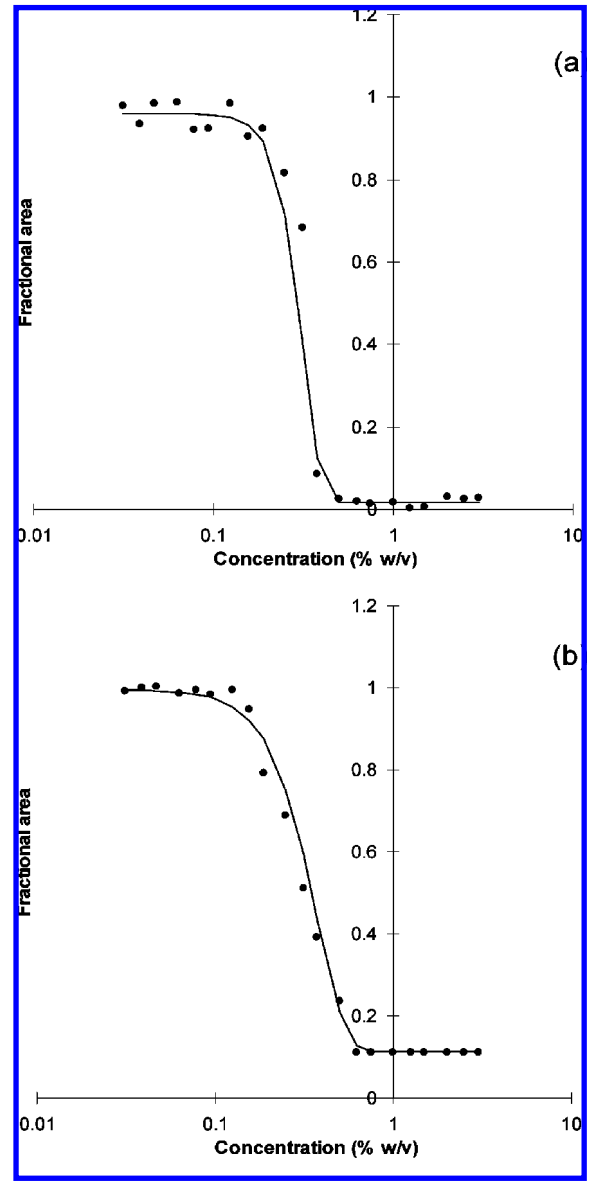

Figure 2. Inhibition profile of Voidomato seeds extract against *Listeria monocytogenes* with conductance technique (**a**) and count technique (**b**): (●) observed fractional area; (—) predicted fractional area.

min). The reaction tubes were incubated at 30 °C for 30 min and subsequently inoculated with 0.2 mL of an 18 h culture (10^3 CFU/mL) of *L. monocytogenes* grown at optimum temperature in the same growth medium. Each experiment was carried out in triplicate. The tubes were incubated for 48 h at 30 °C.

Method of Analysis. The analyses were carried out on a Malthus system (*22*), according to an experimental setup based on a method developed by Lambert and Pearson (*23*). Three stock solutions were used (3, 2.5, and 2% w/v), which by seven 2-fold serial dilutions—using the growth medium—covered a range from 3 to 0.03125% w/v of the extracts.

The basis of this methodology is the comparison of the area under the conductance/time curve (*22*). The effect on growth was manifested by the observed reduction in the area under the conductance/time curve at a specified time, in relation to a control (*23*). Thus, the calculation of this area (using the trapezoidal rule) results in the determination of the relative amount of growth (as the ratio of the test area to control), which is known as the fractional area (FA; *23*). At counts technique, the FA was determined as the count quotient for each concentration, with respect to the control count. The count for each concentration was measured after 48 h of experiment in Malthus tubes.

MIC Measurement. Data in the form of log concentration versus fractional area can be processed by using a modified Gompertz

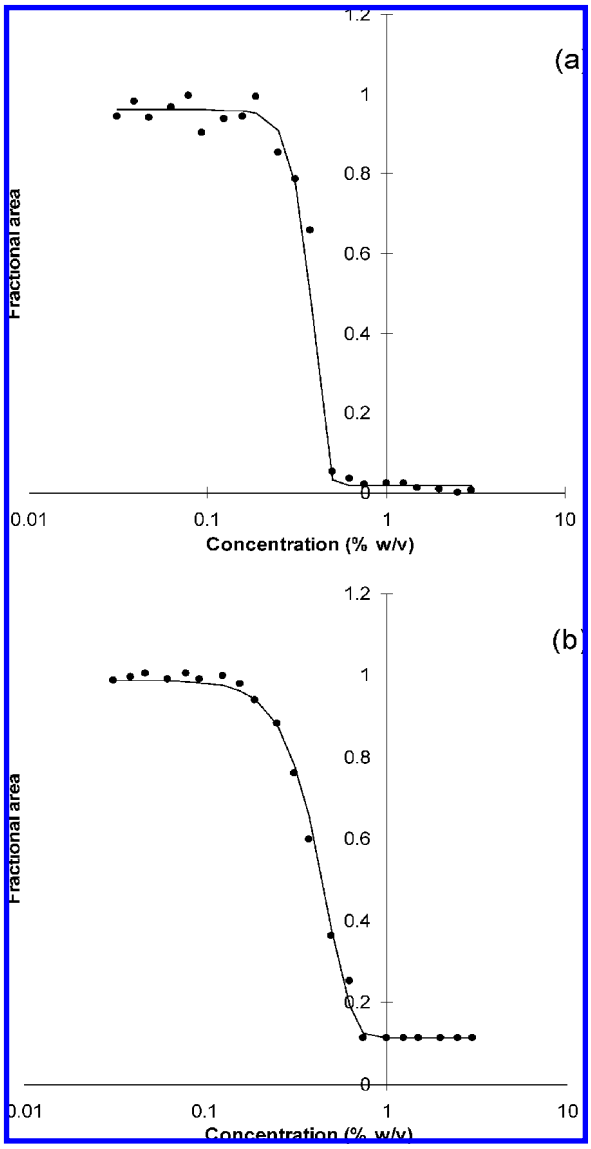

Figure 3. Inhibition profile of Mandilaria stems extract against *Listeria monocytogenes* with conductance technique (**a**) and count technique (**b**): (●) observed fractional area; (—) predicted fractional area.

Table 4. MIC Values and Their Standard Errors (Percent, v/v, Concentration of Antimicrobial in BH Broth) against *Listeria monocytogenes* Scott A at 10^3 CFU/mL Level of Inoculation

	MIC value	
extract[a]	conductance technique	count technique
Voidomato seeds	0.261 ± 0.008	0.261 ± 0.022
Mandilaria stems	0.339 ± 0.014	0.345 ± 0.019

[a] Experiments were carried out in triplicate for 48 h, and the standard error is given.

equation (*23–25*). In this study, we have used the altered Gompertz function that relates the fractional area (y) to the log of antimicrobial concentration (x), as shown in eq 1.

$$y = A + C\,e^{-e^{B(x-M)}} \quad (1)$$

A corresponds to the lower asymptote of y, B the slope parameter, C the distance between the upper and lower asymptote, and M the log concentration of the inflection point.

The MIC values were defined as the intersection of the lines $y = A + C$, with the equation of the line tangential to the point [M ($A + C\,e^{-1}$)] (*23*).

$$\mathrm{MIC} = 10^{(M+1/B)} \quad (2)$$

The values of A, C, B, and M were obtained from a nonlinear fitting procedure (*23*). The values were estimated with statistical package Figure.P 2.5.

RESULTS AND DICUSSION

Polyphenol Content. Table 1 summarizes the total polyphenolic content and the bioactive polyphenol quantitation of samples assayed. The concentration of each polyphenol is expressed as milligrams per gram of extract, whereas TPC is presented as milligrams per gram of gallic acid equivalents.

Grape Berries. The bulk of noncolored polyphenols detected in grape berries consisted of flavonoids and 3-*O*-flavonol glycosides (**Table 1**). More specifically, the monomers of (+)-catechin and (−)-epicatechin were the most abundant, found in quantities ranging from 43.69 to 151.67 mg/g and from 17.97 to 55.14 mg/g, respectively. Epicatechin was also assayed in considerable amounts in the form of epicatechin gallate. Flavonols were detected only as 3-*O*-flavonol glycosides, mainly as galactoside and glucoside derivatives. Similar findings have been reported in the literature for quercetin 3-*O*-galactoside in grape varieties Concord and Chanauc (*26*), Cabernet Sauvignon (*27*), and pomace extracts from Cabernet Sauvignon and Temranillo varieties (*28, 29*) and quercetin 3-*O*-glucoside in grapes (*30*) and pomace extracts from Arinto and Muscatel varieties (*31*). Finally, it must be noted that in all examined samples the sum of procyanidins B2 and B3 was always constant, regardless the variety tested.

ε-Viniferin—a *trans*-resveratrol dimer—was the only detected stilbene. This phytoalexin is present in grapes as a consequence of environmental stress, botrytis infections, and/or UV irradiation (*32–34*).

Seeds. As shown in **Table 1**, seeds are particularly rich in monomeric flavan-3-ols (+)-catechin and (−)-epicatechin, epicatechin gallate, and dimeric procyanidins B2 and B3. They also have high TPC, averaging from 325 to 812 mg/g gallic acid equivalents. Their polyphenolic profile is comparable with that of the grape berries from which they originated. These results correlate well with previous studies on seed polyphenols from other Greek grape varieties (*35, 36*).

Pomace and Stems. Grape pomace and stems were found to contain considerable amounts of diverse polyphenols, such as flavonoids, stilbenes, and phenolic acids (**Table 1**). In particular, stems—a scarcely studied class of grape byproduct—are characterized as rich in the bioactive stilbenes *trans*-resveratrol and ε-viniferin. For example, Mandilaria stems were found to contain 17.6 and 31.4 mg/g of *trans*-resveratrol and ε-viniferin, respectively. These results correlate well with previous papers indicating that some grape stems contain large amounts of stilbenes (*34, 37*). Furthermore, stems and pomace contain significant amounts of flavonoids, especially (+)-catechin and procyanidin B3. On the contrary, stems contain only limited amounts of (−)-epicatechin. These findings are in line with a literature report (*38*) on Merlot variety stems indicating that they contain only traces of (−)-epicatechin and reduced amounts of (+)-catechin. Finally, various flavonols were assayed in stems and pomace as aglycon and/or glycoside forms, with quercetin 3-*O*-galactoside as the most abundant.

It must be pointed out that according to the results of this study, the grapes, stems, seeds, and pomace of Asyrtiko, a traditional white variety of Greece, contain considerably large amounts of bioactive polyphenols (as individual compounds and/or total polyphenols), in volumes that are comparable with those

of red varieties. It was also established that grape stems might be considered to be an excellent source of bioactive polyphenols.

Antilisterial Assays. In this study the assessment of antilisterial activities was performed using the following two methods: (i) the traditional microbiological method of measuring the viable counts and (ii) the measurement of indirect indicators that evaluate *L. monocytogenes* growth (e.g., optical density or conductance). The latter was performed in a liquid growth medium containing the antibacterial compound. The respective results are depicted in **Table 2**, indicating that the tested extracts of grape berries and vinification byproduct are active against the tested strain, especially at 1% w/v concentration. On the other hand, the antilisterial activities of the principal polypenols present in the extracts—in various concentrations— were also assessed (**Table 3**). The respective results are in agreement with previous papers (*39*), indicating that the antilisterial properties of the individual ploy phenols are significantly lower as compared to the respective extracts. This observation may be rationalized considering the possible synergistic activity among polyphenols that is applied in the extracts. The most intense antilisterial activity was obtained from extracts of Voidomato seeds and Mandilaria stems and correlated well with their increased polyphenolic content. Thus, they were further evaluated as natural antibacterials through the determination of their minimum inhibitory concentration. The latter is essential to apply the minimum essential concentration capable of preventing bacterial growth (*23*).

Among the various methods used to assay the MIC of antibacterials, a method developed by Lambert and Pearson (*23*) constitutes an automated technique that combines the absorbance measurements with the common dilution method (*22, 25*). This method has the advantage of allowing the quick and efficient MIC assay and is applicable on single compounds (e.g., thymol) and/or preservative mixtures (*25*). A significant improvement of this method is the replacement of the absorbance with conductance measurements (*22*). The latter has the advantage of being independent from the active state of cells (including their shape and size) and is not affected by the antimicrobial agent oxidation (*22*). In this paper, this method was applied on a Malthus apparatus, which allows the measurement of cell metabolism rate—thereby showing the inhibition caused by the essential oil—as a delay in bacteria metabolism compared to the control. The accuracy of MIC determinations was ensured via the mathematical process of data (*22, 23, 25*). More specifically, the areas under the microsiemens/time curves were calculated using the trapezoidal rule (**Figure 1**) and the relative amount of growth—termed the fractional area (FA)—was obtained as the ratio of the test area to control (*22*). On the counts technique, the FA was determined as the quotient of the count for each concentration relative to the control count. A plot of the inhibitor concentration with the fractional area (on a logarithmic scale) gave a characteristic sigmoid-shaped curve (**Figures 2** and **3**). The MIC was assigned as the concentration above which no growth was observed relative to the control (*22, 23, 25*).

The experimental data were modeled using eq 1, and the MIC values were calculated from eq 2. The obtained data have been summarized in **Table 4**, indicating that both seed and stem extracts possess significant bactericidal properties, comparable to those of well-established antimicrobial agents such as essential oils (*1, 40, 41*), and constitute the first antilisterial studies for stem extracts. The results obtained indicated that these extracts may provide valuable alternative pathways for the prevention of microbial growth, but further research is needed to evaluate the effectiveness of these extracts in the food ecosystem and to establish their role as antimicrobial agents in food safety. Finally, because the MIC values obtained via both techniques were similar, the described conductance method may be considered as an alternative rapid means for the MIC estimation.

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