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Screening for Bioactive Compound Rich Pomegranate Peel Extracts and Their Antimicrobial Activities

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Abstract

In this work, seven different extracts from pomegranate (*Punica granatum* L., cv. Hicaz nar) peel were prepared by using different solvents (ethanol, methanol, either alone or in combination with acid, acetone and water). The phenolics (punicalagins and ellagic acid), organic acids (citric acid and malic acid) and sugars of pomegranate peel extracts were determined. The highest amounts of punicalagins and ellagic acid were detected by ethanol-acid extract as 13.86% and 17.19% (w/v) respectively, whereas the lowest levels were obtained with acetone and water extracts. Moreover, the methanol-acid (3.19% malic acid) and ethanol-acid (1.13% citric acid) extracts contained the highest levels of organic acids. The antimicrobial activities of extracts were investigated by agar well diffusion method. Methanol-acid and ethanol-acid extracts exhibited the highest antimicrobial effects on all tested microorganisms, giving inhibition zones ranging in size

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from 17 to 36 mm. Although similar antimicrobial activities were observed by ethanol, methanol, and acetone extracts (up to 24 mm), the lowest antimicrobial activities were attained by water extract (0-15 mm). All extracts were generally more effective against Gram-positive bacteria: *Enterococcus facealis*, *Bacillus subtilis*, *Bacillus cereus* than Gram-negative ones: *Escherichia coli* and *Enterobacter aerogenes*. It was shown that extracts from pomegranate peels represent a good source of bioactive compounds.

Key words: antimicrobial activity, bioactive compounds, biorecovery, extraction, pomegranate peel.

1. Introduction

Punica granatum L. is a tree belonging to Punicaceae family grown in Iran, Afghanistan, Turkey, USA and Far East countries. The world pomegranate production is estimated as 1.5 million tonnes, annually (1). Pomegranate juice is popular because it includes strong antibacterial, anticancer, antioxidant, antiallergic and anti-inflammatory compounds (2, 3). Recent reports also showed its potential use in the treatments of cardiovascular diseases and diabetes (4).

The pomegranate peel, amounts to more than half of the weight of the pomegranate, is a fruit processing-waste but it could be used as a source of antioxidants, phenols, flavonoids and organic acids. Extracts of pomegranate peels including rind, husk, pericarp and membranes are rich in polyphenols (ellagitannin and punicalagins), gallic acid, flavones, flavanones and anthocyanidins (5-7). These phenolic compounds and organic acids can be extracted by using different solvents. However, the efficiency of extraction is mostly depended on the type of solvent, time and temperature. Therefore, it is important to

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determine the best solvent and extraction method to obtain the best bioactive compound rich extracts. Many extraction methods are reported for extraction of phenolic compounds from pomegranate by using ethanol, methanol, acetone, ethyl acetate and water (8, 9). The presence of punicalagins, punicalin, ellagic acid and gallic acid in pomegranate peel extracts could determine their antimicrobial activities on microorganisms (10, 11). Pomegranate peel extracts prepared by using ethanol, methanol or their mixtures with water were shown to be effective on *Staphylococcus aureus*, *Enterobacter aerogenes*, *Klebsiella pneumoniae* and *Salmonella typhi* strains (10). Abdollahzadeh et al. (12) reported that the methanolic extract of pomegranate peel exhibited antimicrobial activities against oral pathogens including *S. aureus* and *Staphylococcus epidemidis* strains. Moreover, pomegranate peel extracts containing polyphenols, tannins and other secondary metabolites showed effective antibacterial activity against shiga toxin producing *E. coli* (STEC) (13). Extracts from other parts of pomegranate (such as rinds, membranes and seeds) also had antimicrobial effects on *S. aureus* and *Bacillus megaterium* (14). To the best of our knowledge there is no report for evaluation of antimicrobial activities of different pomegranate peel extracts for Turkish Hicaz variety. Therefore, in the present study the most efficient extraction methods were investigated to obtain potential natural antimicrobial compounds from Turkish Hicaz pomegranate peel that can be used as a source of safe preservatives in food industry. For this, different solvents (ethanol, methanol, either alone or in combination with acid, acetone and water) were used for extraction of phenolics and organic acids from pomegranate fruit peels obtained as a waste from fruit juice processing industry. Extracts were then screened for their antimicrobial activities against some important microorganisms.

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2. Materials and methods

2.1. Preparation of pomegranate waste

Pomegranate (*Punica granatum* L., cv. Hicaznar) peels were obtained from fruit processing industry in Turkey and stored at 4°C. The peels were lyophilized in a freeze dryer (Virtis Ultra Wizard 2.0, Warminster, USA) by freezing at -30°C for 5 h and drying under 10 Pa pressure at 20°C for 24 h. The freeze-dried peels were grounded to powder (No. 48 sieve) using a grinder and stored at 4°C until use.

2.2. Chemicals

Ellagic acid, punicalagins (A and B forms) and gallic acid standards were purchased from Fluka (Buchs, Switzerland). Malic acid and citric acid standards were obtained from Merck (Darmstadt, Germany). Ethanol (99%, v/v), methanol (99%, v/v), acetone (99%, v/v), and hydrochloric acid (HCl; %37, v/v) were used as solvents (Merck, Darmstadt, Germany).

2.3. Preparation of pomegranate peel extracts (PPEs)

5 g of pomegranate peel powder was mixed with 100 mL of different solvents and incubated at 50°C for 30 min or 2 hours in an ultrasonic water bath (150 W, 40 KHz) (E1: ethanol for 30 min, E2: ethanol for 120 min, EA: ethanol with HCl, M: methanol, MA: methanol with HCl, A: acetone and W: distilled water, for 30 min, Table 1). The mixtures were centrifuged at 4°C at 4000 rpm for 15 min as described by Zhang et al. (15) and Türkyılmaz et al. (14) with slight modifications (Schema 1). The supernatants were filtered with Whatman no. 1 paper and then evaporated in a rotary evaporator (Heidolph,

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Schwabach, Germany) at 50°C under a vacuum of 400 mb. The extracts were stored at 4°C until used for further studies.

2.4. Qualitative analysis of phenolics and sugars by TLC

Sugar and phenolic contents of PPEs were screened by TLC. About 1 µL of each extract and standards were applied on TLC plate. For screening of phenolics, the plate was run into the ethyl acetate: glacial acetic acid: formic acid: distilled water (100:11:11:5, v/v; adapted from Kumar et al. (16)). The plates were then sprayed with 5% (w/v) ferric chloride reagent (13). For sugars, the plate was run into the acetonitrile: water (85:15, v/v) solvent system and then stained with α-naphthol (0.5%, w/v) dissolved in ethanol solution acidified with H_2SO_4 (5%, v/v), followed by heating at 110°C for 10 min (17). The colors of the spots were identified. An individual Rf value for each spot was measured and compared with standard reference sugars and phenolic compounds run in the same respective solvent systems.

2.5. Quantitative analysis of phenolics, sugars and organic acids by HPLC

Detection and quantification of phenolics, organic acids and sugars were carried out by HPLC system. Each sample was centrifuged and then filtered through 0.22 µm membrane filter before HPLC analysis.

Ellagic acid and punicalagin separations were achieved at 30°C on a C18 column (150 mm × 4.6 mm, 5 µm, GL Sciences Inc.). HPLC analysis was performed using Schimadzu Corp., Columbia, MD, USA Class VP, 20 AD series equipped with Photodiode-array detector (PDA) and an autosampler. The mobile phase consisted of formic acid (1%) and acetonitrile with gradient mode elution (0-18 min, 15% v/v acetonitrile, 20 min 65% (v/v)

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acetonitrile, 25 min 5% (v/v) acetonitrile, and 30 min 5%, v/v acetonitrile) at a flow rate of 1 mL/min. The injection volume was 10 µL. The quantitation wavelength was set at 255 nm (18). 1000 µg/mL of ellagic acid, gallic acid and punicalagins were prepared by dissolving in 5 mL of HPLC grade methanol for standards. The solutions were stored at -20°C. The calibration curves were established from the standards of punicalagins and ellagic acid at concentrations between 0.005-0.02% and 0.25-1%, respectively.

Sugars (glucose and sucrose) were determined by using NH_2 column (250 mm × 4.6 mm, 5 µm, GL Sciences Inc.). The column temperature was 25°C. The eluted samples were detected by RID detector. The mobile phase consisted of acetonitrile (60%) and ultra-pure water (40%). Flow rate was 1 mL/min and injection volume was 20 µL (19). The standards of glucose and sucrose (5, 10, 20, 40 and 50 mg/mL) were used for calibration curves.

Organic acids (citric and malic acids) were determined with a UV-VIS detector (Schimadzu SPD-10 AV VP, Schimadzu Corp., Columbia, MD, USA). All organic acid analyses were carried out with a C18 column (Kromasil, 5µm, 4.6x156 mm). The mobile phase was prepared by using 0.005 N H_2SO_4. Injection volume was 50 µL and the column temperature was 25°C. Flow rate was 0.3 mL/min. The data were recorded at 210 nm (20). Citric and malic acid standards (0.1-2%, w/v) were used to prepare calibration curves.

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2.6. Antimicrobial analysis

The antimicrobial efficacies of PPEs were evaluated against *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (ATCC 11778), *Pseudomonas aureginosa* (ATCC 27853), *Streptococcus uberis*

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(ATCC 700407), *Enterobacter aerogenes* (ATCC 13048) and *Candida albicans* (ATCC 10231) by using agar well diffusion method (Schema 1). Each microbial culture was incubated in MHB (Mueller Hinton Broth, Merck, Germany) for overnight. The OD_{600} values of cultures were adjusted to 0.1 and then 100 µL of each microbial culture was spread on petri dishes containing 17 mL of MHA (Mueller Hinton Agar, Merck, Germany). The media were punched with 7 mm diameter wells and these wells were filled with 40 µL of each extract. The plates were then incubated for 24 hours at 37°C. After incubation, inhibition zones for microorganisms for each extract were measured in millimeters. Each extract was tested three times.

2.7. pH measurements

The pH levels of extracts were measured by a pH meter (Hanna Instruments HI 2211 pH/ORP Meter, Bedfordshire, UK).

2.8. Statistical analysis

Values are expressed as mean ± SD. Data were analyzed by student t-test. The level of statistical significance was accepted as $p < 0.05$.

3. Results and Discussion

3.1. Phenolics

Punicalagins (punicalagin A and B) and ellagic acid were identified in all extracts by TLC. However, gallic acid was not determined in all extracts according to both TLC and HPLC analyses.

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The highest levels of punicalagins and ellagic acid were detected in the EA extract as 13.86% and 17.19% respectively, whereas the lowest levels were obtained with W extract ($p<0.05$) (Figure 1) by HPLC analyses. This is consistent with previous results which reported that the solvents could affect the phenolic contents of plant extracts (21, 22). The difference in phenolic contents of extracts depends on the solvent polarity that affects solubility of selected groups found in antimicrobial bioactive compounds (8). Water, ethanol and methanol are polar solvents while acetone is an intermediate polar solvent that can solve both polar compounds including phenolics and inpolar compounds. In addition, the extracts obtained by mixture of solvents (combination of acid and ethanol or methanol) could be more radical scavenger than the pure solvents (23) by changing polarity that affects antimicrobial activities. It was also observed that ethanol alone or ethanol-acid combination could be more effective than other solvents to obtain high levels of phenolic compounds. The antimicrobial activities of phenolics (ellagic acid and punicalagins) were shown previously (24, 25). These polyphenols found in PPEs can work as antimicrobial agents by forming complex with bacterial cell to cause death or by inhibiting protein activities. The position and the number of hydroxyl groups on the phenolic components may also increase this inhibitory effect on the microorganisms (26, 27).

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3.2. Sugars

The highest total sugar contents (glucose and sucrose) were obtained with MA (5.95%), E2 (4.98%) and EA (4.93%) extracts significantly ($p<0.05$) (Table 2). MA and EA extracts exhibited the highest antimicrobial activities (Figure 2). Sugars might help antimicrobial efficacies of these extracts due to the osmotic effects of carbohydrates on

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microorganisms. However, there was no clear consistency for the sugar contents and antimicrobial activities of other extracts.

3.3. Organic acids

The organic acid contents of PPEs determined by HPLC were presented in Table 2. MA extract had the highest malic acid (3.19%) content whereas the highest citric acid level (1.13%) was detected with EA extract ($p<0.05$) (Table 2). Organic acids could affect integrity of the cell membrane, activities of enzymes or biosynthesis of macromolecules and cellular homeostasis (28, 29).

3.4. pH values

The lowest pH level (0.26) was measured with MA extract while the highest pH (3.55) value was determined with W extract as expected (Table 2). The highest inhibition zones for all tested microorganisms were determined with MA and EA extracts with the low level of pH (almost zero) that might also affect microbial growth.

3.5. Antimicrobial activities of PPEs

The antimicrobial effects of different extracts obtained by using different solvents were evaluated against food associated microorganisms. The antimicrobial activities were assessed by the presence or absence of inhibition zones and zone diameters. The results were given in Figure 2. The data of the study showed that MA and EA extracts of pomegranate peels had the highest antibacterial activities against all tested microorganisms ($p<0.05$) (Figure 2b). The inhibition zone diameters were found to be 17-36 mm with MA extract (including the diameter of the wells). EA extract, the second

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most efficient extract, resulted in 17-32 mm inhibition zones (Figure 2a). The extracts E1, E2, M and A showed similar antimicrobial effects (Figure 2). Increasing extraction time from 30 min to 120 mins for E extract did not enhance ($p>0.05$) antimicrobial efficacies almost in most cases. The lowest inhibitions for all microorganisms were detected with W extract in the range of 0-15 mm ($p<0.05$) (Figure 2a).

In general, the antimicrobial effects of extracts could be attributed to their phenolics (30) and organic acid contents (31, 32). Therefore, according to results obtained in this work, the highest levels of organic acids (MA and EA) and phenolics (EA) could contribute antimicrobial activities of these extracts.

In previous works, Gram positive bacteria were more sensitive to plant extracts than Gram negative ones (33, 34) consistent with the results obtained from this study. It was found that *B.subtilis*, *B. cereus*, *E. faecalis* and *S. uberis* (Gram positive) strains were more sensitive than *E. coli* and *E. aerogenes* (Gram negative) strains (Figure 2). The cell walls of Gram positive bacteria were shown as more sensitive to antimicrobial compounds compared with Gram negative bacteria (35, 36) due to the lipopolysaccharide layer and periplasmic space present in Gram negative bacterial cell wall. However, Hama et al. (37) found that pomegranate juice had antibacterial activity on both Gram positive (*S. aureus*) and Gram negative bacteria including *E. coli* and *Pseudomonas aeruginosa.* The multilayered peptidoglycan was shown as the main factor for antimicrobial resistance (37). According to results obtained in this work, the antibacterial activities of extracts were similar for *P.aureginosa* (Gram negative) and *S. uberis* (Gram positive) strains. The reason for this is not known, but might be related with some spesific properties of these microorganisms and/or extraction methods. Therefore no clear correlation was found between the cell wall structures and the antibacterial activities of the extracts. Previously,

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methanol extracts of peel were shown the greatest activities on different bacteria depending on the pomegranate variety tested (38,39). It was indicated that ethanol extracts of pomegranate had hydrolysable tannins including punicalagins, ellagic acid and gallic acid (30). In this work, punicalagins and ellagic acids were found in all extracts but gallic acid was not determined that may be linked to the variety of the pomegranate or extraction techniques.

In the present study, the inhibition zones for *C. albicans* were about 8-25 mm diameters. The highest inhibition zone was obtained with MA extract (25 mm). It was reported that the methanolic PPEs inhibited *C. albicans* with the inhibition zones of 6-6.5 mm (12) which were much lower than found in this study. Punicalagins were shown as antifungal components of ethanol extract of pomegranate peels. *C. albicans* treated with punicalagins extracted from pomegranate peels exhibited morphological alterations in cell structure and abnormal budding (40). The aqueous extract of pomegranate peels also showed inhibitory activity on *C. albicans* (41) which was consistent with the results obtained with this study.

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4. Conclusion

In the present work, PPEs were prepared from Turkish Hicaz pomegranate variety by using ethanol, methanol, or their combinations with acid, acetone and water. All extracts were found to have antimicrobial effects on different bacteria and a fungus. Amongst the evaluated extracts, MA and EA exhibited the largest inhibition zones for all tested microorganisms. It was shown that high amounts of organic acids (for MA and EA) and phenolics (for EA) could be responsible for the antibacterial activities. Further studies are required to isolate other potential bioactive compounds of the PPEs and to identify their

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molecular mechanisms of actions. In addition, potential PPEs from different varieties of pomegranate should also be investigated to obtain the most valuable bioactive compounds that could be used as safe food preservatives in food industry.

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Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Tables

Table 1. Extraction methods (E1: ethanol for 30 min, E2: ethanol for 2 h, EA: ethanol-acid, MA: methanol-acid, A: acetone and W: water) for different PPEs used in this study.

Extract	Solvents	Solvent ratio (%, v/v)	Extraction time (min)
E1	EtOH:Dw	60:40	30
E2	EtOH:Dw	60:40	120
EA	EtOH:HCl:Dw	60:5:35	30
M	MeOH:Dw	80:20	30
MA	MeOH:HCl:Dw	80:5:15	30
A	Aseton:Dw	70:30	30
W	Distilled water (Dw)	100	30

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Table 2. The organic acids (malic and citric,%), total sugar (glucose and sucrose,%) contents and pH levels of PPEs (E1: ethanol for 30 min, E2: ethanol for 2 h, EA: ethanol-acid, MA: methanol-acid, A: acetone and W: water). Data are given as mean values ± standard deviations (n=3).

Extracts	pH	Malic acid (%)	Citric acid (%)	Total Sugar (%)
E1	3.10 ± 0.05	0.01 ± 0.00	0.44 ± 0.03	3.72 ± 0.24
E2	3.10 ± 0.04	0.03 ± 0.01	0.62 ± 0.07	4.98 ± 0.21
EA	0.30 ± 0.02	1.59 ± 0.12	1.13 ± 0.14	4.93 ± 0.14
M	3.40 ± 0.04	0.00 ± 0.00	0.69 ± 0.03	4.76 ± 0.29
MA	0.26 ± 0.02	3.19 ± 0.14	0.97 ± 0.04	5.95 ± 0.07
A	3.50 ± 0.05	0.37 ± 0.04	0.65 ± 0.13	4.00 ± 0.13
W	3.55 ± 0.07	0.04 ± 0.01	0.10 ± 0.01	1.69 ± 0.25

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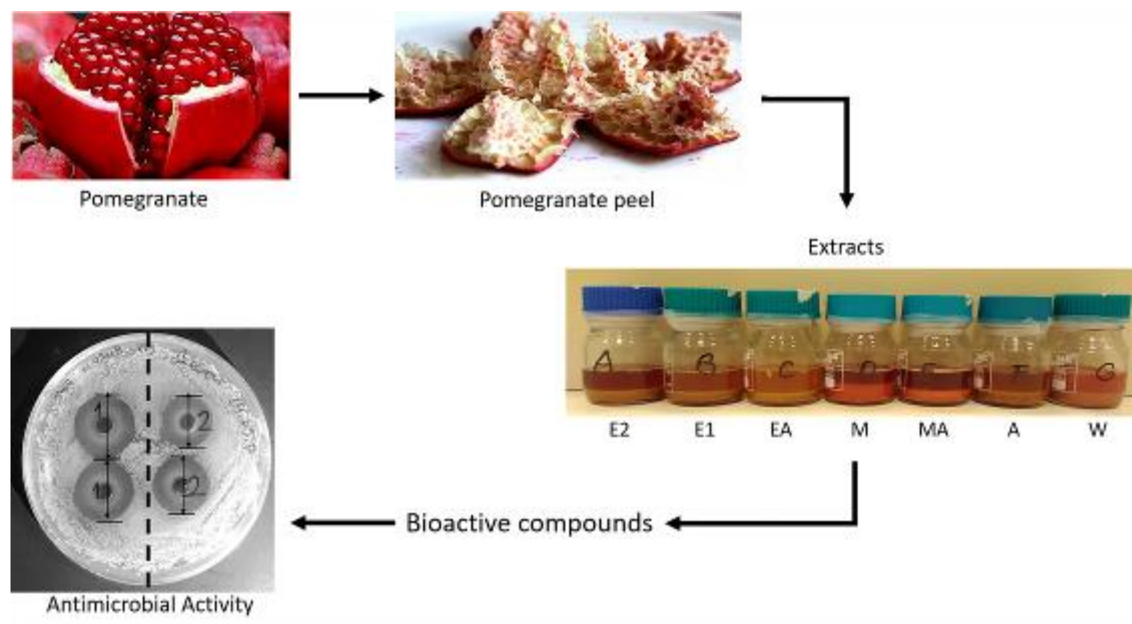
Figures



Schema 1. Schematic representation for preparation of PPEs and determination of their antimicrobial activities.

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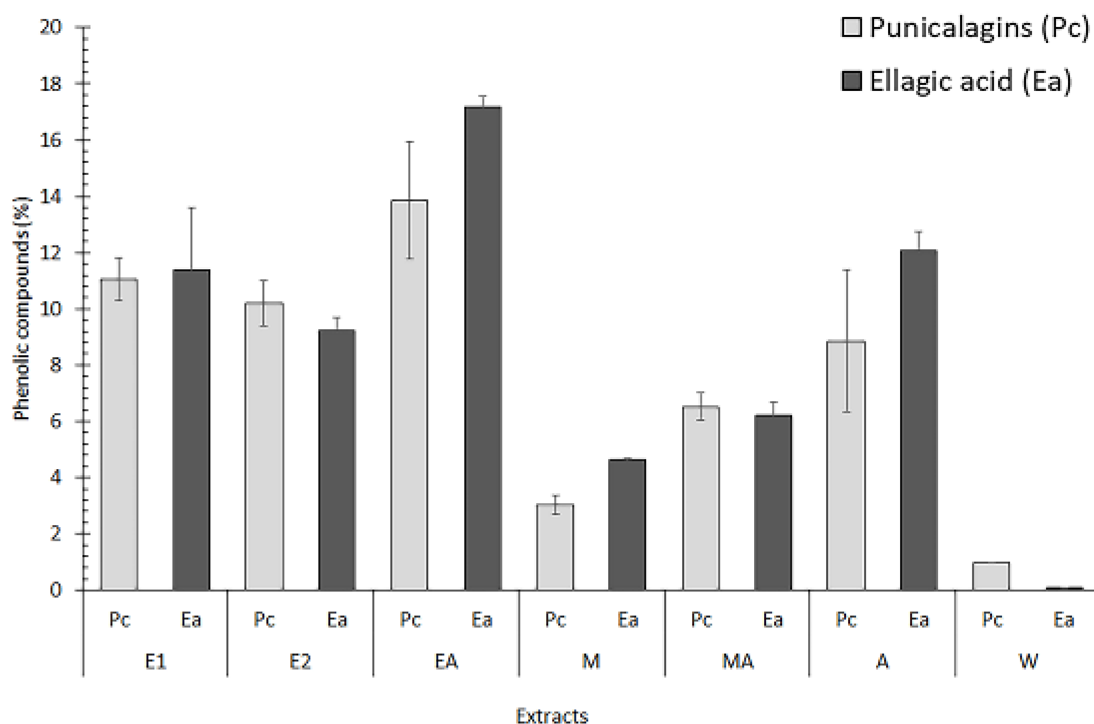

Figure 1. The phenolic contents (punicalagins and ellagic acid, %) of PPEs (E1: ethanol for 30 min, E2: ethanol for 2 h, M: methanol, EA: ethanol-acid and MA: methanol-acid, A: acetone and W: water). Values are the averages of three determinations; error bars represent standard deviations.

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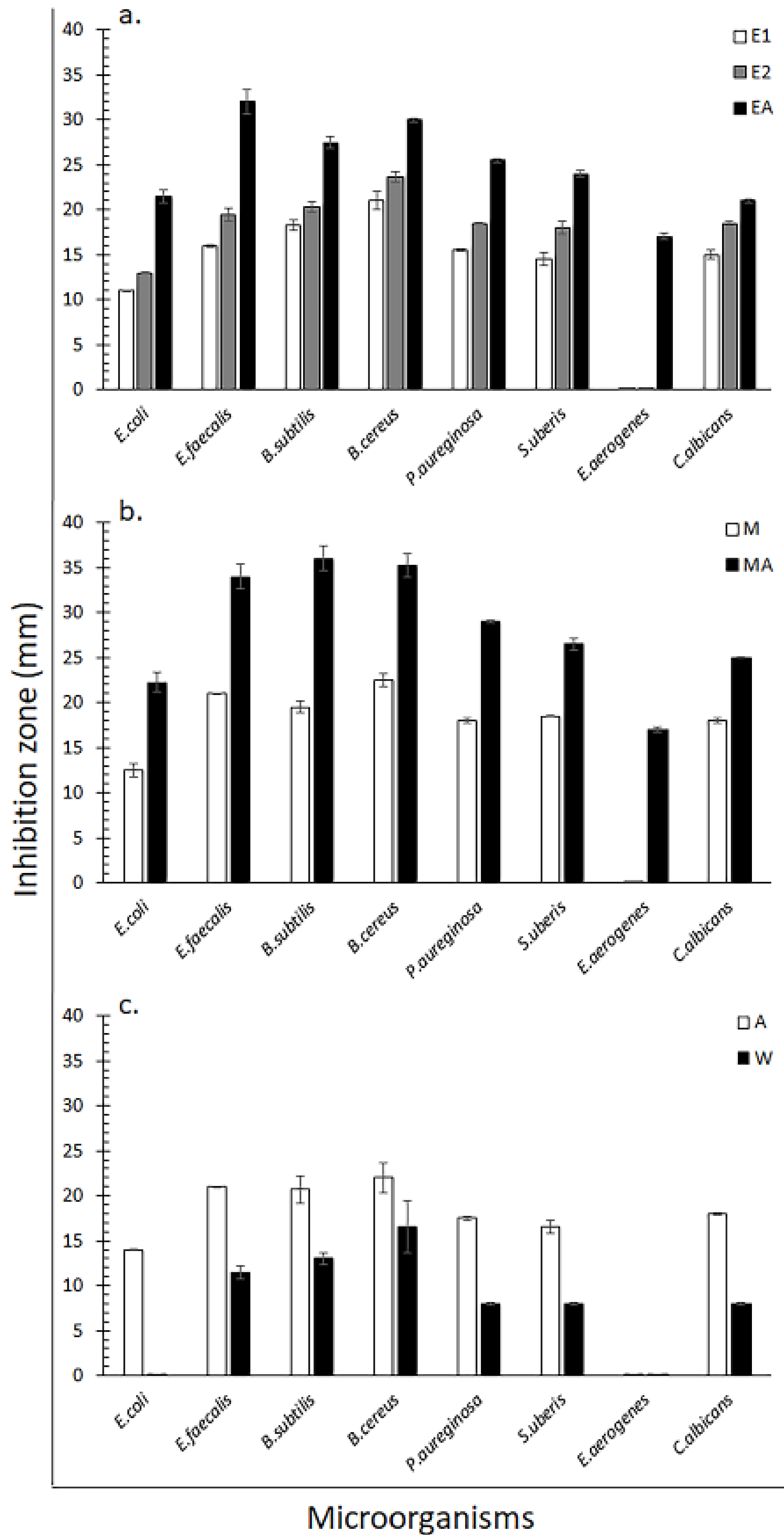

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Figure 2. Inhibition zones (mm) exhibited by different PPEs (a, E1: ethanol for 30 min, E2: ethanol for 2 h, EA: ethanol-acid; b, M: methanol, and MA: methanol-acid, c; A: acetone and W: water) against several microorganisms. Values are the averages of three determinations; error bars represent standard deviations.

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The Authors

Merve Balaban received her MSc from the Department of Molecular Biology and Genetics at Gebze Technical University under the supervision of Prof. Meltem Yesilcimen Akbas. During her master thesis she worked on extraction strategies to recover bioactive *compounds* from fruit proccessing wastes and identify their antimicrobial and antibiofilm effects on microorganisms.

Cansel Koç received her MSc from the Department of Molecular Biology and Genetics at Gebze Technical University under the supervision of Prof. Meltem Yesilcimen Akbas. During her master thesis she worked on *bioactive compounds* and antimicrobial and antibiofilm potential for polyphenol-*rich* fruit processing waste *extracts on food related pathogenic bacteria.*

Taner Sar is a Guest Postdoctoral Researcher at Högskolan i Borås, Sweden. He received his Ph.D. at Gebze Technical University, Turkey in the group of Prof. Akbas. His current research interests are recovery of nutrients from food processing wastes, production of protein-rich microbial biomass and bioactive compounds as antimicrobial and antibiofilm agents. His research also focuses on enhancement of bioethanol production from industrial wastes by using *Vitreoscilla* Hemoglobin gene.

Meltem Yesilcimen Akbas is currently working as a Professor of Molecular Biology and Genetics at Gebze Technical University, Turkey. Her research interests span many aspects of industrial microbiology and microbial biotechnology including engineering of bacteria using bacterial hemoglobin to improve growth and productivity and using food processing waste extracts for antimicrobial and antibiofilm agents.

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