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Lycopersicon esculentum Seeds: An Industrial Byproduct as an Antimicrobial Agent

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Lycopersicon esculentum (tomato) fruit is a widely studied matrix. However, only few works focus their attention on its seeds, which constitute a major byproduct of the tomato processing industry. In this study the antimicrobial potential of ten different tomato seed extracts from "Bull's heart" and "Cherry" varieties were analyzed against Gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Enterococcus faecalis* and *Bacillus cereus*) and Gram-negative (*Proteus mirabilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) bacteria and fungi (*Candida albicans*, *Aspergillus fumigatus* and *Trichophyton rubrum*). Regarding antibacterial capacity, the different extracts were revealed to be active only against Gram-positive bacteria, *E. faecalis* being the most susceptible one (MIC: 2.5−10 mg/mL). Concerning antifungal activity, "Bull's heart" extracts were the most active. In a general way *C. albicans* was the most susceptible species (MIC: 5−10 mg/mL). The chemical composition of the extracts was also pursued, concerning organic acids, phenolics and fatty acids, in order to establish a possible relationship with the observed antimicrobial effect.

KEYWORDS: ***Lycopersicon esculentum*; seeds; byproducts; antimicrobial activity**

INTRODUCTION

Lycopersicon esculentum Mill (tomato) production is widespread throughout the world, both for fresh consumption and industrial processing. In 2008 the total world production exceeded 130 million tons (*1*), thus representing one of the most relevant crops in terms of employment and wealth generation. The industrial processing of tomato leads to several byproducts, namely, tomato seeds and peels. These byproducts represent a major disposal problem for the industry. In fact, seeds account for approximately 10% of fruit and 60% of the total waste (*2*). They are often used as feed or as fertilizer. However, demand for feed may be varying and dependent on agricultural yields. Thus, the search for an efficient, inexpensive and environmentally sound application of this material is becoming more important (*3*).

Industrial byproducts usually constitute a promising source of compounds that can be used for their nutritional properties and biological potential (*2*). One alternative route could be their use as dietary supplements and in food fortification, since it is generally assumed that consumers prefer natural functional ingredients. In addition, an increasing interest has been devoted to the biopreservation of food systems, in the pursuit of shelf life extension and enhancement of food safety by using natural antimicrobial compounds (*4*). Thus, another possible utilization of those byproducts could be as food preservative, to be applied in the food industry (*5*).

In spite of being poorly studied, tomato seeds are known to present several classes of metabolites, such as fatty acids, carotenoids, saponins (*2*), phenolic compounds (*6*), proteins (*7*), amino acids and vitamins (*8*). This seems to point to the potential of seed extracts as a source of bioactive compounds. Regarding the biological potential of tomato seeds, a significant proliferation inhibition of the rat basophile leukemia (RBL-2H3) cell line and an acetylcholinesterase inhibitory activity were already described. The same was noticed regarding antioxidant capacity evaluated against several reactive species (*6*). This matrix already revealed antifungal activity against *Cladosporium cucumerinum* (*9*). However, as far as we know, no study concerned the antibacterial spectrum of these seeds.

This work aimed to screen the antimicrobial potential of tomato seeds for possible application in several industries. In order to try to establish a possible relationship between the biological activity and the chemical composition of the analyzed extracts, phenolic compounds, organic acids and fatty acids were determined by high-performance liquid chromatography coupled to diode array detector (HPLC/DAD), high-performance liquid chromatography coupled to ultraviolet detector (HPLC/UV) and gas chromatography coupled to mass spectrometry (GC/MS),

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respectively. The antibacterial and antifungal activity of both *L. esculentum* seeds extracts and the determined chemical components were evaluated by broth dilution assays.

MATERIALS AND METHODS

Standards and Reagents. All chemicals used were of analytical grade. Reference compounds were purchased from various suppliers: quercetin-3-*O*-rutinoside (rutin), kaempferol-3-*O*-rutinoside, and isorhamnetin-3-*O*-rutinoside were from Extrasynthése (Genay, France). The studied organic acids were obtained from the following source: oxalic, citric, fumaric, malic, acetic, aconitic and pyruvic acids from Sigma-Aldrich (Steinheim, Germany). Pentadecanoic, palmitic, palmitoleic, heptadecanoic, stearic, oleic and linoleic acids methyl esters, and boron trifluoride (BF_3) 10% methanolic solutions were purchased from Sigma-Aldrich (Steinheim, Germany). Ciprofloxacin, fluconazole, methanol, chloridric, acetic and sulfuric acids were obtained from Merck (Darmstadt, Germany). Mueller Hinton Broth (MHB) and Mueller Hinton Agar (MHA) media were purchased from Liofilchem (Teramo, Italy) and Saboraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) from Bio-Mèrieux (Marcy L'Etoile, France). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

Plant Material. Seeds of *L. esculentum* Mill. from "Bull's heart" (RJS, Portugal) and "Cherry" (Galassi Sementi, Italy) varieties were purchased from local commerce. These two cultivars correspond to the most consumed ones in Portugal. No antibacterial or antifungal agents were added by the producer for conservation. Seeds were washed with water, then surface sterilized with 10% sodium hypochlorite solution, rinsed with sterile distilled water and were air-dried at room temperature. The samples were ground into a fine powder.

Preparation of Extracts. Seeds were extracted using five different solvents: methanol, chloroform, ethyl acetate, hexane and sulfuric acid 0.005 M. Eight grams of powdered seeds was extracted with 200 mL of each solvent with sonication (5 min), followed by 24 h of extraction at room temperature with mechanic stirrer (200 rpm). The resulting extracts were filtered through a Buchner funnel and evaporated to dryness under reduced pressure using a rotary evaporator, at 40 °C. The extracts were redissolved in 4 mL of methanol and preserved at −20 °C until analysis.

HPLC/DAD Phenolic Compounds Analysis. For identification and quantification of phenolic compounds, 20 μL of redissolved extract was analyzed using a HPLC/DAD unit (Gilson) and a Spherisorb ODS2 (25.0 × 0.46 cm; 5 μm particle size) column. The mobile phase consisted of two solvents: water/acetic acid (1%) (A) and methanol (B), starting with 5% B and using a gradient to obtain 50% at 30 min and 80% at 37 min. Spectral data from all peaks were accumulated in the range of 200−400 nm, and chromatograms were recorded at 350 nm. The different phenolic compounds were identified by comparing their chromatographic behavior and UV−vis spectra in the 200−400 nm range with authentic standards and with published data (*6*). Data were processed on Unipoint system software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities. Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Because standards of all identified compounds were not commercially available, kaempferol derivatives were quantified as kaempferol-3-*O*-rutinoside, quercetin derivatives as quercetin-3-*O*-rutinoside, and isorhamnetin derivatives as isorhamnetin-3-*O*-rutinoside. Kaempferol-3-*O*-sophoroside plus isorhamnetin-3-*O*-sophoroside and kaempferol-3-*O*-(2-sophorosyl)glucoside plus quercetin-3-*O*-(2-pentosyl)rutinoside were quantified together as kaempferol-3-*O*-rutinoside.

HPLC/UV Organic Acids Analysis. Twenty microliters of redissolved extract was analyzed on an analytical HPLC unit (Gilson), using an ion exclusion column Nucleogel Ion 300 OA (300 × 7.7 mm), in conjunction with a column heating device set at 30 °C. Elution (70 min) was carried out at a solvent flow rate of 0.2 mL/min, isocratically, with sulfuric acid 0.005 M as the mobile phase. Detection was performed with a Gilson UV detector at 214 nm. The organic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards.

GC/MS Fatty Acid Analysis. *Derivatization.* Fatty acid derivatization was performed according to a described procedure (*10*). Briefly, sterified fatty acids in the extracts were hydrolyzed with KOH methanolic solution (11 g/L), at 90 °C for 10 min. The free fatty acids originally present and those resulting from the alkaline hydrolysis were derivatized to their methyl ester forms with BF_3 methanolic solution (10%), at 90 °C for 10 min. The methyl ester derivatives were extracted with isooctane, and anhydrous sodium sulfate was added to ensure the total absence of water. The resulting extract was evaporated to dryness under a stream of nitrogen and redissolved in isooctane. All the assays were performed in triplicate.

Gas Chromatography/Mass Spectrometry Conditions. Standard mixture/sample extracts (1 μL) were analyzed using a Varian CP-3800 gas chromatograph (USA) equipped with a VARIAN Saturn 4000 mass selective detector (USA) and a Saturn GC/MS workstation, software version 6.8. AVF-5 ms (30 m × 0.25 mm ×0.25 μm) column (VARIAN) was used. The injector port was heated to 250 °C. Injections were performed in split mode, with a ratio of 1/40. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min. The oven temperature was set at 40 °C for 1 min, then increasing 5 °C/min to 250 °C, 3 °C/min to 300 °C and held for 15 min. All mass spectra were acquired in the electron impact (EI) mode. Ionization was maintained off during the first 4 min, to avoid solvent overloading. The ion trap detector was set as follows: transfer line, manifold and trap temperatures were 280, 50, and 180 °C, respectively. The mass ranged from 50 to 600 *m/z*, with a scan rate of 6 scan/s. The emission current was 50 μA, and the electron multiplier was set in relative mode to auto tune procedure. The maximum ionization time was 25,000 μs, with an ionization storage level of 35 *m/z*. The analysis was performed in full scan mode. Identification of compounds was achieved by comparison of their retention time and mass spectra with those from pure standards injected under the same conditions, and from NIST 05 MS Library Database. The amount of fatty acid methyl esters (FAME) present in the extract samples was achieved from the calibration curve of the respective FAME standards. The FAME values were then converted into their respective fatty acid contents.

Antimicrobial Activity. *Microorganisms.* Nine bacterial species were used for the experiment: *Staphylococcus aureus* (ATCC 20231), *Staphylococcus epidermidis* (ATCC 20044), *Salmonella typhimurium* (ATCC 43971), *Proteus mirabilis* (ATCC 4479), *Escherichia coli* (ATCC 30083), *Pseudomonas aeruginosa* (ATCC 50071), *Bacillus cereus* (ATCC 31), *Enterococcus faecalis* (ATCC 20477) and *Micrococcus luteus* (ATCC 20030). Cultures were obtained from the Department of Microbiology, Faculty of Pharmacy, Porto University, Portugal. The same organisms were also tested against the reference antibacterial drug ciprofloxacin. Stock cultures were maintained on MHA at 4 °C.

Antifungal activity was checked against *Candida albicans* (ATCC 10231), *Aspergillus fumigatus* (ATCC 46645) and *Trichophyton rubrum* (CECT 2794). The same organisms were also tested against the reference antifungal drugs fluconazole (*C. albicans*) and voriconazole (*A. fumigatus* and *T. rubrum*) for comparison of results. *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used for quality control. All strains were stored in SDB with 20% glycerol at −70 °C and subcultured in SDA before each test, to ensure optimal growth conditions and purity.

Antibacterial Effect. Bacterial inocula were prepared by growing cells in MHB for 24 h, at 37 °C. Cell suspensions were diluted in sterile MHB to provide initial cell counts of about 10^6 colony-forming unit per mL (CFU/mL). The minimum inhibitory concentration (MIC) of seeds extracts, organic acids and rutin were determined by 2-fold serial dilution method, in 96-well plates. All tests were performed in MHB. The initial concentration was 20 mg/mL for the seed extracts and all organic acids, with the exceptions of aconitic (10 mg/mL) and fumaric (2.5 mg/mL) acids. Rutin was tested at a maximum concentration of 69 μg/mL. The final concentration of methanol did not exceed 1% (v/v). Briefly, 90 μL of MHB and 10 μL of a suspension containing 10^6 CFU/mL were added in each well, which contained 100 μL of extract/compound. Negative controls in MBH alone and with 1% methanol (v/v) and sterility and growth controls were included. A positive control with ciprofloxacin was also performed. Plates were incubated for 24 h, at 37 °C, and then examined by a binocular microscope. The MIC for bacteria was determined as the lowest concentration of seed extracts or standard compounds (organic acids or rutin) inhibiting the visual growth of the test culture on the microplate. The experiments were performed in duplicate and repeated independently three times, yielding essentially the same results (a range of values is presented when different results were obtained).

Antifungal Effect. Broth microdilution methods based on the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) reference

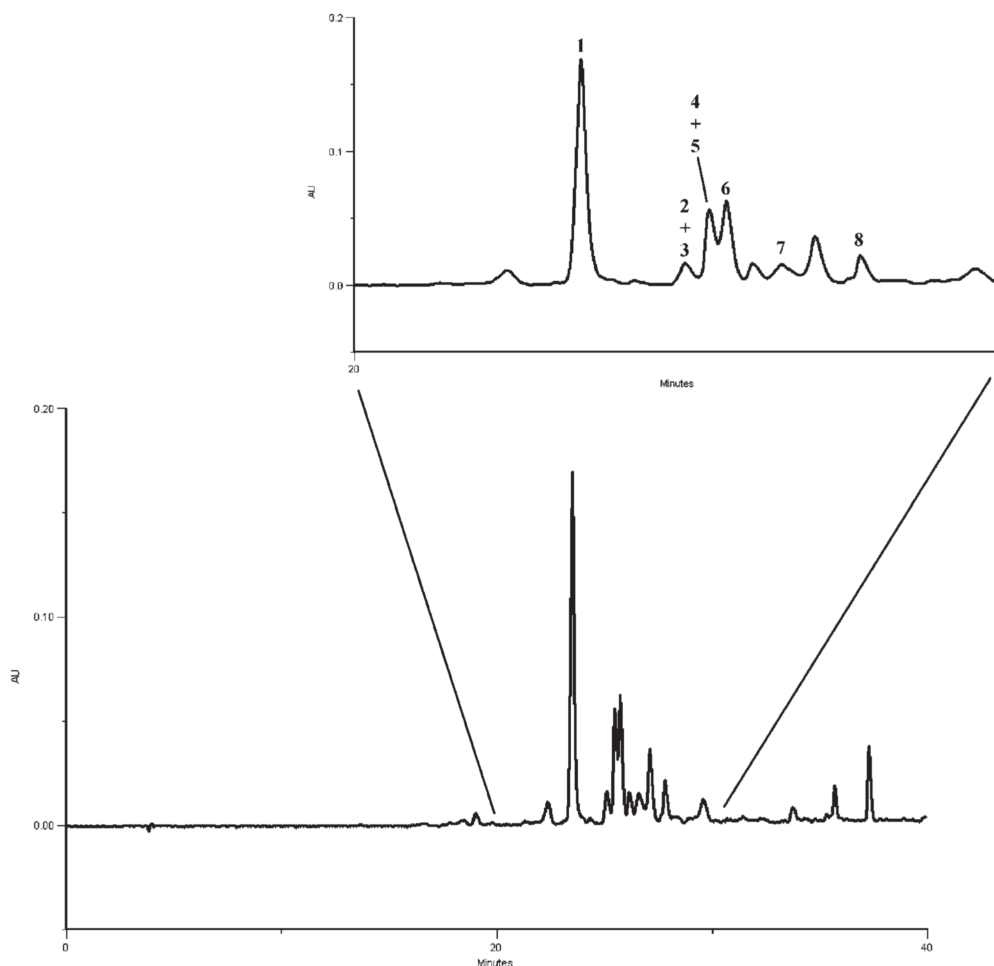

Figure 1. HPLC/DAD phenolic profile of *L. esculentum* seeds ("Bull's heart" variety). Detection at 350 nm. Compounds: (**1**) quercetin-3-*O*-sophoroside; (**2**) kaempferol-3-*O*-(2-sophorosyl)glucoside; (**3**) quercetin-3-*O*-(2-pentosyl)rutinoside; (**4**) kaempferol-3-*O*-sophoroside; (**5**) isorhamnetin-3-*O*-sophoroside; (**6**) isorhamnetin-3-*O*-gentiobioside; (**7**) quercetin-3-*O*-rutinoside; (**8**) kaempferol-3-*O*-(2-pentosyl)glucoside.

documents M27-A3 and M38-A2 (*11*, *12*), for yeast and filamentous fungi, respectively, with minor modifications, were used to determine MICs. Briefly, inoculums suspensions were prepared from SDA cultures at the final required density in RPMI-1640 medium (with glutamine, without bicarbonate, and with phenol red as a pH indicator).

Seed extracts were dissolved in 50% methanol (v/v), and organic acids and rutin were dissolved in RPMI-1640 broth. 2-fold serial dilutions were prepared in RPMI-1640 broth, starting from 20 mg/mL for the seed extracts and organic acids, with the exceptions of aconitic (10 mg/mL) and fumaric (2.5 mg/mL), and 0.21 mg/mL for rutin. The solutions and cell suspensions in the test medium were then distributed into sterile 96-well plates. Maximum methanol concentrations were kept at 1% (v/v). The plates were incubated at 35 °C in a humid atmosphere, without agitation, for 48 h for *C. albicans* and *A. fumigatus*, and for four days for *T. rubrum*. MICs were the lowest concentrations resulting in 100% growth inhibition. Fluconazole MICs for *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) were determined as quality controls, and the results were within the recommended limits (*13*). Sterility and growth controls in RPMI-1640 medium alone and with 1% of methanol (v/v) were included. The experiments were performed in duplicate and repeated independently three times, yielding essentially the same results (a range of values is presented when different results were obtained).

RESULTS AND DISCUSSION

Phenolic Compounds. The analysis by HPLC/DAD of the different *L. esculentum* seeds extracts revealed the presence of phenolic compounds only in the methanolic extracts (**Figure 1**). The phenolic profile of the "Cherry" variety was analyzed for the first time. Both varieties exhibited the same phenolic profile: eight flavonoid glycosides were identified, which included quercetin, kaempferol and isorhamnetin derivatives, previously reported in the "Bull's heart" tomato variety seeds (*6*).

Table 1. Phenolic Composition of *L. esculentum* Seeds Methanolic Extracts (mg/kg of Seeds)[a]

	Compound[b]	Bull's heart variety	Cherry variety
1	Q-3-Soph	20.30 ± 1.80	14.44 ± 0.12
2 + 3	K-3-(Soph)Glc + Q-3-pent-Rut	2.40 ± 0.28	nq
4 + 5	K-3-Soph + I-3-Soph	6.16 ± 0.32	10.76 ± 0.20
6	I-3-Gtb	13.04 ± 0.40	2.92 ± 0.20
7	Q-3-Rut	2.88 ± 0.20	0.72 ± 0.04
8	K-3-Pent-Glc	5.28 ± 0.04	0.44 ± 0.08
	$\sum$	50.42	29.28

[a] Results are expressed as means ± standard deviation of three determinations; nq, not quantified; $\sum$, sum of the determined flavonoids. [b] Q: quercetin. K: kaempferol. I: isorhamnetin. Soph: sophoroside (glucosyl(1→2)glucoside). Gtb: gentiobioside (glucosyl(1→6)glucoside). Rut: rutinoside (rhamnosy(1→6)glucoside). Glc: glucoside. Rh: rhamnoside. Pent: pentoside.

In what concerns the quantification of these compounds, "Bull's heart" variety exhibited a higher amount of phenolics than the "Cherry" variety (**Table 1**). In both varieties quercetin-3-*O*-sophoroside was the major component, representing ca. 45% of the identified compounds, while the pair kaempferol-3-*O*-(2-sophorosyl)glucoside plus quercetin-3-*O*-(2-pentosyl)rutinoside represented the minor components (**Table 1**).

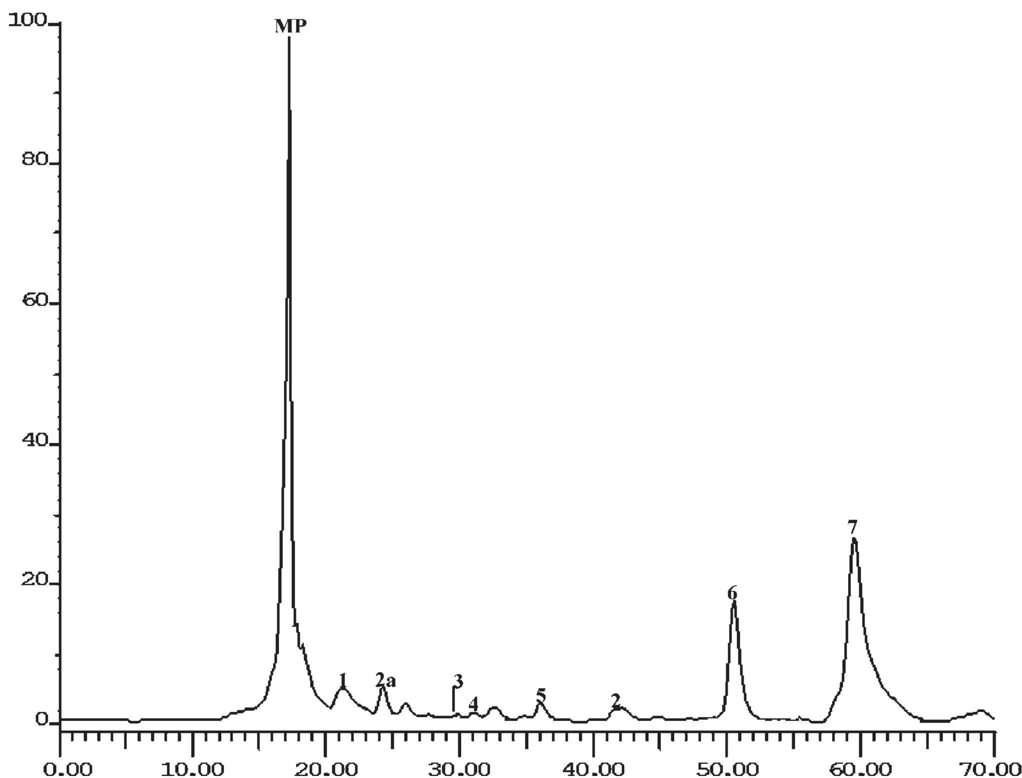

Figure 2. HPLC/UV organic acid profile of *L. esculentum* seed sulfuric acid extract ("Bull's heart" variety). Detection at 214 nm. Peaks: (MP) Mobile phase; (1) oxalic acid; (2a+b) aconitic acid; (3) citric acid; (4) pyruvic acid; (5) malic acid; (6) acetic acid; (7) fumaric acid.

Table 2. Organic Acid Composition of *L. esculentum* Seed Sulfuric and Methanolic Extracts (mg/kg of Seeds)[a]

		Bull's heart		Cherry	
	compound	sulfuric acid	methanol	sulfuric acid	methanol
1	oxalic	98.80 ± 0.70	7.70 ± 0.60	38.60 ± 0.40	6.30 ± 0.90
2	aconitic	5.00 ± 0.00		4.20 ± 0.50	
3	citric	75.70 ± 0.10		66.30 ± 0.20	
4	pyruvic	25.80 ± 0.90		22.80 ± 0.10	
5	malic	234.10 ± 2.00		262.60 ± 1.30	
6	acetic	56.20 ± 0.20	19.10 ± 0.00	39.50 ± 0.30	18.20 ± 0.20
7	fumaric	7.90 ± 0.00		8.20 ± 0.10	
	∑	503.50	26.80	442.20	24.50

[a] Results are expressed as means ± standard deviation of three determinations; ∑, sum of the determined organic acids.

Organic Acids. The two tomato seed varieties contained organic acids in both methanolic and sulfuric acid extracts (**Figure 2** and **Table 2**). Sulfuric acid extracts presented oxalic, aconitic, citric, pyruvic, malic, acetic and fumaric acids. In the methanolic extracts only oxalic and acetic acids were noticed (**Figure 2**). Citric, pyruvic, malic and fumaric acids were already reported in tomato seeds (*14*), while oxalic, aconitic and acetic acids were described in tomato fruits and leaves (*14*, *15*).

The total organic acid content in sulfuric acid extracts was nearly twenty times higher than that found in the methanolic extracts (**Table 2**). The "Bull's heart" variety exhibited higher levels of these compounds (**Table 2**). Malic acid was the main compound in sulfuric acid extracts (ca. 53%), while acetic acid predominated in the methanolic ones (ca. 73%) (**Table 2**).

Fatty Acids. Fatty acids were present in all extracts analyzed in this study, with the exception of the sulfuric acid (**Table 3** and **Figure 3**). Chloroform provided the highest fatty acid contents (**Table 3**), which is not surprising considering the low polarity of this solvent. Seven compounds were identified, which can be grouped in saturated fatty acids (pentadecanoic, palmitic, heptadecanoic and stearic acids), monounsaturated fatty acids (palmitoleic and oleic acids) and polyunsaturated fatty acids (linoleic acid). Pentadecanoic acid was the only previously described fatty acid in tomato fruit (*16*). The remaining compounds were already reported in tomato seed oil (*17*). Palmitic acid was the major compound in all analyzed extracts, except in the chloroformic extract from the "Cherry" variety, in which linoleic acid was present in highest amounts (**Table 3**).

The presence of glycoalkaloids in tomato seeds, which could also contribute to the antimicrobial activity (*18*), was discarded in all extracts, by using the general alkaloid precipitation tests: negative results with Dragendorff's (solution of potassium bismuth iodide), Mayer's (potassium mercuric iodide solution) and Bertrand's (silicotungstic acid solution) reagents were obtained.

Antimicrobial Potential. In order to evaluate the antimicrobial potential of *L. esculentum* seeds the different extracts were screened against Gram-positive and Gram-negative bacteria and fungi. Additionally, in order to try to establish a relationship with the chemical composition, the effect of all identified organic acids and rutin was also assessed. Rutin was the only phenolic compound tested because it is the only one commercially available among the identified phenolics. Fatty acid standards were not assayed, because they are insoluble in water and, consequently, in both RPMI and MHB media.

Antibacterial Activity. Concerning the effect against Gram-positive bacteria, it was possible to determine some MICs, which are indicative of the antimicrobial potential of tomato seeds (**Table 4**). Regarding the different solvents used, we can observe that hexane, sulfuric acid and ethyl acetate seem to be the most promising extracts (**Table 4**), although none of the tested extracts can be clearly highlighted.

Comparing the extracts of "Bull's heart" and "Cherry" varieties, no relevant differences were found, *E. faecalis* being the most susceptible species (**Table 4**). *E. faecalis*, present in the

Table 3. Fatty Acid Composition of *L. esculentum* Seed Extracts (mg/kg of Seeds)[a]

compound		Bull's heart[b]				Cherry[b]			
		Chlor	Met	EtAc	Hex	Chlor	Met	EtAc	Hex
1 pentadecanoic acid	C15:0	0.93 ± 0.17							
2 *cis*-9-hexadecenoic acid (palmitoleic acid)	C16:1	2.42 ± 0.16							
3 hexadecanoic acid (palmitic acid)	C16:0	12354.54 ± 1522.12	1.50 ± 0.01	4750.46 ± 99.51	1.94 ± 0.07	12334.39 ± 949.68	0.73 ± 0.01	2648.18 ± 37.38	2.45 ± 0.27
4 heptadecanoic acid	C17:0	nq	nq	nq	nq	nq	nq	nq	nq
5 *cis*-9,12-octadecadienoic acid (linoleic acid)	C18:2	764.75 ± 29.41	nq	0.04 ± 0.00	nq	28634.22 ± 361.04	0.62 ± 0.01	949.28 ± 52.54	nq
6 *cis*-9-octadecenoic acid (oleic acid)	C18:1	0.51 ± 0.01	nq				nq	0.06 ± 0.00	
7 octadecanoic acid (stearic acid)	C18:0	1.10 ± 0.12	0.128 ± 0.01	0.25 ± 0.01		0.24 ± 0.01	0.07 ± 0.02	0.05 ± 0.01	
	SFA[c]	12356.57	1.50	4750.71	1.94	12334.63	0.74	2648.24	2.45
	MUFA[d]	2.93	0.13	nq	nq	nq	nq	0.06	nq
	PUFA[e]	764.75	nq	0.40	nq	28634.22	0.62	949.28	nq
	$\sum$	13124.56	1.63	4751.11	1.94	40968.85	1.36	3597.58	2.45

[a] Results are expressed as means ± standard deviation of three determinations; nq, not quantified; $\sum$. sum of the determined fatty acids. [b] Chlor: chloroform. Met: methanol. EtAc: ethyl acetate. Hex: hexane. [c] SFA: saturated fatty acids. [d] MUFA: monounsaturated fatty acids. [e] PUFA: polyunsaturated fatty acids.

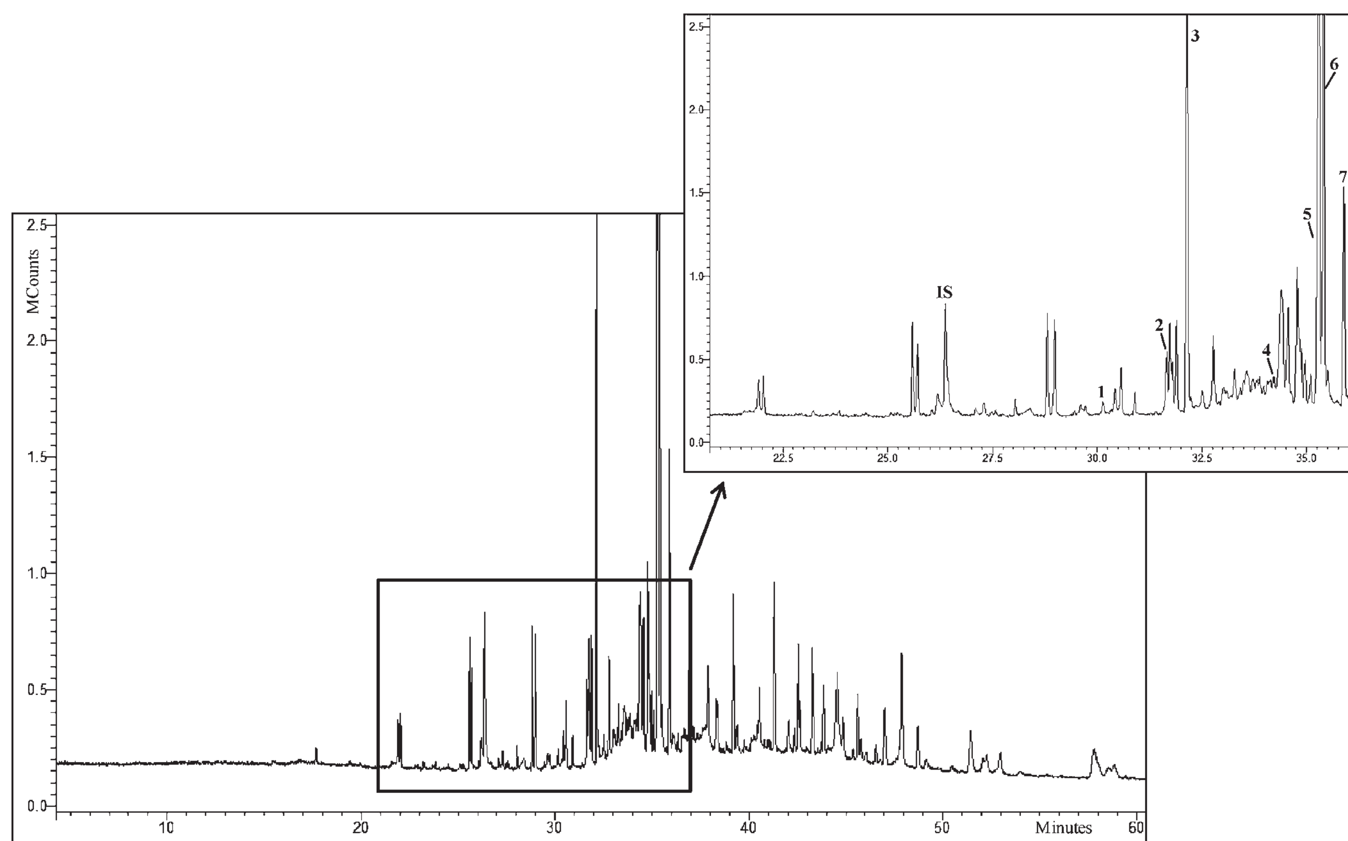

Figure 3. GC/MS fatty acids profile of chloroformic *L. esculentum* seeds extract ("Bull's heart" variety). Full scan. (IS) Internal standard; (1) pentadecanoic acid; (2) *cis*-9-hexadecenoic acid; (3) hexadecanoic acid; (4) heptadecanoic acid; (5) *cis*-9,12-octadecadienoic acid; (6) *cis*-9-octadecenoic acid; (7) octadecanoic acid.

gastrointestinal tract of humans, can cause several infections, especially in nosocomial environments (*19*). The hexane extract from "Cherry" variety and the ethyl acetate extract from both varieties were revealed to be the most active against this bacterial species (MIC = 2.5 mg/mL) (**Table 4**).

The "Bull's heart" ethyl acetate extract and the hexane extracts from both varieties were the most active against *S. aureus* (**Table 4**), which constitutes an important pathogenic agent in both nosocomial and community environments, being also frequently involved in cases of food poisoning (*20*).

Considering the protection against *S. epidermidis*, "Bull's heart" sulfuric acid, "Cherry" ethyl acetate and hexane extracts from both varieties were the most effective, presenting a MIC corresponding to 10 mg/mL (**Table 4**). Sulfuric acid extract from "Cherry" variety was the most active against *M. luteus* and *B. cereus* (**Table 4**). *S. epidermidis* and *M. luteus* are important pathogens in immunocompromised patients (*21*). *B. cereus* is responsible for food borne illness (*22*, *23*).

Under the tested concentrations all Gram-negative bacteria were found to be resistant to the different extracts (**Table 4**).

Table 4. MIC of *L. esculentum* Seed Extracts against Selected Bacteria[a]

bacteria	Bull's heart (mg/mL) Chlor	Met	EtAc	Hex	Sulf	Cherry (mg/mL) Chlor	Met	EtAc	Hex	Sulf	ciprofloxacin (μg/mL)
					Gram-Positive						
S. aureus	20	20	5	5	>20	20	>20	10	5	20	1.25
S. epidermidis	>20	>20	>20	10	10	20	>20	10	10	>20	0.6
M. luteus	>20	>20	>20	20	10	20	20	>20	10	5	1.25
E. faecalis	10	5	2.5	5	10	5	10	2.5	2.5	5	5
B. cereus	20	20	20	20	10	10	>10	20	>20	5	10
					Gram-Negative						
P. mirabillis	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	1.25
E. coli	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	1.25
P. aeruginosa	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	2.5
S. typhimurium	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	0.6

[a] Chlor: chloroform. Met: methanol. EtAc: ethyl acetate. Hex: hexane. Sulf: sulfuric acid.

Previous studies indicate that Gram-negative bacteria appear to be less sensitive to the action of many natural extracts (*24*), which is in agreement with our results. These results can probably be related to the physical differences between the cell walls of Gram-positive and Gram-negative bacteria. The presence of the external membrane with high content of lipopolysaccharide surrounding the cell wall in Gram-negative bacteria is considered to be the major contributor to its ability to resist chemical stress (*25*, *26*).

To check whether the observed antibacterial activity could be due to the presence of the identified organic acids and phenolics, the same assays were performed with the available standards. All organic acids presented antibacterial activity at the tested concentrations, with the exception of pyruvic acid. Fumaric acid was generally the most active compound against all tested bacteria (**Table 5**). Gram-negative bacteria were more resistant to organic acids than Gram-positive bacteria (**Table 5**), which agrees with the results obtained with the seed extracts. Thus, the activity found for tomato seeds can, in part, be due to the presence of this class of compounds.

Organic acids have commonly been used as food preservatives and have been reported to exhibit general antimicrobial activities. Wu et al. (*5*) demonstrated that the antimicrobial effect of organic acids is linked to pH: organic acids affect bacteria by lowering both environmental and intracellular pH (*27*). To check if the antibacterial effect of organic acids was correlated with their capacity to decrease the medium pH, three different solutions with pH 2.2, 3.0, and 4.0 were tested. The smaller value corresponds to that of the organic acid solution with the lowest pH. All bacteria were able to grow only at pH 4.0 (data not shown), which confirms that the antibacterial activity may be linked to the decrease in pH.

Rutin was also tested, presenting activity against all bacteria, except for *S. aureus*, *P. mirabilis* and *P. aeruginosa* (**Table 5**). Phenolics in seeds are related to their defense against predators and pathogens, including bacteria and fungi (*28*). Since they are known to be synthesized by plants in response to microbial infection (*29*), it is not surprising that they have been found to be effective antimicrobial substances against a wide array of microorganisms *in vitro*. The antibacterial activity of rutin was already noticed in previous works (*30*). Additionally, other extracts, also characterized by the presence of quercetin, kaempferol and isorhamnetin glycosides, have been shown to possess antimicrobial capacity (*31*). Thus, phenolic compounds also seem to give some contribution to the antibacterial properties of tomato seed extracts. The antimicrobial activity of phenolic compounds can be concentration dependent. At low concentration, phenols

Table 5. MIC of Organic Acids and Rutin against Selected Bacteria and Fungi[a]

microorganism	organic acids (mg/mL) pyruvic	aconitic	citric	malic	acetic	oxalic	fumaric	rutin (μg/mL)
				Gram-Positive Bacteria				
S. aureus	>10	1.25	1.25	1.25	1.25	1.25	0.625	>69
S. epidermidis	>10	1.25	1.25	1.25	1.25	1.25	0.625	69
M. luteus	>10	1.25	1.25	1.25	1.25	1.25	1.25	69
E. faecalis	>10	1.25	1.25	1.25	1.25	1.25	1.25	69
B. cereus	>10	1.25	1.25	1.25	1.25	1.25	0.625	69
				Gram-Negative Bacteria				
P. mirabillis	>10	1.25	2.5	1.25	1.25	1.25	1.25	>69
E. coli	>10	2.5	2.5	2.5	1.25	1.25	2.5	69
P. aeruginosa	>10	1.25	1.25	1.25	1.25	1.25	1.25	>69
S. typhimurium	>10	2.5	2.5	2.5	1.25	2.5	2.5	69
				Fungi				
C. albicans	>20	>10	>20	>20	10	10	>2.5	>69
A. fumigatus	>20	>10	>20	>20	10	10	>2.5	>69
T. rubrum	>20	>10	20	20	5	10	>2.5	>69

[a] MICs (μg/mL) for fluconazole were 0.25 (*C. albicans* ATCC 10231); for voriconazole 0.25 (*A. fumigatus* ATCC 46645) and 0.03 (*T. rubrum* CECT 2794). MICs for ciprofloxacin against bacteria were similar to those in **Table 4**.

affect enzyme activity, particularly those associated with energy production, while at high concentration they cause protein denaturation (*26*). The antimicrobial effect of phenolic compounds may be due to their ability to alter microbial cell permeability, thereby allowing the loss of macromolecules from the interior. They can also interfere with membrane function and interact with membrane proteins, causing deformation in structure and functionality (*26*). All these processes are independent of pH (*31*).

Also fatty acids may participate in the observed effect, as their bactericidal potential is well-known (*32*). Fatty acids and derivatives are most effective against Gram-positive bacteria. Gram-negative organisms are generally not affected, since they are less sensitive to lipophilic agents. This natural resistance can be due to the complexity of their cell walls (*33*). The mechanism of bactericidal action of long chain fatty acids and derivatives is ascribed to a balance between the hydrophilic and hydrophobic parts of the molecule. Regardless of the polarity of the hydrophilic portion, optimum chain length is between C12 and C16 (*33*). In addition, palmitoleic, palmitic, linoleic, oleic and

Table 6. MIC of *L. esculentum* Seed Extracts against Selected Fungi[a]

	Bull's heart (mg/mL)					Cherry (mg/mL)				
microorganism	Chlor	Met	EtAc	Hex	Sulf	Chlor	Met	EtAc	Hex	Sulf
C. albicans	5	5−10	>20	10	5−10	>20	>20	10	>20	>20
A. fumigatus	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
T. rubrum	10	5−10	>20	20	20	>20	>20	10	>20	>20

[a] Chlor: chloroform. Met: methanol. EtAc: ethyl acetate. Hex: hexane. Sulf: sulfuric acid. MICs (μg/mL) for fluconazole were 0.25 (*C. albicans* ATCC 10231); for voriconazole 0.25 (*A. fumigatus* ATCC 46645) and 0.03 (*T. rubrum* CECT 2794).

stearic acids, present in our extracts, are known for their antibacterial activity (*32*).

Antifungal Activity. Tomato seed extracts displayed antifungal properties, although they seemed to be less effective in comparison to Gram-positive bacteria. In a general way, the extracts from "Bull's heart" variety were more active than "Cherry" extracts (**Table 6**). Under the tested concentrations *A. fumigatus* was resistant to the action of all extracts (**Table 6**).

Candida species, especially *C. albicans*, is a causal agent of opportunistic oral and genital infections in humans. Candidoses have emerged as important causes of morbidity and mortality in immunocompromised patients (*19*). All "Bull's heart" extracts were active against *C. albicans*, except for the ethyl acetate extract. The chloroformic extract was the most active (**Table 6**).

T. rubrum is the commonest causative agent of dermatophytoses worldwide, and inhabits soil, humans or animals (*33*). The methanolic extract from "Bull's heart" variety was revealed to be the most active against this species (**Table 6**).

Tomato seeds exhibited inhibitory activity against some microorganisms implicated in the pathogenesis of skin diseases, such as *C. albicans* and *T. rubrum*, providing some scientific basis for the possible treatment of skin pathologies.

In addition, rutin displayed no activity against *C. albicans*, *A. fumigatus* or *T. rubrum* in the concentrations tested (**Table 5**). Regarding the antifungal activity of organic acids, best results were obtained for acetic and oxalic acids (**Table 5**). Although, as referred above, pH is an important factor for antibacterial activity, fungi are more resistant to pH variation, being able to grow under lower values. To verify the effect of pH in fungus growth, solutions with different pH values were also assayed, as indicated above for bacteria. All fungi were able to grow at pH 2.2 (data not shown). These results seem to indicate that the antifungal activity is due not solely to low pH but also to the specific organic acid molecule. Antifungal activity of fatty acids has already been reported, being related with their lipophilicity as it happened against bacteria (*32*).

Although organic acids and rutin alone have been shown to be active, synergistic and/or antagonistic interactions between these and other components have not been evaluated and cannot be excluded. In fact, the precise mechanism of antimicrobial action of the extracts is difficult to elucidate, due to complex interactions between different compounds (*34*). As observed with other matrices, the inherent activity of an extract can also be expected to relate with the chemical configuration of its components and the proportion in which they are present (*35*). Other extractive methods, such as supercritical fluid extraction or sequential extraction, can be used in order to accumulate more active components and improve the antibacterial and antifungal activity.

In conclusion, tomato seed extracts revealed activity against Gram-positive bacteria and fungi. The obtained results are encouraging, increasing the possibility of taking profit from the great wastes produced by the tomato processing industry. The antimicrobial properties of tomato seed extracts may be exploited by food and pharmaceutical industries for preservation and prevention of microbial spoilage. They can also be important agents for incorporation in antimicrobial packaging, from where they migrate through diffusion and partitioning, or in edible antimicrobial films. Additionally, for the possible use of these extracts as food preservatives it should be taken into account the presence of off-flavors, which may condition consumer's acceptance. In alternative, these extracts may have value as antimicrobials for topical use.

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