Antimicrobial activity of cold pressed citrus seeds oils, some citrus flavonoids and phenolic acids

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This study aims at determining the antimicrobial activity of cold pressed lemon, orange, and grapefruit seed oils, and to compare their activities with some antibiotic disks, flavonoid and phenolic acid standards. These oils had inhibition zones ranging from 6.62 to 11.00 mm against fifteen tested pathogenic bacteria. Only lemon seed oils and orange seed oil showed some inhibition against *Candida utilis* yeast. None of the oils had measurable inhibition zone against *Micrococcus luteus* ATCC 4698. Although most oils showed no growth inhibition even at a 100% concentration, *Escherichia coli* ATCC 25922 and *Salmonella* Enteriditis ATCC 13076 inhibited at 100% oil concentrations. Cold pressed and solvent extracted lemon seed oils inhibited growth of *Staphylococcus aureus* RSKK 1009 at 2% level, and cold pressed and microwave treated-cold pressed orange seed oil inhibited growth of *Klebsiella pneumonia* ATCC 700603 at 16 and 50%, respectively. The antibiotic disks ampicillin, sulbactam, piperacillin, tobramycin, mezlocillin, amoxicillin and cyclohexiimide presented 3-4 fold larger inhibition zones (10.24 - 47.00 mm) than the oil samples. Similarly, flavonoid standards naringin, naringenin, hesperidin, neohesperidin, catechin and kaempherol; and phenolic acids gallic, syringic, *tr*-ferulic, rosmarinic, *tr*-2-hydroxycinnamic and chlorogenic acids had inhibition zones like the seed oil samples. Hence citrus seed oils pose moderate levels of antimicrobial activity and could be used as antimicrobial aids.

Keywords: antimicrobial activity, antibiotic disk, citrus seed, cold press oil, minimum inhibition concentration.

1. INTRODUCTION

Edible common plant seed and kernel oils are largely produced by industrial screw presses, solvent extraction systems or a combination of both for the main vegetable oil supply chain. Whereas, cold pressing and supercritical CO_2 extraction techniques are usually used to extract some special and less common seeds, kernels and other oil bearing plant tissues to produce the so-called 'cold press oils' that are totally natural, solvent and chemical free, unrefined, fully flavoured and phytochemicals rich sources. It is not expected that cold press oils replace the industrially produced and refined oils for the mass consumption, but they fulfil the demands of nutraceuticals and functional foods, cosmetics, aromatherapy, and other applications [2, 19, 30].

Since cold pressed oils are produced from very clean, homogeneous, safe and high-quality materials, the oils are also high in sensory, nutritional and aroma quality. Furthermore, cold pressed oils retain the bioactive molecules originating from the starting plant material and infiltrated during the pressing into the oil; hence, they might have some functional properties including antimicrobial activity [13, 18].

It was indicated that plant extracts are rich sources of phenolics and other

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bioactive compounds, and can pose some antimicrobial, antioxidant, and antimutagenic activities; hence, they could be used as alternative natural antimicrobial agents [21]. It was also demonstrated that foodborne pathogens are a major concern of safety and could yield very high costs of illness and economical loss, not to mention death. Among others *Campylobacter*, *Salmonella*, *Listeria monocytogenes*, *Escherchia coli* O157:H7 and non-O157 STEC are the most prevalent foodborne pathogens [21, 22, 28].

Since cold pressed oils are natural, unrefined and bio active rich sources, they may pose some antimicrobial activity. Some studies considered this hypothesis [1, 13, 18, 22, 27, 28]. Keceli and Robinson [18] extracted the phenolic compounds from virgin olive oil, and tested their antimicrobial effects against some bacteria and fungus. They indicated that phenolic extract inhibited the growth of *Streptococcus thermophilus*, *Kluyveromyces marxianus* and *Penicillium frequentens*, and the effect was dependent on concentration, pH, time and type of microorganism. In another study [27], cumin seed and black cumin seed cold press oils were tested for antimicrobial activity against the bacteria species of *E. coli, P. aeruginosa, S. aureus, B. subtilis*, and mold species of *A. niger, A. flavus, C. albicans, S. cerevisiae*. The results showed that cumin seed oil inhibited growth of all tested microorganisms, while black cumin seed oil inhibited growth of all tested microorganisms except *A. niger* and *A. flavus*. In a similar study [28], cold pressed clove oil was tested against the bacteria of *E. coli, P. aeruginosa, S. aureus, B. subtilis* and molds of *A. niger, A. flavus, C. albicans, S. cerevisiae*. Results indicated that all tested microorganisms were inhibited for growth.

In literature, no study was found on the antimicrobial activity of citrus seed oils, but some studies on the antimicrobial activities of various citrus essential and/or peel oils are present. Mainly citrus essential oils are produced from fruit peels, and they are concentrated volatile, aromatic mixtures of biologically active substances in different chemical natures. Hence, essential oils are not triglyceride lipids, and totally different from the citrus seed cold press oils studied here. In an early study [25], the cold pressed terpeneless Valencia orange oil was tested against *E. coli* O157:H7, and shown to inhibit the microorganism at 0.2 to 4.6% concentrations at various temperatures effectively. In another study [15], the essential oils from orange, tangerine, pomelo, and lime were tested against pathogenic fungi *M. hiemalis, P. expansum and F. proliferatum*, and results indicated significant inhibitory effects. Phi *et al*. [26] studied different varieties of lime, orange and pomelo essential oils for composition and antimicrobial activity. They were shown to mainly include α-pinene, sabinene, β-pinene, myrcene, α-terpinene, limonene, terpinolene, γ-terpinene and linalool as the main volatiles. Lime and pomelo essential oils performed significant inhibition against *S. aureus*, while other orange and lime oils had significant inhibition against *B. cereus, S.* Typhi and *P. aeruginosa*. Furthermore, these oils inhibited growth of *A. flavus* and *F. solani* molds. Lately Federman *et al*. [11] studied the cold pressed terpeneless citrus derived oils against the growth of *S. aureus*. The minimum inhibitory concentration was 0.025%, and complete elimination was achieved at 0.25% concentration and 3 h incubation. In addition, various studies reported the antimicrobial effects of flavonoids and phenolic acids [7, 8, 9, 16].

The objectives of this study were to determine the antimicrobial activities of cold pressed citrus seed oils, and to compare the antimicrobial activity of the oils with some commercial flavonoid and phenolic acid standards (which are found in the same oils) and commercial antibiotic disks. Hence, the findings of this study may define new usage areas of these unconventional oil sources.

2. MATERIALS AND METHODS

2.1. MATERIALS

In this study, cold pressed oils from lemon seeds (*Citrus limon*), orange seeds (*Citrus sinensis*), and grapefruit seeds (*Citrus paradise*) were used. All oils were cold pressed in our laboratory, and the properties of the seeds, cold pressing conditions and oil general properties were already published in our previous studies [3, 4, 31, 32]. The general properties of the examined oils could be found in Table I [31] (Fig. 1).

The bacteria cultures of *Staphylococcus aureus* (ATCC 29213, ATCC 25923, RSKK 1009, ATCC 6538P), *Micrococcus luteus* (ATCC 4698), *E. coli* 0157:H7 (ATCC 43895), *Bacillus cereus* (NCIMB 7464), *Bacillus cereus Holl.* (No. 8), *Escherichia coli* (ATCC 25922, ATCC 8739), *Salmonella* Typhimurium (ATCC 51812, ATCC 14028), *Pseudomonas aeroginosa* (ATCC 27853), *Salmonella* Enteriditis (ATCC 13076), *Klebsiella pneumoniae* ATCC 700603; and the yeast cultures of *Saccharomyces cerevisiae* (ATCC 9763), *Candida albicans* (ATCC 10231), *Candida utilis* were used. All cultures were purchased from Microbiologics firms (USA) or obtained from Food Engineering department of Canakkale Onsekiz Mart University. The cultures were stored individually at –20°C in tryptic soy broth medium (TSB) (Biolife, Italy) and Saboroud Dextrose Broth medium (SDB, Biolife, Italy) with 25% glycerol. Strains were subcultured in twice. Yeast were striked to TSA and SDA. A single colony of strains were transferred to appropriate broth incubated overnight at 37°C, and for yeast cultures at 25°C. During the period of this study, they were maintained in TSB and SDB at 4°C and renewed bimonthly.

The Tryptic Soy Broth (TSB, Biolife, Italy) and Sab-

Table I - Main characteristics of the cold pressed citrus seed oils used [31]

	Lemon seed Oil	Orange seed Oil	Grapefruit seed Oil
Specific gravity (g/mL, 25 °C)	0.94±0.01	0.92±0.01	0.92±0.01
Refractive index (25 °C)	1.47±0.01	1.47±0.01	1.47±0.01
Viscosity (cP, 25 °C)	56.50±1.10	59.50±0.50	58.40±0.10
Color L value	10.62±0.92	9.75±1.03	9.50±0.92
a* value	28.10±0.90	33.50±3.00	35.80±1.75
b* value	4.35±0.40	15.62±6.10	17.05±4.50
Free fatty acidity (linoleic acid %)	0.60±0.01	0.27±0.02	0.37±0.05
Peroxide value (meqO_2/kg)	9.40±1.40	13.10±3.20	13.80±1.73
Iodine value (gl/100 g)	117.50±2.85	117.90±0.90	125.01±25.00
Saponification number (mg KOH/g)	199.95±1.80	203.89±1.30	202.55±2.45
Unsaponifiable matter (%)	1.05±0.23	0.70±0.15	0.80±0.05
Fatty Acid Composition (%)			
Palmitic acid	20.85±0.04	25.68±0.24	28.15±0.17
Palmitoleic acid	0.27±0.01	0.52±0.01	0.26±0.01
Stearic acid	4.28±0.02	5.75±0.11	3.65±0.01
Oleic acid	30.85±0.20	25.35±0.13	20.74±0.05
Linoleic acid	33.75±0.15	36.50±0.19	40.53±0.12
Linolenic acid	8.35±0.13	4.94±0.02	5.21±0.013

Figure 1 - The lemon, orange and grapefruit seed oils produced by cold pressing and different treatment techniques.

oroud Dextrose Broth (SDB, Biolife, Italy) were also purchased. The antimicrobial disks (ampicillin, sulbactam cefoperazone, piperacillin, tobramycin, mezlocillin, amoxicillin) were bought from Bioanalyse (Turkey). Cycloheximide, flavonoid and phenolic acid standards were purchased from Sigma Chem Co. (St. Louis, USA).

2.2. PREPARATION OF INOCULUM

The methods of Oliveira *et al*. [24] and EUCAST [10] were followed with some modifications to prepare the microbial inoculum. The bacterial strain was first activated in a Tryptic Soy Broth (TSB, Biolife, Italy) for 24 h at 37 ± 2°C in an incubator (Nüve İnkubator EN 055, İzmir, Turkey), and then 1 mL of each grown culture was transferred in 50 mL TSB medium and incubated at 37°C until they reached 8 log cfu/mL. Then 5 mL volume of that culture were centrifuged (5000 xg, 15 min, 4°C) (Sartorius, Sigma 2-16K) and washed by saline solutions three times. Then microbial density was adjusted to Macfarland 0.5. Meantime, the strains were inoculated into the Tryptic Soy Agar (TSA) by spread plate technique, and incubated at 37 ± 2°C for 24 ± 2h. The colonial counts and absorbance values were compared. Finally, they were used in the analyses.

Yeast cultures inoculated to Saboroud Dextrose Broth (SDB, Biolife, Italy) and incubated at 25°C for 48-72 h, 1 mL of each grown culture was transferred in 50 mL of SDB medium (SDB, Biolife, Italy) and incubated at 25°C until they reached 8 log cfu/mL. 5 mL volume of that cultures were centrifuged (5000 xg, 15 min, 4°C) (Sartorius, Sigma 2-16K) and washed by saline

solutions three times. Then microbial density was adjusted to Macfarland 0.5 and also determined by plating on Sabraoud Dextrose Agar (SDA) at 25 ± 2°C for 48 - 72h. Finally, they were used in the analyses.

2.3. ANTIMICROBIAL ACTIVITY

2.3.1. Determination of Antimicrobial Activity by Disk Diffusion Assay

The Agar disk diffusion method determined the antimicrobial effect of citrus seed oils. In this method, 0.1 mL from stock cultures of each culture (10^8 cfu/mL) was spread to Muller Hinton Agar (MHA, Biolife) plates to ensure the 10^6 cfu/mL MHA level. Sterile filter paper discs (6 mm diameter) were used for addition of 15 µL of each oil and kept at room temperatures for absorption, then 3 discs for each oil was placed on MHA plates with the help of tweezers. Negative control (sterile saline - 0.85% NaCl) and positive control (ampicillin, sulbactam, cefaperasone, piperacillin, tobramycin, mezlocilline, and amoxicillin antibiotics and cycloheximide for yeast cultures) discs were also used. Plates were incubated at 37°C for 24 hours for bacterial cultures, and at 25°C for 24-48 hours for yeast cultures. The diameters of the inhibition zones were measured including the diameter of paper discs. Tests were performed four times and results were presented as averages. The citrus flavonoids and phenolic acids standards were prepared at 500 ppm aqueous solution and tested in the same way [5, 6].

2.3.2. Determination of Minimum Inhibition Concentration Values

In the determination of MIC values, broth microdilution method was used. In this method, cultures were grown at appropriate medium and temperature until they reach 10^8 cfu/mL. Geometric dilutions ranging from 0.0312% to 16% of seed oils were prepared in Muller Hinton Broth (MHB) containing test tubes. 25% v/v stock solutions of seed oils prepared in DMSO to obtain the desired concentration. Then, 20 µL cultures and 180 µL oil containing medium were added in 96-U well microtiter plate. One growth control (only MHB) and one sterility control (MHB+test oil) was also done. Plates were incubated at 37°C for 24 ± 2h [5]. For yeast culture Saboroud Dextrose Broth was used and incubations were made at 25°C for 48±2h [12]. The optical density (OD) of each well were read on microplate reader (Thermo Scientific, Multiscan FC) at a wavelength of 620 nm. After determining the OD values, 20 µL of a 1% solution of sterile 2,3,5-triphenyl tetrazoliumcloride was added to each well, and incubated for 20 min at room temperature. The change in medium colour was controlled and the formation of a pink colour was used as an indicator of viable cells. For conformation, inoculations from each well that did not show any change in colour were made on TSA and SDA using the drop plate method. Plates were incubated at 37°C for 24 ± 2 and 25°C 48 h for the control of colony formation. All results were compared to determine the MIC value and the evaluation of MIC values was carried out in three separate plates with duplicate wells for each microorganisms and concentration [5, 6, 12].
The same assays were followed with the Saboroud Dextrose Broth (SDB) medium for the yeast strains [12]. After observing that the MIC values were higher than the tested concentrations, more tests with 25, 50 and 100% concentrations were also accomplished. Higher concentrations (25, 50 and 100%) were used directly.

2.4. STATISTICAL ANALYSES

All tests were performed as six separate measurements. The results were given as an average of six determinations with standard deviation values.

3. RESULTS AND DISCUSSION

3.1. THE DISK DIFFUSION ASSAY

The antimicrobial activities of the six citrus seed oils against 15 bacteria and 3 yeast species were tested by the disk diffusion assay, and the results are presented in Table II. If the zone of inhibition was smaller than the standard size of 6 mm, the measurements were not reported. The lemon seed oils were most effective against *S. aureus* ATCC 6538P, *S. aureus* RSKK 1009, *Escherichia coli* ATCC 25922, and *S.* Enteriditis ATCC 13076 bacteria with inhibition zones ranging from 8.50 to 10.62 mm size. The minimum zones were 7.33 mm for the *K. pneumonia* ATCC 700603 with cold pressed, and 7.00 mm for the *S.* Typhimurium ATCC 51812 with solvent extracted lemon seed oils, respectively. Very similar findings were evident (Table II) for the orange and grapefruit seed oils. Generally, the inhibition zones of all samples were ranged from the minimum value of 7.00 mm to the maximum value of 11.00 mm. Cold pressed orange seed oil was most effective against *S. aureus* ATCC 6538P (9.50 mm) and *S. aureus* RSKK 1009 (9.25 mm), while microwave treated-cold pressed orange seed oil was most effective against *E. coli* ATCC 25922 (11.00 mm) and *K. pneumonia* ATCC 700603 (10.21 mm), respectively. Similarly, *E. coli* ATCC 25922 (11.00 mm) and *P. aeroginosa* ATCC 27853 (9.87 mm), and *P. aeroginosa* ATCC 27853 (10.87 mm) and *S. aureus* ATCC 6538P (10.25 mm) had the largest inhibition zones by the cold pressed grapefruit seed oil and enzyme treated-cold pressed grapefruit seed oil, respectively. Furthermore, except for both lemon seed oils and microwave treated-cold pressed orange seed oil, none were effective against

Table II - Antimicrobial activity of cold pressed citrus seed oils against bacteria and yeast species tested by disc diffusion assay

Microorganism	Lemon seed oil		Orange seed oil		Grapefruit seed oil	
	Cold Pressed	Solvent Extracted	Cold Pressed	Microwave Treated	Cold Pressed	Enzyme Treated
	Inhibition zone diameter (mm)					
Staphylococcus aureus ATCC 29213	8.62±1.50	8.38±0.92	8.00±0.76	9.50±0.76	8.00±1.07	8.25±1.83
Staphylococcus aureus ATCC 25923	8.12±0.99	9.25±1.58	8.38±1.76	8.75±0.71	7.75±0.89	8.00±0.93
Staphylococcus aureus RSKK1009	9.75±0.70	10.12±0.64	9.25±1.75	9.25±0.70	9.00±0.92	9.25±1.49
Staphylococcus aureus ATCC 6538P	10.62±0.92	9.75±1.03	9.50±0.92	8.75±1.30	9.50±0.76	10.25±0.46
Micrococcus luteus ATCC 4698	-*	-	-	-	-	-
Bacillus cereus NCIMB 7464	-	-	-	7.87±1.46	-	-
Bacillus cereus Holl. No. 8	-	-	6.62±0.52	6.50±0.84	-	-
E.coli 0157:H7 ATCC 43895	7.75±0.46	7.62±0.52	7.62±0.52	7.12±0.35	7.37±0.52	7.50±0.53
Escherichia coli ATCC 25922	9.12±0.99	9.37±1.30	8.37±1.30	11.00±2.88	11.00±3.25	8.37±1.40
Escherichia coli ATCC 8739	7.75±0.76	8.00±0.76	7.12±.35	7.50±0.76	8.00±0.93	7.50±0.76
Salmonella Typhimurium ATCC 51812	7.50±0.93	7.00±0.76	-	7.50±0.53	7.25±1.16	7.62±1.30
Salmonella Typhimurium ATCC 14028	7.75±0.70	7.25±0.46	7.50±0.53	7.50±0.53	7.62±0.74	7.25±0.46
Salmonella Enteritidis ATCC 13076	8.62±1.26	8.50±1.26	7.94±0.68	7.75±1.00	7.37±0.62	7.50±0.76
Pseudomonas aeroginosa ATCC 27853	8.00±1.09	8.25±1.26	8.00±1.31	9.62±2.85	9.87±1.89	10.87±0.83
Klebsiella pneumoniae ATCC700603	7.33±1.50	7.37±0.92	8.75±1.75	10.12±2.59	7.50±1.41	9.37±1.60
Saccharomyces cerevisiae ATCC 9763	-	-	-	-	-	-
Candida albicans ATCC 10231	-	-	-	-	-	-
Candida utilis	9.92±1.93	8.67±1.50	-	9.75±2.26	-	-

(*) The zone of inhibition was smaller than the standard size (6 mm). All values was expressed as the average of four determinations ± standard deviation.

the yeasts tested. These three oils showed some inhibition against *Candida utilis* yeast.
There are some studies in literature with seed oils against the microbial growth by similar agar inhibition tests performed. In one study [27], cold pressed black cumin and cumin oils were tested against four bacteria and four mold species. The results revealed that cumin oil inhibited growth of all tested microorganisms, while black cumin oil inhibits all except *A. niger* and *A. flavus*. Inhibition zones ranged from 8.00 to 23.00 mm. Hence, cumin seed oils seem more effective antimicrobials than our citrus seed oils. Cold pressed clove oil was also tested against microbial growth of four bacteria and four molds [28]. The inhibition zones were larger for molds than for the bacteria, and all ranged from 17 to 29 mm. In another study [22], cold pressed black cumin seed oil was added into Domiati cheese at 0.1 and 0.2%, and the growth inhibition of *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* Enteriditis were followed during storage. Higher concentrations were more effective, and shelf life and sensory properties of the cheese improved significantly. Eight different cold pressed seed and kernel oils (argan, pomegranate, safflower, grape, walnut, date, flax, and golden berry) were tested for antimicrobial activity [13]. Different amounts of these oils (10, 25, and 50 ml) were added directly into the agar plates' wells, and the inhibition zones were measured. The inhibition zones were ranged from 6.5 mm to 25.5 mm, and increased as the oil amount increased. The most effective oils were pomegranate, argan and walnut oils. Since this study used directly oils in the agar medium like ours, it is more suitable to compare with our results. Generally, citrus seed oils had lower inhibition zones than the oils listed above. However, we used 15 ml of 100% citrus seed oils. In another study, 4, 20 and 50% of argan, pomegranate, safflower, grape, walnut, date, flax and golden berry seed and kernel oils were tested for antimicrobial activity. Only almond and pomegranate seed oils were found to have antimicrobial activity [17].
Although there is no study published with citrus seed oils antimicrobial activities to compare with our results, there are some studies accomplished with citrus peel and/or essential oils. Since some minor constituents of citrus seed oils and citrus peel oils are similar, it would be worth to compare the findings. The cold pressed peel oils of lemon, grapefruit, bergamot, bitter orange, sweet orange, and tangerine were tested against 9 bacteria and 5 yeast species [20]. It was shown that the oils had strong antimicrobial activity against all tested microorganisms, and lemon and bergamot oils were slightly better than the rest. In another study [26], lime, orange and pomelo essential oils from Vietnam were tested against 4 bacteria and 2 mold species. Inhibition zones of 17.1-28.2 mm were reported. Clearly, citrus essential oils seem to have higher antimicrobial activities than our citrus seed cold press oils tested.

3.2. THE MINIMUM INHIBITION CONCENTRATIONS (MIC)

The minimum inhibition concentrations (MIC) of the citrus seed oil were also reported (Table III) in our study. Most oils showed no inhibition to any microorganism even at 100% concentration applied. Only *E. coli* ATCC 25922 were inhibited by all six oils tested at 100% concentration. Similarly, *S.* Enteriditis ATCC 13076 was inhibited at 100% concentration by all six oils except enzyme treated-cold pressed grapefruit seed oil. The minimum concentration of 2% of both lemon seed oils was inhibited only for the *S. aureus* RSKK 1009. Cold pressed orange seed oil at 16% concentration, and microwave treated-cold pressed orange seed oil at 50% concentration inhibited the growth of *K. pneumoniae* ATCC 700603. Generally, cold pressed lemon, orange and grapefruit seed oils were not highly effective against tested organisms in terms of complete growth inhibition. In one study [13], (2015), eight different cold pressed seed oils were tested against two strains of *Aspergillus parasiticus* at 0.075, 0.15 and 0.30% concentrations. Around 8.3-22.1% inhibition was presented, and when added oil concentration increased, % inhibition also increased. This result indicates that their cold press oils were more effective, but they did not test them against bacteria. Ramadan *et al*. [27] reported 1-3 mL/L medium for bacteria, and 2-4 mL/L medium for molds as the MIC values of black cumin and cumin seed oils. Similarly, cold pressed clove oils showed to have 0.6-1.0 mL/L, and < 0.6 mL/L MIC values for the tested bacteria and molds, respectively [28]. Terpeneless orange oils [25] showed to have around 0.2-0.6% MIC values against *E. coli* O157:H7 isolated from beef. Orange, tangerine, pomelo and lime essential oils [15] were tested against *Mucor hiemalis, Penicillium expansum*, and *Fusarium proliferatum*, and inhibition values ranging from 37.1% to 100% were reported. Lastly citral, linalool, decanal and valencene as the major components of terpeneless orange oil were tested against *S. aureus* for the MIC values [11]. Citral and linalool showed 0.02% and 0.12% MIC values, whereas decanal and valencene were ineffective.
Overall, the MIC values of the cold press produced lemon, orange and grapefruit seed oils in this study were much lower than those reported and discussed above in literature for various seeds and citrus peel oils. Hence, in terms of total growth inhibition of most foodborne pathogens, citrus seed oils were not really effective. But they can be used in combination with other natural antimicrobials such as nisin.

3.3. ANTIMICROBIAL ACTIVITY OF SOME ANTIBIOTIC DISKS

Some antimicrobial disks (ampicillin, sulbactam, cefoperazone, piperacillin, tobramycin, mezlocillin,

Table III - Antimicrobial activity of cold pressed citrus seed oils against bacteria and yeast species tested by minimum inhibition concentration (MIC) assay

Microorganism	Lemon seed oil		Orange seed oil		Grapefruit seed oil	
	Cold Pressed	Solvent Extracted	Cold Pressed	Microwave Treated	Cold Pressed	Enzyme Treated
	Concentration (%)					
Staphylococcus aureus ATCC 29213	-*	-	-	-	-	-
Staphylococcus aureus ATCC 25923	-	-	-	-	-	-
Staphylococcus aureus RSKK1009	2	2	-	-	-	-
Staphylococcus aureus ATCC 6538P	-	-	-	-	-	-
Micrococcus luteus ATCC 4698	-	-	-	-	-	-
Bacillus cereus NCIMB 7464	-	-	-	-	-	-
Bacillus cereus Holl. No. 8	-	-	-	-	-	-
E.coli 0157:H7 ATCC 43895	-	-	-	-	-	-
Escherichia coli ATCC 25922	100	-	100	100	100	100
Escherichia coli ATCC 8739	-	-	-	-	-	-
Salmonella Typhimurium ATCC 51812	-	-	-	-	-	-
Salmonella Typhimurium ATCC 14028	-	-	-	-	-	-
Salmonella Enteritidis ATCC 13076	100	-	100	100	100	-
Pseudomonas aeroginosa ATCC 27853	-	-	-	-	-	-
Klebsiella pneumoniae ATCC700603	-	-	16	50	-	-
Saccharomyces cerevisiae ATCC 9763	-	-	-	-	-	-
Candida albicans ATCC 10231	-	-	-	-	-	-
Candida utilis					-	-

* No inhibition at the maximum concentration (100% oil) used. All values was expressed as the average of six determinations ± standard deviation.

amoxcillin and cycloheximide) were tested with the same microorganisms by disk diffusion technique, inhibition zones were measured (mm) and the results are given in Table IV. Cycloheximide was tested only for the yeasts, while the rest of the antibiotic disks were tested for most of the microorganisms selected for this study. The reason behind these measurements was the use as a positive control and to compare the antimicrobial effectiveness of citrus seed oils. Clearly, antimicrobial disks had larger inhibition zones. For all tested disks, the inhibition zones ranged from 10.25 to 47.00 mm, but most common values were between 20 and 30 mm. Compared to the inhibition zone values of the tested citrus seed oils (Table II), the antimicrobial disks had around 3-4-fold higher values. This result is quite expected since the disks contain the given concentrations (Table IV) of actual commercial antibiotics. Most importantly these results together indicate that citrus seed oils pose some level of antimicrobial activity, which would add to their usage value. In this respect, not only the antimicrobial effects but also other cellular effects (antitumor, antivirus etc.) of these unexplored oils need to be researched. Although it is not a common test in oil antimicrobial studies, in one study (Kirbaslar *et al*., 2009), the antimicrobial activity of citrus peel oils was compared with six different antibiotics, and only the ampicillin was a common factor between our study and theirs.

3.4. ANTIMICROBIAL ACTIVITY OF CITRUS FLAVONOIDS AND PHENOLIC ACIDS

Antimicrobial activities of 6 common citrus flavonoids (naringin, naringenin, hesperidin, neohesperidin, catechin and kaempherol) and six common citrus phenolic acids (gallic, syringic, *tr*-ferulic, rosmarinic, *tr*-2-hydroxucinnamic and chlorogenic acids) were determined as the agar inhibition zones and presented in Table V and VI. For this determination, the commercially available flavonoid and phenolic acid standards were used. The reason for selecting these particular standards is that the same compounds were already measured in our oil samples [3, 31]. Since the flavonoid and phenolic acids compositions of the same oils used in this study were already published, it would not be possible to use the same composition data in table format. Except catechin in grapefruit seed oil, all flavonoids and phenolic acids were quantified in the oil samples. In lemon seed oils, eriocitrin, hesperidin, gallic acid and *tr*-ferulic acid were in higher concentrations, while in orange seed oils, hesperidin, naringin and *tr*-ferulic acid were dominated. Likewise, very high concentrations of naringin, hesperidin, neohesperidin, *tr*-ferulic acid and gallic acid were quantified in the grapefruit seed oils. As the sum of all flavonoids and phenolic acids considered, grapefruit seed oil contained the highest amounts

Table IV - Antimicrobial activity of some antibiotic disks against bacteria and yeast species tested by disc diffusion assay

Microorganism	Ampicillin (10 µg)	Sulbactam, Cefoperazone (10.5 µg)	Piperacillin (100 µg)	Tobramycin (10 µg)	Mezlocillin (75 µg)	Amoxicillin (30 µg)	Cycloheximide (% 0.1)
	Inhibition zone diameter (mm)						
Staphylococcus aureus ATCC 29213	29.25±4.35	-	45.00±5.77	24.75±0.96	38.50±1.91	40.25±0.50	nt
Staphylococcus aureus ATCC 25923	-*	33.00±0.82	-	15.25±0.96	10.25±0.96	nt	nt
Staphylococcus aureus RSKK 1009	11.00±0.82	26.00±5.88	17.00±4.24	21.00±1.15	17.60±4.39	30.00±0.01	nt
Staphylococcus aureus ATCC 6538P	31.75±3.4	41.00±1.41	-	26.00±1.15	33.25±3.95	31.00±1.15	nt
Micrococcus luteus ATCC 4698	38.00±2.88	38.00±2.88	40.00±0.01	21.00±1.82	38.25±2.36	nt	nt
Bacillus cereus NCIMB 7464	-	15.00±0.81	nt	19.50±0.58	18.00±1.41	10.25±0.96	nt
Bacillus cereus Holl. No. 8	-	28.00±2.00	22.75±0.96	19.00±1.41	nt	nt	nt
E.coli 0157:H7 ATCC 43895	nt	25.50±2.51	24.50±1.00	23.00±2.58	25.00±2.58	16.25±0.50	nt
Escherichia coli ATCC 25922	nt	28.00±1.41	23.75±2.63	22.00±0.82	20.75±0.96	20.00±0.01	nt
Escherichia coli ATCC 8739	nt	32.75±0.56	-	21.00±0.81	21.75±1.26	nt	nt
Salmonella Typhimurium ATCC 51812	nt	31.00±2.58	26.50±0.71	18.75±0.5	22.25±0.50	nt	nt
Salmonella Typhimurium ATCC 14028	nt	28.75±1.75	24.50±0.58	15.50±0.581	17.75±0.50	24.50±1.29	nt
Salmonella enteritidis ATCC 13076	nt	25.75±9.6	25.00±2.71	20.75±2.06	22.25±2.87	23.50±058	nt
Pseudomonas aeroginosa ATCC 27853	nt	26.75±1.26	30.25±1.26	24.75±3.40	18.00 ±1.41	nt	nt
Klebsiella pneumoniae ATCC 700603	nt	24.25±1.25	11.50±2.38	13.00±0.01	11.25±1.29	14.50±0.58	nt
Saccharomyces cerevisiae ATCC 9763	nt	nt	nt	nt	nt	nt	37.50±1.50
Candida albicans ATCC 10231	nt	nt	nt	nt	nt	nt	47.00±1.29
Candida utilis	nt	nt	nt	nt	nt	nt	36.80±7.87

(*) The zone of inhibition was smaller than the standard size (6 mm). All values was expressed as the average of four determinations ± standard deviation. nt: not tested.

Table V - Antimicrobial activity of some flavonoids against bacteria and yeast species tested by disc diffusion assay

Microorganism	Flavonoids					
	Naringin	Naringenin	Hesperidin	Neohesperidin	Catechin	Kaempherol
	İnhibition zone diameter (mm)					
Staphylococcus aureus ATCC 29213	9.25±2.21	10.25±1.26	10.67±1.15	10.75±1.50	11.25±0.96	8.00±0.82
Staphylococcus aureus ATCC 25923	10.50±0.58	-	-	-	10.00±0.01	-
Staphylococcus aureus RSKK 1009	9.50±0.58	10.50±0.58	10.75±0.96	10.25±0.50	11.00±0.82	9.75±1.26
Staphylococcus aureus ATCC 6538P	13.50±0.71	11.50±071	-	11.25±0.96	12.50±0.71	9.00±0.82
Micrococcus luteus ATCC 4698	13.10±1.83	13.00±0.82	13.50±1.29	20.50±1.29	12.75±1.71	11.00±0.82
Bacillus cereus NCIMB 7464	-*	-	-	9.50±0.71	-	-
Bacillus cereus Holl. No. 8	10.00±1.41	-	9.50±1.29	10.75±2.97	9.75±1.26	-
E.coli 0157:H7 ATCC 43895	12.50±058	10.25±0.96	11.00± 1.64	10.50±2.38	11.25±0.96	10.50±0.58
Escherichia coli ATCC 25922	9.25±0.96	9.25±1.58	14.00±1.73	10.75±1.71	12.50±1.29	9.00±0.01
Escherichia coli ATCC 8739	-	-	8.25±0.96	9.25±2.22	6.67±0.58	-
Salmonella Typhimurium ATCC 51812	12.75±1.71	16.25±0.50	13.75±0.50	13.25±3.30	13.00±2.94	12.00±1.41
Salmonella Typhimurium ATCC 14028	9.75±0.96	10.75±2.06	11.75±3.69	10.50±1.87	10.50±0.58	10.75±2.75
Salmonella Enteritidis ATCC 13076	12.00±1.83	-	10.75±0.50	10.67±1.15	11.34±1.53	12.25±0.96
Pseudomonas aeroginosa ATCC 27853	11.25±0.50	8.00±0.81	11.25±1.50	8.25±0.96	10.00±1.15	7.33±0.58
Klebsiella pneumoniae ATCC700603	8.00±0.82	11.25±0.96	11.25±1.26	11.50±1.29	8.00±0.01	11.50±1.73
Saccharomyces cerevisiae ATCC 9763	-	-	-	-	-	-
Candida albicans ATCC 10231	7.50±-0.58		-	-	8.5±0.50	-
Candida utilis	9.75±0.50	-	11.25±0.96	10.50±0.50	10.00±0.50	-

(*) The zone of inhibition was smaller than the standard size (6 mm). All values for bacteria was expressed as the average of four determinations ± standard deviation. Results for yeast were expressed as the average of two determinations and standard deviation.

Table VI - Antimicrobial activity of some phenolic acids against bacteria and yeast species tested by disc diffusion assay

Microorganism	Phenolic acids					
	Gallic acid	Syringic acid	*tr*-Ferulic acid	Rosmarinic acid	*tr*-2- Hydroxycinnamic acid	Chlorogenic acid
	Inhibition zone diameter (mm)					
Staphylococcus aureus ATCC 29213	7.50±0.58	9.00±1.82	9.00±0.82	10.50±1.50	8.25±1.26	10.75±0.96
Staphylococcus aureus ATCC 25923	-*	9.34±2.08	8.75±1.50	8.00±1.00	8.00±1.00	-
Staphylococcus aureus RSKK1009	9.50±1.29	9.50±1.29	10.75±0.96	9.62±1.60	10.00±2.16	9.75±0.96
Staphylococcus aureus ATCC 6538P	-	11.00±1.41	10.50±071	11.50±0.71	9.75±0.5	9.50±0.58
Micrococcus luteus ATCC 4698	10.00±0.82	14.50±1.29	18.25±1.71	12.25±0.50	16.50±0.58	15.50±2.52
Bacillus cereus NCIMB 7464	-	9.50±0.71	10.25±0.50	9.50±0.71	7.34±0.58	9.25±1.26
Bacillus cereus Holl. No. 8	12.50±1.73	-	10.25±1.56	10.00±1.41	8.25±1.25	9.50±1.29
E.coli 0157:H7 ATCC 43895	11.00±2.45	10.25±1.26	10.25±1.26	10.75±0.50	12.25±0.96	10.75±1.50
Escherichia coli ATCC 25922	-	9.00±1.15	9.50±1.73	10.34±1.53	9.25±0.96	10.25±0.96
Escherichia coli ATCC 8739	7.75±0.96	10.50±0.58	11.50±1.29	-	10.00±2.16	-
Salmonella Typhimurium ATCC 51812	10.75±0.96	15.00±1.15	13.00±1.15	11.25±0.57	11.00±0.82	12.25±0.96
Salmonella Typhimurium ATCC 14028	11.50±3.69	9.75±1.50	10.25±2.06	10.00±1.83	9.75±0.96	11.00±2.16
Salmonella Enteritidis ATCC 13076	11.75±0.50	11.00±0.50	10.00±1.64	10.00±0.81	12.75±2.22	10.50±0.71
Pseudomonas aeroginosa ATCC 27853	10.25±1.71	10.50±1.73	9.50±1.29	9.25±0.96	9.25±0.96	9.50±1.29
Klebsiella pneumoniae ATCC 700603	10.00±1.63	8.75±1.26	9.50±1.29	12.75±0.5	11.50±0.71	11.00±1.41
Saccharomyces cerevisiae ATCC 9763	-	-	-	-	-	-
Candida albicans ATCC 10231	7.50±0.58		-	-	8.5±0.50	15.25±0.96
Candida utilis	9.75±0.50	-	10.25±0.96	-	10.00±0.50	18.25±0.50

(*) The zone of inhibition was smaller than the standard size (6 mm). All values for bacteria was expressed as the average of four determinations ± standard deviation. Results of yeasts were given as the average of two determinations.

(around 2300-3600 mg/kg) followed by lemon seed oil (around 2500-2800 mg/kg) and orange seed oil (1700-2000 mg/kg), respectively [3, 31].
Naringin had the largest inhibition zones (13.50 and 13.10 mm) against *S. aureus* ATCC 6538P and *M. luteus* ATCC 4698, while the minimum value was for *Klebsiella pneumoniae* 700603 (8.00 mm) (Table V). Similarly, naringenin had the highest zone against *M. luteus* ATCC 4698 (13.00 mm), and the lowest for *P. aeroginosa* ATTC 27853 (8.00 mm). The maximum and minimum inhibition zones for hesperidin and neohesperidin were 14.00 to 8.25 mm, and 20.50 to 8.25 mm, respectively. Catechin showed inhibition zones between 6.67 and 12.75 mm, while the same ranges were 7.33 and 12.25 mm for the kaempherol (Table V). Among all tested flavonoids, the largest inhibition zone was observed with neohesperidin for *M. luteus* ATCC 4698 (20.50 mm), and the lowest was measured with catechin for *E. coli* ATCC 8913 (6.67 mm). In literature, some studies exist for the flavonoids and phenolic acids as pure substances or components of plant extracts for the antimicrobial activity determination. Tripoli *et al*. [29] reported that several polymethoxylated flavones strongly inhibit bacterial lipopolysaccharide-induced expression of TNF-a. Particularly hesperidin said to significantly inhibit inflammation and inhibit growth of *S. Typi* and *S. Typhimurium*. In a review [16], it was indicated that hesperidin was very effective against *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Salmonella* Typhimurium and *Enterobacter cloacae*, while tangeretin and nobiletin were nearly inactive. Furthermore, neohesperidin, hesperetin, neoeriocitcin, eriodictyol, naringin and naringenin aglycones exhibited MIC values around 250-1000 mg/mL. Also, it was indicated that hesperidin did not impact human intestinal bacteria (*Bacteroides galacturonicus, Lactobacillus sp., Enterococcus caccae, Bifidobacterium catenulatum, Ruminococcus gauvreauii and E. coli*), but hesperetin inhibited all of them. Similarly, in another review [14] almost all plant phenolics indicated to have various degrees of antimicrobial activities towards many pathogens. Mandalari *et al*. [23] used bergamot extract against Gram-negative bacteria (*Escherichia coli, Pseudomonas putida, Salmonella enterica*), Gram-positive bacteria (*Listeria innocua, Bacillus subtilis, Staphylococcus aureus, Lactococcus lactis*) and the yeast *Saccharomyces cerevisiae*, and found that they were active against all Gram-negative bacteria, and potency increases after enzymatic deglycosylation. Also, the MIC values of pure flavonoids, neohesperidin, hesperetin (aglycone), neoeriocitrin, eriodictyol (aglycone), naringin and naringenin (aglycone), were found to be in the range 200 to 800 mg/mL.
Clearly literature indicates the presence of some antimicrobial activity of the citrus flavonoids and phenolic acids against a wide range of microorganisms, either as extracts or as pure substances. Hence, our results in Table V and VI concur with literature. As could be observed from both tables, the inhibition zone values of the flavonoids and phenolic acids are within the same range. Although diverse results reported in literature, it could be concluded that citrus flavonoids and phenolic acids represent moderate levels of antimicrobial activities. In fact, when we compare the antimicrobial activities of the citrus seed oils (Table II) and those tested pure flavonoid and phenolics standards (Table V and VI), it is evident that the activities are quite similar. Hence, it would be said that most of the antimicrobial activity of the citrus oils originate from their own flavonoids, phenolic acids, and perhaps other micro components. Since cold pressed oils retain all the minor components infiltrated during the pressing into the oil, and not refined, they might to be used as antimicrobial additives in various food enrichments, feed, pharmaceutical and cosmetic areas.

4. CONCLUSIONS

Citrus processing creates significant masses of citrus seeds as waste materials. Among others, cold pressed seed oils are one of the important way to valorise seeds. We already have cold pressed lemon, orange and grapefruit seeds, and have determined their composition and all the main physicochemical properties. Some results of our studies have already been published. We realized the importance of measuring the antimicrobial activity of the citrus seed oils to possibly explore new usage areas. In this study, agar diffusion and MIC assays tested six citrus seed oil samples. All oils had some antimicrobial activities against 15 pathogenic bacteria, and some had antimicrobial activity against yeasts. Compared to other cold press seed and kernel oils, and citrus peel oils in literature, our oils had generally lower antimicrobial activities. But in comparison with the pure substances of citrus flavonoids and phenolic acids standards, it was clear that there were similar levels of antimicrobial activities. Furthermore, commercial antimicrobial disks were tested in the same way, and showed to have 3-4 fold higher antimicrobial activities than those of the oil samples. Hence, it could be concluded that citrus seed oils pose some antimicrobial activity against common foodborne pathogens. They might have applications in food processing as antimicrobial aid agents. Further applications of these unexplored, very new oil sources in food, feed, pharmaceutical and cosmetic sectors are foreseen.

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