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Antimicrobial and functional properties of the proteins extracted from lemon, orange and grapefruit seeds press meals

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RESEARCH ARTICLE

Abstract

The aim of this study was to determine the antimicrobial activities and functional properties of the proteins extracted from lemon (*Citrus limon* var. Kütdiken), orange (*Citrus sinensis* var. Dörtyol) and grapefruit (*Citrus paradisi* var. Beyaz) seeds press meals. The ethanolic extracts of the proteins showed around 6.3-15.8 mm inhibition zones against some foodborne pathogenic bacteria. Although this range was 3-4 fold lower than the commercial antibiotic disk (sulbactam cefoperazone, 10.5 µg), it still indicates presence of some antimicrobial activity. Likewise, the minimum inhibition concentration values were determined against the same bacteria. Although some pathogens were not inhibited at highest used concentration (5%) of protein ethanolic extracts, some extracts even were effective at 0.16-0.63% concentration levels. Hence, these extracts could inhibit the growth of some pathogenic bacteria. These citrus seed protein extracts exhibited good water and oil holding capacities and some foaming abilities, although their emulsification properties were lower. Overall, citrus seed proteins could be used in food products or other areas for their antimicrobial and functional properties.

Keywords: citrus seeds, protein extract, antimicrobial activity, functional properties

1. Introduction

Since world population increases steadily and 15% of population is starving, it is imperative to manage world food supply together with waste valorisation and effective utilisation. It was indicated that food waste covers all food life cycles, and 42% originates from household, 39% losses occur in food manufacturing, 14% pertains to food sector and 5% is lost along the distribution. This situation results in great economical losses and enormous environmental challenges. Hence, waste utilisation and bio-refinery become a future proposition (Matharu *et al.*, 2016; Mirabella *et al.*, 2014).

Among other sectors, citrus processing is a huge one and creates wastes like seeds, peels and pulp. Citrus peels and pulps have been utilised industrially for the production of essential oils and aromas, pectin, dietary fibre, molasses, juice sacks and others, while valorisation of the citrus seeds are fairly limited. Studies indicate that citrus seeds are rich sources of oil, protein, fibre, flavonoids, limonoids, and other phenolic compounds (El-Adawy *et al.*, 1999a,b; Malacrida *et al.*, 2012). It was reported that (El-Adawy *et al.*, 1999a) orange seed includes 17.01% protein, 2.01% non-protein nitrogen, 42.59% oil, 3.17% ash, 22.53% fibre, 14.70% total carbohydrate and 8.70% moisture. Another study (Anwar *et al.*, 2008) reported 6.43, 3.90, 5.56, and 9.56% protein (on dry weight) for lemon, grapefruit, sweet orange and mandarin. Also, these seeds showed to contain around 27.0-36.5% oil, 5.0-8.5% fibre and 4.6-5.6% ash. Recently, we have cold pressed the citrus seeds, and characterised the oils in detail (Aydeniz Güneşer and Yilmaz, 2017a,b; Yılmaz, 2017; Yilmaz and Aydeniz Güneşer, 2017).

There are very limited numbers of studies about the citrus seed proteins in literature (El-Adawy *et al.*, 1999a,b; El-Safy *et al.*, 2012). El-Adawy *et al.* (1999a,b) showed that citron, orange and mandarin seeds include 19.93, 17.01 and 15.87% protein. Furthermore, their seed flours shown to include 18 amino acids, of which glutamic acid, arginine and

aspartic acid were the major components, while tryptophan, methionine and isoleucine were the limiting amino acids. Likewise, citrus seed proteins shown to be minimum soluble at pH 4.0-4.5, and had bands at around 37, 23 and 17 kD on the electrophoresis. In another study (El-Safy *et al.*, 2012), orange seed flour was shown to contain 3.06% protein (dry weight), and 17 amino acids with glutamic acid, arginine and aspartic acid at the highest concentration. Clearly some differences exist based on genetic and environmental variabilities.

Recently (Karabiber and Yılmaz, 2017), the main properties of the seed proteins extracted from the defatted press meals of lemon, orange and grapefruit seeds were published. Ultrasound-assisted alkaline process resulted 34.47-80.95% protein extraction yields. The maximum protein solubilities were observed at around pH 11-12 and below 2 for these extracted-lyophilized proteins. While asparagine, cysteine, hydroxyproline and tryptophan were absent, sixteen other amino acids were quantified. Gel electrophoresis indicated four distinct protein bands at around 10, 15-20, 20, and 25-37 kD. Also thermal properties (denaturation temperature and enthalpy of denaturation) of the proteins were reported. After determining the main physicochemical properties, it was imperative to determine their potential functional and antimicrobial properties.

Literature lacks the antimicrobial activity of citrus seed proteins. But in one study (Cvetnic and Vladimir-Knezevic, 2004), antibacterial and antifungal activity of grapefruit seed and pulp ethanolic extracts were determined. The extracts exhibited antimicrobial activity against *Salmonella* Enteritidis. In another study (Nile and Park, 2014), seed extracts of Yuzu fruit showed some antimicrobial activity. On the other hand, for various food proteins or their extracts antimicrobial activities were reported (Abd El-Aziz and Abd El-Kalek, 2011; Dhaval *et al.*, 2016; Jean *et al.*, 2016; Nile and Park, 2014; Przybylski *et al.*, 2016; Rai *et al.*, 2016).

The aim of this study was to determine the antimicrobial activity and functional properties of extracted citrus seed proteins. For the first time in literature, the antimicrobial activity against some foodborne bacteria, and functional properties like water and oil holding capacities, emulsification and foaming capacities and stabilities, and gelling properties of the citrus seed proteins are reported. Citrus seed proteins emerge as a new and unexplored protein source to be utilised in various areas.

2. Materials and methods

Materials

In this study, the extracted-lyophilized seed proteins from lemon (*Citrus limon* var. Kütdiken), orange (*Citrus sinensis* var. Dörtyol) and grapefruit (*Citrus paradisi* var. Beyaz) press meals were used as the materials. Protein extraction was done according to Karabiber and Yılmaz (2017). Briefly, defatted press meals and deionized distilled water were mixed at 1:20 (w/v) ratio, and ultratoraxed (Yellow line D125 basic) at 13,500 rpm for 5 min, before adjusting pH to 8.8 by 1 N NaOH solutions. The slurry was mixed for additional 5 min at 13,500 rpm and pH adjusted to 9.0. Then the slurry was sonicated with an ultrasound apparatus (VCX750; Sonics, Newtown, CT, USA) at max 70% amplitude until max 40 °C heat reached by 5 sec apply-5 sec pause mode. Finally, it was filtered through 0.053 mm mesh sieve to remove the insoluble particles, and centrifuged at 3,000×*g* for 20 min (2-16K; Sigma, Osterode, Germany) to collect the extracted proteins. From the six different defatted press meals, six different protein extracts were collected. Cold pressed and solvent extracted lemon seed meals were extracted to get the cold pressed lemon seed protein (CPLP) and solvent extracted lemon seed protein (SELP) samples. Similarly, cold pressed orange seed protein (CPOP) and microwave roasted-cold pressed orange seed protein (MROP), and cold pressed grapefruit seed protein (CPGP) and enzyme treated-cold pressed grapefruit seed protein (ETGP) were extracted from their defatted press meals, and used in this study.

Staphylococcus aureus (ATCC 29213, ATCC 25923, RSKK 1009, ATCC 6538P), *Bacillus cereus* (NCIMB 7464), *Bacillus cereus* Holl., *E. coli* 0157:H7 (ATCC 43895), *Escherichia coli* 0157:H7 EDC, *E. coli* (ATCC 25922, ATCC 8739), *Salmonella* Typhimurium (ATCC 51812, ATCC 14028), *Salmonella* Enteriditis (ATCC 13076), and *Klebsiella pneumoniae* (ATCC 700603) were purchased from Microbiologics (St Cloud, MN, USA) or obtained from Food Engineering Department of Canakkale Onsekiz Mart University (Çanakkale, Turkey). The cultures were stored individually at -20 °C in tryptic soy broth medium (TSB) (Biolife, Milan, Italy) with 25% glycerol. TSB and the antibiotic disk sulbactam cefoperazone (Bioanalyse, Ankara, Turkey) were also purchased. All other standards and chemicals were of analytical grade and purchased from Sigma Chem Co. (St. Louis, MI, USA), and Merck (Darmstadt, Germany).

Preparation of the ethanolic extracts from the proteins

Self-made ethanolic extract from the citrus seed protein samples was prepared from the lyophilized protein extract samples. For microbiological test, 5% (w/v) extract was prepared using 80% ethanol solution. After filtering the

ethanolic extracts from 0.45 µm sterile syringe filters, they were collected into sterile flacons, and stored at -20 °C until the analyses (Burt and Reinders, 2003).

Preparation of the inoculum

The methods of Oliveira *et al.* (2011) and EUCAST (2013) were modified for inoculum preparation. The bacterial cultures were inoculated to TSB and incubated at 37 °C for 24±2 h, then 1 ml of each grown culture was transferred into 20 ml of TSB medium and incubated at 37 °C until they reached 8 log cfu/ml. The level of microorganism was determined by plating at 30 min intervals. A standard curve was prepared to establish a correlation between the colony forming unit concentration (cfu/ml) and the optical density of the dilutions at MacFarland turbidimeter (Grant Instruments, Cambridge, UK). The time which cultures reached to 8 log cfu/ml was determined and incubations were made according to this determined time. Finally, the culture (5 ml) was centrifuged (5,000×*g*, 15 min, 4 °C) (Sigma 2-16K; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) and washed three times by saline solutions. The microbial density was adjusted to MacFarland 0.5, and also determined by plating on tryptic soy agar (TSA; Biolife) at 37±2 °C for 24±2 h.

Determination of antimicrobial activity by disk diffusion assay

The antimicrobial activity of the ethanolic protein extracts was determined by agar disk diffusion method. In this method, 0.1 ml from stock cultures of each culture (10^8 cfu/ml) was diluted and spreaded over 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 protein extract and kept at room temperatures for absorption, and then 2 discs for each extracts was placed on MHA plates with the help of tweezers. Negative control (80% ethanol) and positive control (sulbactam cefaperasone) discs were also used. Since the disks were kept at room temperature, solvent (80% ethanol) was evaporated, and the effect of it was omitted. Plates were incubated at 37 °C for 24 h. Finally, the diameters of the inhibition zones were measured including the diameter of paper discs (Burt and Reinders, 2003).

Determination of minimum inhibition concentration values

To determine the minimum inhibition concentration (MIC) values of the protein extracts, the broth microdilution method was used (Burt and Reinders, 2003; Gammariello *et al.*, 2014). First, the cultures were grown at appropriate medium and temperature until they reach 10^8 cfu/ml. Geometric dilutions ranging from 0.042% to 5% of the protein extracts were prepared in muller hinton broth (MHB; Biolife) containing test tubes. Then, 20 µl cultures and 180 µl extract containing medium were added in 96-U well microtiter plate. One growth control (only MHB) and one sterility control (MHB + test extract) was also prepared. Plates were incubated at 37 °C for 24±2 h (Burt and Reiders, 2003). The absorbances of each well were read on microplate reader (Multiscan FC; Thermo Scientific, New York, NY, USA) at a wavelength of 620 nm. After determining the absorbance 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 formation of pink colour was used as an indicator of viable cells. For conformation, inoculations from each well that not show any change in colour was made on TSA by drop plate method. Plates were incubated at 37 °C for 24±2 for control of colony formation. All results were compared to determine the MIC value and the evaluation of MIC values was carried out in triplicate.

Protein functional properties

All protein functional properties were determined by the methods described in our previous studies (Yılmaz and Dündar Emir, 2016; Yılmaz and Hüriyet, 2017).

Briefly, water holding capacity (WHC) of the samples was determined by dispersing 0.5 g protein in 10 ml deionized (DI) water, adjusting pH to 7.0, vortexing vigorously, waiting at room temperature for 30 min, centrifugating at 2,290×*g* for 15 min, and finally decanting the supernatant, draining the solids 30 min on filter paper and weighing. The weight difference of initial and end proteins was used to calculate the WHC, and results are expressed as g water adsorbed/g protein.

To measure oil holding capacity (OHC), 0.5 g protein and 5 ml sunflower oil were mixed, vortexed, waited 30 min at room temperature, before centrifugation at 2,291×*g* for 15 min. Finally, 5 min more centrifugation at 19,890×*g* was accomplished before inverting the tubes to drain the free oil out for 1 h. Finally, the OHC was calculated from the weight difference and was reported as g oil adsorbed/g protein.

Emulsifying activity (EA) was determined by dissolving 0.5 g protein in 10 ml DI water, adjusting pH to 7.0, and mixing with 10 ml of sunflower oil. The mixture was centrifuged at 2,291×*g* for 15 min and EA calculated by:

$$EA = 100 \times \frac{\text{height of emulsion layer}}{\text{total height of mixture in tube}} \quad (1)$$

Then emulsion stability (ES) was determined by heating the tubes in a water bath (80 °C, 30 min), then cooling

quickly under tap water, and centrifugating at 2,291×*g* for an additional 5 min. ES was calculated by:

$$ES = 100 \times \frac{\text{height of remaining emulsified layer}}{\text{total height of mixture in tube}} \quad (2)$$

The foaming capacity (FC) and foam stability (FS) were also determined. Protein dispersions (1%) in DI water prepared, pH set to 7.0, and then the mixture was whipped at high speed in a Waring blender (Warring blender 7011S; Warring Laboratory, Torrington, CT, USA) for 3 min, and immediately poured into 100 ml volumetric cylinder. FC was reported by:

$$FC(\%) = 100 \times \frac{\text{volume after agitation-volume prior to agitation}}{\text{volume prior to agitation}} \quad (3)$$

The same samples were standed for an additional 30 min at room temperature to estimate the FS by:

$$FS(\%) = 100 \times \frac{\text{residual foam volume}}{\text{total foam volume}} \quad (4)$$

The least gelation concentration (LGC) was determined by preparing protein suspensions of 2, 4, 6, 8, and 10% from the 20% (w/v) stock solution, adjusting their pH to 7.0, and heat denaturing the samples at 100 °C in a water bath for 30 min, before quickly cooling under tap water and on ice. Finally, the samples were kept at 4 °C for 18 h, and visually examined for the gelation condition. LGC was determined as the lowest protein concentration (%, w/v) at which the samples did not move (solid gel) when the tubes were inverted.

Statistical analysis

The whole study was replicated twice, and all analyses within each replicate were completed at least two or three times. The results are presented as means with standard deviation values. The comparison of the functional properties and antimicrobial activities by disk diffusion assay were accomplished by the ANOVA and Tukey's tests with Minitab v.16.1.1 software (Minitab, 2010).

3. Results and discussion

Antimicrobial activity of the citrus seed proteins

Antimicrobial activities of the ethanolic extracts collected from the citrus seed proteins are tested by disc diffusion method and results as inhibition zones (mm) are presented in Table 1. Sulbactam cefoperazone (10.5 µg) was the positive control sample for comparison. No inhibition zones observed with negative controls (ethanol solvent) since alcohol was evaporated during holding at room temperature so effects of alchocol were omitted. The antimicrobial disk sulbactam cefoperazone was the most effective against all tested microorganisms compared with the protein extracts. It was an expected result, but the protein extracts still had some antimicrobial activities. Among all samples, the highest antimicrobial activity was measured against *E. coli* 0157:H7 ATCC 43895 with SELP extract (15.8 mm), while the lowest value (6.3 mm) was with MROP extract against *S. aureus* RSKK 1009. Compared to the antimicrobial disk sulbactam cefoperazone (17.0-34.5 mm), the inhibition zones of the extracts were 3-5 fold lower. When the protein extracts were compared with each other except the sulbactam cefoperazone disk, it was shown that there was no statistically significant difference for the *S. aureus* ATCC 25923 and 6538P, *B. cereus* Holl., *E. coli* 0157:H7 EDC and *Salmonella* Enteritidis ATCC 13076 ($P \leq 0.05$). The extract from MROP sample yielded the significantly lowest ($P \leq 0.05$) inhibition zone values for *S. aureus* ATCC 29213 (8.4 mm), *S. aureus* RSKK1009 (6.3 mm), *B. cereus* NCIMB 7464 (7.6 mm), *Escherichia coli* ATCC 8739 (7.6 mm), *Salmonella* Typhimurium ATCC 51812 (7.0 mm), and *Salmonella* Typhimurium ATCC 14028 (7.5 mm) among all extracts. While extracts from the SELP sample produced the lowest inhibition zones for *E. coli* ATCC 25922 (8.5 mm) and *K. pneumonia* ATCC700603 (6.9 mm), it also provided the highest inhibition zones for *S. aureus* ATCC 29213 (14.0 mm) and *E.coli* 0157:H7 ATCC 43895 (15.8 mm). Clearly, ethanolic extract from MROP sample had lower antimicrobial activities compared with other extracts. Hence, it can be claimed that seed microwave pretreatment might had some effects on its proteins to decrease the antimicrobial activity of the extract. The measured antimicrobial activities of the seed protein extracts would be arised from some peptides and/or other bioactive substances present in the protein samples. We have determined the molecular weights of the electrophoresis bands of these same protein samples, and published the results previously (Karabiber and Yılmaz, 2017). All of our extracted citrus seed proteins had molecular weights at around 10, 15-20, 20, and 25-37 kD. Also, it was quite evident that band intensities of MROP and ETGP were significantly lower than the others. Most probably the seed pretreatments microwave roasting and enzyme (naringinase and hesperinidase) treatment caused some protein losses. But eventually, only the microwave roasting pretreatment changed the antimicrobial activity.

The MIC values of the protein extracts were also determined and reported (Table 2). The MIC values of all samples ranged from 0.16 to >5%. Generally, *S. aureus* species were inhibited at around 0.1-0.6%, while *B. cereus* and *E. coli* O157:H7 species could not be inhibited at highest concentration of (5%) extracts. Similarly, *Salmonella*

Table 1. Antimicrobial activity of the citrus seed proteins ethanolic extracts by disc diffusion method.[1,2]

	Inhibition zone (mm)						
	CPLP	SELP	CPOP	MROP	CPGP	ETGP	Sulbactam cefoperazone (10.5 µg)
Staphylococcus aureus ATCC 29213	9.3±0.9^{c}	14.0±0.7^{b}	12.2±2.1^{c}	8.4±0.5^{d}	11.0±1.0^{c}	11.1±2.9^{c}	32.5±1.0^{a}
Staphylococcus aureus ATCC 25923	12.6±2.3^{b}	12.5±1.3^{b}	10.7±0.9^{b}	9.0±2.4^{b}	9.4±3.1^{b}	11.2±1.6^{b}	26.3±1.7^{a}
Staphylococcus aureus RSKK1009	11.2±0.8^{b}	12.3±2.1^{b}	14.5±1.3^{b}	6.3±0.5^{c}	13.8±2.1^{b}	14.2±2.2^{b}	26.0±5.9^{a}
Staphylococcus aureus ATCC 6538P	11.6±0.9^{b}	9.2±3.1^{b}	14.0±1.8^{b}	12.2±1.8^{b}	9.5±2.4^{b}	10.6±1.8^{b}	34.5±3.3^{a}
Bacillus cereus NCIMB 7464	14.0±0.9^{b}	8.0±1.9^{c,d}	9.6±2.1^{c}	7.6±0.5^{d}	9.8±2.6^{c}	11.3±1.9^{c}	18.0±1.3^{a}
Bacillus cereus Holl.	12.3±1.7^{b}	10.7±1.4^{b}	8.8±3.2^{b}	9.8±1.9^{b}	9.2±1.9^{b}	11.0±0.8^{b}	17.0±2.0^{a}
Escherichia coli 0157:H7 ATCC 43895	12.0±2.3^{b}	15.8±2.9^{b}	10.4±2.1^{b,c}	12.5±1.3^{b}	8.8±2.9^{c}	13.8±1.3^{b}	25.5±2.51^{a}
Escherichia coli 0157:H7 EDC	13.4±3.5^{a}	9.6±3.2^{a}	12.0±2.8^{a}	11.8±2.6^{a}	10.0±1.8^{a}	12.4±3.4^{a}	–
Escherichia coli ATCC 25922	10.8±2.3^{b}	8.5±1.3^{c}	14.5±1.8^{b}	10.0±1.8^{b}	9.7±0.50^{b}	14.0±3.9^{b}	29.3±0.9^{a}
Escherichia coli ATCC 8739	9.3±1.7^{b}	8.6±2.4^{b}	10.0±2.2^{b}	7.6±1.5^{c}	10.3±3.4^{b}	10.2±2.1^{b}	22.7±0.9^{a}
Salmonella Typhimurium ATCC 51812	12.0±4.1^{b}	11.5±0.6^{b}	11.5±1.7^{b}	7.0±0.8^{c}	10.5±1.3^{b}	11.5±2.4^{b}	25.5±2.6^{a}
Salmonella Typhimurium ATCC 14028	14.0±2.5^{b}	15.0±3.9^{b}	12.3±1.3^{b}	7.5±1.7^{c}	9.5±2.4^{b}	11.0±2.0^{b}	22.0±1.6^{a}
Salmonella Enteritidis ATCC 13076	8.4±1.9^{b}	9.0±0.7^{b}	10.0±0.7^{b}	10.0±1.6^{b}	7.6±2.2^{b}	8.6±1.3^{b}	25.8±0.9^{a}
Klebsiella pneumonia ATCC700603	10.4±1.1^{b}	6.8±0.8^{c}	10.6±2.1^{b}	8.8±1.6^{b}	9.2±1.5^{b}	9.4±2.9^{b}	24.3±1.3^{a}

[1] Means in the same row followed by different superscript letters were significantly different ($P \leq 0.05$, n=4).
[2] CPLP = cold pressed lemonseed protein; SELP = solvent extraction lemonseed protein; CPOP = cold pressed orangeseed protein; MROP = microwave roasted orangeseed protein; CPGP = cold pressed grapefruitseed protein; ETGP = enzyme treated grapefruitseed protein.

Table 2. Antimicrobial activity of the citrus seed proteins ethanolic extracts by minimum inhibition concentration test.[1]

	Concentration (%)					
	CPLP	SELP	CPOP	MROP	CPGP	ETGP
Staphylococcus aureus ATCC 29213	0.16	0.08	0.63	0.63	0.16	0.16
Staphylococcus aureus ATCC 25923	0.23	0.16	0.16	0.63	0.31	0.16
Staphylococcus aureus RSKK1009	0.16	0.16	0.16	0.16	0.16	0.16
Staphylococcus aureus ATCC 6538P	0.31	0.16	0.16	0.63	0.31	0.16
Bacillus cereus NCIMB 7464	>5	>5	>5	>5	>5	>5
Bacillus cereus Holl.	>5	>5	>5	>5	>5	>5
Escherichia coli 0157:H7 ATCC 43895	>5	>5	>5	>5	>5	>5
Escherichia coli 0157:H7 EDC	0.63	0.31	0.31	0.31	0.31	0.31
Escherichia coli ATCC 25922	0.31	0.16	0.16	0.16	0.31	0.16
Escherichia coli ATCC 8739	0.47	0.16	0.16	0.16	0.63	0.16
Salmonella Typhimurium ATCC 51812	0.16	0.63	0.16	0.63	0.31	0.16
Salmonella Typhimurium ATCC 14028	0.16	1.3	0.16	0.63	0.31	0.16
Salmonella Enteritidis ATCC 13076	0.16	0.63	0.31	0.31	0.31	0.31
Klebsiella pneumoniae ATCC700603	0.16	0.16	0.16	0.16	0.16	0.16

[1] CPLP = cold pressed lemonseed protein; SELP = solvent extraction lemonseed protein; CPOP = cold pressed orangeseed protein; MROP = microwave roasted orangeseed protein; CPGP = cold pressed grapefruitseed protein; ETGP = enzyme treated grapefruitseed protein (n=4).

species and *K. pneumoniae* were inhibited at 0.1-1.3% concentrations. Clearly, most tested bacteria could be inhibited at fairly low concentrations of the protein extracts, despite their relatively lower inhibition zone values. King *et al.* (2008) reported that results between the disc diffusion and broth microdilution method are not always comparable.

Othman *et al.* (2011) stated that results obtained from broth microdilution method are considered more accurate. In addition, results depends polarity and some other characteristics of extracts (Tan and Lim, 2015). Hence, these extracts pose some antimicrobial activity potential, and this could be used in various food products, since the proteins are edible and do not contain any offensive substance.

There are some studies reporting antimicrobial activity for food proteins or their extracts (Abd El-Aziz and Abd El-Kalek, 2011; Dhaval *et al.*, 2016; Jean *et al.*, 2016; Nile and Park, 2014; Przybylski *et al.*, 2016; Rai *et al.*, 2016). Abd El-Aziz *et al.* (2011) studied the antimicrobial activities of proteins extracted from pumpkin seeds, rinds and pulps, and found around 1-10, 2-25 and 1-12 mm inhibition zones, respectively. Also, seed radiation treatment up to 10 kGy did not impart antimicrobial activity. In another study (Jean *et al.*, 2016), lactoferrin protein was digested with pepsin and peptides <10 kDa were extracted and tested. Results indicated that cationic peptides were active against avian pathogens. Recently, Przybylski *et al.* (2016) tested antimicrobial effect of bovine cruor, a slaughterhouse by-product, which is mainly composed of hemoglobin protein and other peptides. The peptide inhibited the growths of total viable colonies, yeasts and molds, but the most striking effect was on the proliferation of the coliform bacteria. In a current review (Rai *et al.*, 2016), natural antimicrobial peptides for food preservation was discussed. Not to mention other sources, plants provide defensins, cyclotides, 2S albumin, lipid transfer proteins, hevein-like proteins knotins and snakins as common antimicrobial peptides. In general, an enormous research area for exploring natural antimicrobial proteins, peptides or their extracts seems available.

Unfortunately, we could not find any literature about the antimicrobial activity of the citrus seed proteins. But in one study (Cvetnic and Vladimir-Knezevic, 2004) antibacterial and antifungal activity of ethanolic extract of grapefruit seed and pulp was examined against 20 bacterial and 10 yeast strains. The extract exhibited strongest effects against *Salmonella* Enteritidis with 2.06% MIC value. Other tested microorganisms showed sensitivity at around 4.13-16.50% level. Health promoting properties of Yuzu fruit was studied (Nile and Park, 2014). Among several other biological activities of this citrus fruit, Yuzu seed extract with 80% methanol showed potent antimicrobial activity against *Micrococcus luteus*, *S. aureus*, *E. coli* and *Salmonella* Enteritidis. Another study (Yao *et al.*, 2012), reported the antimicrobial activity of nobiletin and tangeretin, the polymethoxylated citrus flavones. Of course these are not protein, but citrus flavonoids are widespread in every part of the fruits and could be found in seeds, and hence in seed protein extracts. Both were shown to very effectively destroy the *Pseudomonas fluorescens* and *Pseudomonas aeruginosa in vitro*.

Hence, in the light of the above discussed literature, it could be claimed that citrus seed protein extracts pose moderate levels of antimicrobial activity against several foodborne pathogenic bacteria whether due to the peptides and/or phenolic compounds present in them, and since they are edible source and could easily be produced, they might have some usage areas as antimicrobial aids in food processing or in other areas.

Functional properties of the citrus seed proteins

Food processing functional properties of the citrus seed proteins are presented in Table 3. The processing functionalities of WHC, OHC, EC, ES, FC and FS are the most common properties that determine the suitability of a protein in different food applications. The WHC of the orange and grapefruit seed protein samples (5.54-6.28 g/g) were significantly higher than lemon seed proteins (2.55-3.58 g/g). Clearly seed pretreatments did not have significant effects on this property, except lemon seed

Table 3. The functional properties of the citrus seed proteins.[1,2]

Sample	WHC (g/g)	OHC (g/g)	EC (%)	ES (%)	FC (%)	FS (%)
CPLP	3.58±0.19^{b}	2.41±0.41^{b}	7.37±0.61^{a}	5.80±0.54^{a}	37.50±4.17^{b}	62.50±4.17^{b}
SELP	2.55±0.16^{c}	2.36±0.12^{b}	4.77±0.54^{b}	4.23±0.02^{a}	27.08±2.08^{c}	72.92±2.08^{a}
CPOP	5.54±0.16^{a}	2.99±0.46^{b}	7.37±0.61^{a}	3.69±0.54^{b}	57.92±7.92^{a}	42.08±7.92^{d}
MROP	5.92±0.13^{a}	3.29±0.27^{b}	6.38±0.87^{a}	2.66±0.53^{c}	50.50±14.70^{a}	53.7±11.9^{c}
CPGP	6.28±0.21^{a}	3.96±0.10^{a}	4.77±0.54^{b}	3.18±0.62^{b}	40.13±4.93^{b}	59.87±4.93^{b}
ETGP	6.15±0.05^{a}	4.89±0.12^{a}	4.15±0.02^{b}	3.11±0.61^{b}	32.46±6.03^{b}	67.54±6.03^{a}

[1] Means in the same column followed by different superscript letters were significantly different ($P \leq 0.05$); n=4.

[2] WHC = water holding capacity; OHC = oil holding capacity; EA = emulsification capacity; ES = emulsification stability; FC = foaming capacity; FS = foaming stability; CPLP = cold pressed lemonseed protein; SELP = solvent extraction lemonseed protein; CPOP = cold pressed orangeseed protein; MROP = microwave roasted orangeseed protein; CPGP = cold pressed grapefruitseed protein; ETGP = enzyme treated grapefruitseed protein.

protein. Generally, WHC measures the water retention ability of the sample, and includes those of the bound, hydrodynamic, capillary and physically entrapped waters. Also, it was stated that WHC is mainly determined by the amino acid profile and number of charged residues, conformation, hydrophobicity, temperature, pH and ionic strength and protein concentration (Moure *et al.*, 2006). Although there is no study reporting the WHC of citrus seed proteins, in the early report of El-Adawy *et al.* (1999a), the WHC of orange seed flour was reported around 3.64 g/g, and in El-Safy *et al.* (2012), it was reported as 1.20 g/g. The same studies also provides the protein content of the flours as around 33.14 and 3.06%. Hence, it could be said that citrus seed proteins have good WHC values. In a review about the functional properties of proteins extracted from various oilseed and kernels (Moure *et al.*, 2006), WHC values ranged from 0.34 to 6.60 g/g. Overall, WHC of grapefruit seed and orange seed proteins could be valued as high and lemon seed proteins can be though as moderate capacity samples. WHC is an important functionality for proteins in comminuted meat products, bakery products, food gels, etc.

OHC values of the samples were also measured (Table 3). Among all samples, the highest value was with ETGP sample (4.89 g/g), and like WHC values, OHC values were also higher in grapefruit seed proteins (3.96-4.89 g/g) than the lemon and orange seed proteins (2.36-3.29 g/g). While microwave roasting for orange seeds and enzyme treatment for grapefruit seeds enhanced OHCs a little, solvent extraction for lemon seed reduced it. El-Adawy *et al.* (1999a) and El-Safy *et al.* (2012) reported around 3.13 and 4.90 g/g fat absorption capacity for orange seed flours. Our findings concur with theirs. Likewise, diverse OHC values (0.36-5.87 g/g) were reported for various oilseeds and kernels (Moure *et al.*, 2006). Sharma *et al.* (2010), studied the OHC of ten selected oilseed proteins (almond, Brazil nut, cashew, hazelnut, macadamia, pine nut, pistachio, Virginia peanut, Spanish peanut and soybean), and their values ranged from 2.8 to 7.0 g oil/g protein. Our results are mostly within the reported ranges in the literature. Like WHC, the OHC of the microwave roasted and enzyme treated samples were higher, indicating some positive effects of partial protein denaturation. Partial heat denaturation may partially unfold protein, and this could result extended exposure of the buried amino acids to the mileue to enhance the binding capacities (Khattab and Arntfield, 2009; Sharma *et al.*, 2010).

EC and ES were measured as very important functional properties for various food applications for the proteins (Table 3). The highest EC values were 7.37% for both CPLP and CPOP samples. Grapefruit seed proteins had lower EC values, as does SELP sample. Clearly, solvent extraction of lemon seed reduced EC of its protein significantly. In the other samples, seed pre-treatments reduced the EC values to some degrees as well. The same trend of change was evident for the ES values. The highest ES values were measured in lemon seed proteins (4.23-5.80%), and the lowest value was 2.66% measured in the MROP sample. Hence, it could be indicated that any denaturing effects on the citrus seed proteins reduces their emulsification properties. Generally, control sample of lemon seed proteins had better EC and ES values in this study. EC values as ml oil/g protein were reported around 90 and 50.96 for the orange seed flours in the literature (El-Adawy *et al.*, 1999a; El-Safy *et al.*, 2012). Since different measurement techniques and units of data reported, it would not be proper to compare the results. Emulsion activity index (%) values ranging from 2.20% to 100%, and emulsion stability (%) values from 7.64% to 100% were reported for various seed and kernel proteins (Moure *et al.*, 2006). Generally, both EC and ES values of the citrus seed protein samples in this study could be considered lower levels. It was discussed that emulsification properties of proteins can be affected by their molar mass, level of hydrophobicity and solubility, conformation, charge status as well as physico-chemical factors like pH, ionic strength, temperature, and presence of interfering agents (Moure *et al.*, 2006). Hence, citrus seed proteins may not be very suitable for emulsion type food applications.

For some food processing operations, foaming is an indispensable property for food proteins. FC and FS were also measured and results are presented in Table 3. The highest FC was (57.92%) for CPOP, and the lowest one was (27.08%) for SELP samples. Generally, seed pre-treatments caused a little decreases in FC values. Contrarily, FS values affected in the different patterns, and except enzyme treatments for grapefruit seeds, seed pre-treatments caused significant enhancements in FS values. The highest FS value (72.92%) was for SELP, and the lowest (53.7%) was for MROP sample. Although no report in literature for citrus proteins, foam expansion (%) and foam stability (%) values of orange seed flour were given as 11.72 and 59.77%, respectively (El-Safy *et al.*, 2012). Clearly, citrus seed proteins had better scores than flour samples. FC as percentage volume change, and FS as ml in 1-120 min were reported for various proteins extracted from oilseeds and kernels, and ranged from 10-295% and 7-600 ml (Moure *et al.*, 2006). Foaming capacity (as % overrun) and stability (<1 h) of ten selected edible oilseed proteins (Sharma *et al.*, 2010) were measured. Generally, citrus seed proteins, hence, could be accepted as moderate level foaming proteins. In some food products, foams are formed as air dispersion within a surrounding continuous aqueous medium. Proteins mostly locate at the water-air interface through formation a thin film to reduce the interfacial tension to enhance the foam stability. The thickness, gas permeability and viscoelasticity of the proteins determines its foaming functionalities. Good foaming proteins must rapidly adsorb, rearrange and form as cohesive durable film. Protein

hydrophobicity, net charge and charge distribution and hydrodynamic properties usually determines its foaming properties (Moure *et al.*, 2006; Sharma *et al.*, 2010).

Gelling ability is another intrinsic property of food proteins for gel type product applications (Table 4). While orange seed and grapefruit seed proteins yielded true solid gels at around 8% protein dispersions, lemon seed proteins were gelled at 10% concentrations. Depending on the source, nature, state and environmental conditions, diverse types of protein gels with different LGC, gel structures and gel stabilities could be produced. Moure *et al.* (2006) reported around 4-28% of LGC values for various oilseed and kernel proteins. Similarly, Sharma *et al.* (2010) reported 6-20% LGC values for ten selected edible oilseed proteins. Usually, our results are within the reported range. Hence, citrus seed protein based gels could be produced, if desired. Gelling of any protein is determined by the type and denaturation state of protein, as well as presence of non-protein impurities, salts, pH, ionic strength and presence of reducing agents. Furthermore, it was indicated that partially unfolded and high molecular weight proteins form more stable gels (Moure *et al.*, 2006; Sharma *et al.*, 2010).

4. Conclusions

In this study, proteins extracted from lemon, orange and grapefruit seeds press meals were characterised for antimicrobial activity and functional properties. The ethanolic extracts of the proteins showed around 6.3-15.8 mm inhibition zones against some most important foodborne pathogenic bacteria. Likewise, the MIC values showed that most pathogens were inhibited at around >5% protein ethanolic extracts, some even were effective at 0.16-0.63% concentration levels. Hence, these extracts could inhibit the growth of some bacteria. As functional properties, the WHC and OHC of grapefruit seed and orange seed proteins were significantly higher than lemon seed proteins. On the other hand, EC and ES values of the proteins were generally lower than the reported values. FC and FS values were also good, and partially denatured samples (solvent extracted, microwave and enzyme treated) showed higher foam stability values. All samples yielded stable solid protein gels at around 8-10% concentrations. In conclusion, citrus seed proteins pose some antimicrobial activity and good functional properties to be utilised in various food or other applications.

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References

Abd El-Aziz, A.B. and Abd El-Kalek, H.H., 2011. Antimicrobial proteins and oil seeds from pumpkin (*Cucurbita moschata*). Nature and Science 9: 105-119.

Anwar, F., Naseer, R., Bhanger, M.I., Ashraf, S., Talpur, F.N. and Aladedunye, F.A., 2008. Physico-chemical characteristics of citrus seeds and seed oils from Pakistan. Journal of the American Oil Chemists' Society 85: 321-330.

Aydeniz Güneşer, B. and Yılmaz, E., 2017a. Bioactives, aromatics and sensory properties of cold pressed and hexane extracted lemon (*Citrus limon* L.) seed oils. Journal of the American Oil Chemists' Society 94: 723-731.

Aydeniz Güneşer, B. and Yılmaz, E., 2017b. Effects of microwave roasting on the yield and composition of cold pressed orange seed oils. Grasas y Aceites 68: e175.

Burt, S.A. and Reinders, R.D., 2003. Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. Letters in Applied Microbiology 36: 162-167.

Cvetnic, Z. and Vladimir-Knezevic, S., 2004. Antimicrobial activity of grapefruit seed and pulp ethanolic extract. Acta Pharmaceutica 54: 243-250.

Dhaval, A., Yadav, N. and Purwar, S., 2016. Potential applications of food derived bioactive peptides in management of health. International Journal of Peptide Research and Therapeutics 22: 377-398.

Table 4. The least gelling concentrations of the citrus seed proteins.[1]

Dispersion (%)	CPLP	SELP	CPOP	MROP	CPGP	ETGP
2	Liquid+clot	Liquid+clot	Liquid+clot	Liquid+clot	Liquid+clot	Liquid+clot
4	Liquid+clot	Liquid+clot	Liquid+clot	Liquid+clot	Liquid+clot	Liquid+clot
6	Liquid+clot	Liquid+clot	Liquid+clot	Liquid+clot	Liquid+clot	Liquid+clot
8	Liquid+clot	Liquid+clot	Solid gel	Solid gel	Solid gel	Solid gel
10	Solid gel	Solid gel	Solid gel	Solid gel	Solid gel	Solid gel

[1] CPLP = cold pressed lemonseed protein; SELP = solvent extraction lemonseed protein; CPOP = cold pressed orangeseed protein; MROP = microwave roasted orangeseed protein; CPGP = cold pressed grapefruitseed protein; ETGP = enzyme treated grapefruitseed protein (n=4).

El-Adawy, T.A., Rahma, E.H., El-Bedawey, A.A. and Gafar, A.F., 1999a. Properties of some citrus seeds. Part 1. Physico-chemical characteristics of proteins. Food/Nahrung 43: 374-378.

El-Adawy, T.A., Rahma, E.H., El-Bedawey, A.A. and Gafar, A.F., 1999b. Properties of some citrus seeds. Part 3. Evaluation as a new source of protein and oil. Nahrung/Food 43: 385-391.

El-Safy, F.S., Salem, R.H. and Abd El-Ghany, M.E., 2012. Chemical and nutritional evaluation of different seed flours as novel sources of protein. World Journal of Dairy and Food Science 7: 59-65.

European Committee On Antimicrobial Susceptibility Testing (EUCAST), 2013. EUCAST disk diffusion test manual (Vol. 3.0). EUCAST, Basel, Switzerland. Available at: https://tinyurl.com/y89spoz7.

Gammariello, D., Conte, A., Lucera, A., Mastromatteo, M. and Del Nobile, M.A., 2014. Anti-yeast activity of natural compounds: *in vitro* and *in vivo* tests. Food Packaging and Shelf Life 1: 30-37.

Jean, C., Boulianne, M., Britten, M. and Robitaille, G., 2016. Antimicrobial activity of buttermilk and lactoferrin peptide extracts on poultry pathogens. Journal of Dairy Research 83: 497-504.

Karabiber, E.B. and Yılmaz, E., 2017. Extraction and characterization of lemon, orange and grapefruit seeds press cake proteins. Quality Assurance and Safety of Crops & Foods 9: 357-367.

Khattab, R.Y. and Arntfield, S.D., 2009. Functional properties of raw and processed canola meal. LWT – Food Science and Technology 42: 1119-1124.

King, T., Dykes, G. and Kristianti, R., 2008. Comparative evaluation of methods commonly used to determine antimicrobial susceptibility to plant extracts and phenolic compounds. Journal of AOAC International 91: 1423-1429.

Malacrida, C.R., Kimura, M. and Neuza Jorge, N., 2012. Phytochemicals and antioxidant activity of citrus seed oils. Food Science and Technology Research 18: 399-404.

Matharu, A.S., De Melo, E.D. and Houghton, J.A., 2016. Oppurtunity for high value-added chemicals from food supply chain wastes. Bioresource Technology 215: 123-130.

Minitab., 2010. Statistical software. Minitab Inc., State College, PA, USA.

Mirabella, N., Castellani, V. and Sala, S., 2014. Current options for the valorization of food manufacturing waste: a review. Journal of Cleaner Production 65: 28-41.

Moure, A., Sineiro, J., Dominguez, H. and Parajo, J.C., 2006. Functionality of oilseed protein products: a review. Food Research International 39: 945-963.

Nile, S.H. and Park, S.W., 2014. Bioactive components and health-promoting properties of yuzu (*Citrus ichangensis* × *C. reticulate*). Food Reviews International 30: 155-167.

Oliveria, T.L.C., Soars, R.A., Ramos, E.M., Cardoso, M.G., Alves, E. and Piccoli, R.H., 2011. Antimicrobial activity of *Satureja montana* L. essential oil against *Clostridium perfringens* type A inoculated in mortadella type sausages formulated with different levels of sodium nitrate. International Journal of Food Microbiology 144: 546-555.

Othman, M., San Loh, H., Wiart, C., Khoo, T.J., Lim, K.H. and Ting, K.N., 2011. Optimal methods for evaluating antimicrobial activities from plant extracts. Journal of Microbiological Methods 84: 161-166.

Przybylski, R., Firdaous, L., Châtaigné, G., Dhulster, P. and Nedjar, N., 2016. Production of an antimicrobial peptide derived from slaughterhouse byproduct and its potential application on meat as preservative. Food Chemistry 211: 306-313.

Rai, M., Pandit, R., Gaikwad, S. and Kövics, G., 2016. Antimicrobial peptides as natural bio-preservative to enhance the shelf-life of food. Journal of Food Science and Technology 53: 3381-3394.

Sharma, G.M., Su, M., Joshi, A.U., Roux, K.H. and Sathe, S.K., 2010. Functional properties of select edible oilseed proteins. Journal of Agricultural and Food Chemistry 58: 5457-5464.

Tan, J.B.L. and Lim, Y.Y., 2015. Critical analysis of current methods for assessing the *in vitro* antioxidant and antibacterial activity of plant extracts. Food Chemistry 172: 814-822.

Yao, X., Zhu, X., Pan, S., Fang, Y., Jiang, F., Philips, G.O. and Xu, X., 2012. Antimicrobial activity of nobiletin and tangeretin against *Pseudomonas*. Food Chemistry 132: 1883-1890.

Yılmaz, E., 2017. Production and utilization of the value-added products from citrus seeds. TUBİTAK Project No: COST 114O876. Ankara, Turkey.

Yılmaz, E. and Aydeniz Güneşer, B., 2017. Cold pressed versus solvent extracted lemon (*Citrus limon* L.) seed oils: yield and properties. Journal of Food Science and Technology 54: 1891-1900.

Yılmaz, E. and Dündar Emir, D., 2016. Extraction and functional properties of proteins from pre-roasted and enzyme treated poppyseed (*Papaver somniferum* L.) press cakes. Journal of Oleo Science 65: 319-329.

Yılmaz, E. and Huriyet, Z., 2017. Physico-chemical and functional properties of extracted capia pepperseed (*Capsicum annuum* L.) proteins. Waste and Biomass Valorization 8: 871-881.