Phenolic Compounds and Antimicrobial Properties of Pomegranate (*Punica granatum*) Peel Extracts

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Abstract**—**In recent years, tendency to use of natural antimicrobial agents in food industry has increased. Pomegranate peels containing phenolic compounds and anti-microbial agents, are counted as valuable source for extraction of these compounds. In this study, the extraction of pomegranate peel extract was carried out at different ethanol/water ratios (40:60, 60:40, and 80:20), temperatures (25, 40, and 55 ˚C), and time durations (20, 24, and 28 h). The extraction yield, phenolic compounds, flavonoids, and anthocyanins were measured. Antimicrobial activity of pomegranate peel extracts were determined against some food-borne microorganisms such as *Salmonella enteritidis*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Aspergillus niger,* and *Saccharomyces cerevisiae* by agar diffusion and MIC methods. Results showed that at ethanol/water ratio 60:40, 25 ˚C and 24 h maximum amount of phenolic compounds (349.518 mg gallic acid/g dried extract), flavonoids (250.124 mg rutin/g dried extract), anthocyanins (252.047 mg cyanidin3glucoside/100 g dried extract), and the strongest antimicrobial activity were obtained. All extracts' antimicrobial activities were demonstrated against every tested microorganisms. *Staphylococcus aureus* showed the highest sensitivity among the tested microorganisms.

Keywords**—**Antimicrobial agents, phenolic compounds, pomegranate peel, solvent extraction.

I. Introduction

THE use of some plant extracts as an alternative to synthetic antimicrobials is extensively growing in the food industry [1]. Several recent studies have demonstrated that the peels of pomegranate (*Punica granatum*) have antimicrobial activity against food-borne microorganisms [2]-[7]. The edible parts of pomegranate are utilized for fresh juice and caned beverage [8]. The 40% of pomegranate fruit is peel, and this valuable part of fruit is totally wasted [9]. Pomegranate peel has high amount of total phenolic compounds (TPCs) compared to the other parts such as aril, seed, and leaves [10].
In this study, we used several ratios of water/ethanol as solvent at different temperature and time for extraction of pomegranate peel extracts (PPEs). Amount of TPCs, total flavonoids (TFs), and anthocyanins were measured. Also, PPEs were used against some important food microorganisms including *Salmonella enteritidis, Escherichia coli, Listeria monocytogenes, Staphylococcus aureus, Aspergillus niger*, and *Saccharomyces cerevisiae.*

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II. Materials and Methods

A. Materials

Pomegranate fruit of "Pishras" cultivar was obtained from the local market, Iran (September 2014). Microorganisms strains including *S. enteritidis* (PTCC 1709)*, E. coli* (PTCC 1329)*, L. monocytogenes* (PTCC 1997)*, S. aureus* (PTCC: 1764), *A. niger* (PTCC 5012) and *S. cerevisiae* (PTCC 5177) were bought from the Iranian Research Organization for Science and Technology (IROST).

B. Preparation of Pomegranate Peel

Peels were manually separated and dried at room temperature for eight days. The total moisture content was assessed by maintaining the samples in an oven (Fan Azma Gostar Company, Iran) at 105 °C at constant weight. The moisture content of dried samples was 9.6±0.3% (dry base).

C. Solvent Extraction

5 g of the powdered pomegranate peels was dissolved in 50 ml of solvent. Extraction was carried out at 25, 40, and 55 °C for 20, 24, and 28h, respectively. After filtering, the extracts were centrifuged at 3500 rpm (universal centrifuge, Poya Electronic, Iran) for 10 min, and the supernatant was collected. The liquid extracts after filtering were dried by vacuum oven (Gallenkamp vacuum oven, United Kingdom) at 40 °C.

D. TPCs

The concentration of TPCs was measured by using the Folin-Ciocalteu assay [11]. Briefly, 3 ml of distilled water, 0.3 ml of extract, and 2 ml of aqueous Folin-Ciocalteu solution (100 ml water to 10 ml of Foline-Ciocalteu reagent) were mixed in a 10 ml volumetric flask. After 3 min, 2 ml of 7.5% (w/w) $NaHCO_3$ solution was added to the solution. The final volume of solution was adjusted to 10 ml by the addition of distilled water and was placed in the dark at room temperature for 1 h. Absorbance was measured at 760 nm by using a UV-vis spectrophotometer (Cecil CE 2040). The calibration curve for UV-vis spectrophotometer was acquired by using standard solution of gallic acid with known concentration varied in the range of 0.1 to 1.00 mg/ml. A linear equation with R^2 of 0.995 was established, and TPCs were expressed in milligrams of Gallic acid equivalents per gram of dried extract.

E. TFs

The concentration of TFs was measured by using the colorimetric method described by [12]. Briefly, 1 ml of extract was mixed with 4 ml distilled water and 0.3 ml of 5% (w/w) sodium nitrite solution. After 5 min, 0.3 ml of 10% (w/w)

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aluminum chloride was added to the solution followed by addition of 2 ml of 1 M sodium hydroxide after 6 min. The final volume of solution was increased to 10 ml, using distilled water. The UV spectrophotometer (Cecil CE 2040) was used at wavelength of 510 nm to measure the absorption of TFs. The calibration curve for this measurement was established from standard solution of Rutin (0.1 to 1 mg/ml). A linear equation with R^2 of 0.985 was established, and TFs were expressed in milligrams of Rutin equivalents per gram of dried extract.

F. Anthocyanins

Total anthocyanins content was determined by using pH differential method at pH 4.5 and pH 1.0 [13]. Briefly, 0.4 ml of extract solution mixed with 3.6 ml of pH 1.0 and pH 4.5 buffers separately and was read at both 520 and 700 nm where $A= (A_{520}-A_{700})_{pH=1.0}-(A_{520}-A_{700})_{pH=4.5}$. A blank cell is filled with distilled water. The absorbance was measured within 20–50 min of preparation. Anthocyanin concentration, expressed in cyanidin-3-glucoside equivalents (molar extinction coefficient of 29.6 and molecular weight of 449.2) equivalents per 100 g of dried extract.

G. Preparation of Inoculums

The lyophilized bacteria and yeast were reactivated by inoculation in Muller-Hinton broth and incubation at 37 °C for 48h to obtain approximately 8 log cfu/ml. The fungal inoculums were prepared from 5 to 10 days old culture grown on potato dextrose agar medium. The growth was scraped aseptically, crushed and macerated thoroughly in sterile distilled water, and the fungal suspension was standardized spectrophotometrically to obtain 0.5 McFarland (8 log cfu/ml).

H. Agar Diffusion Method

0.1 ml of freshly grown culture (10^6 cfu/ml) was aseptically spread on to the surface of Mueller-Hinton agar. Paper disks (6 mm) were impregnated with 40 μl of the extracts (200 mg/ml) after filter sterilized (0.45 μm). Ethanol was used as a negative control. The plates were incubated at 37 °C for 24h (bacteria), 28 °C for 48h (yeast), and 28 °C for 72–96h (mold). Diameters (mm) of the inhibitory zones were recorded after incubation.

I. Minimal Inhibitory Concentration (MIC)

A micro-dilution method was used to measure quantitatively *in vitro*. 100 μl of the different concentration of extracts was added in each well of microdilution plate with 96 wells that contained 900 μl Muller-Hinton broth for bacteria and potato dextrose agar for fungi and 100 μl concentration of microorganism suspension. The negative control was prepared with the culture without the extract and microorganism suspension. The positive control was prepared with the culture broth and 100 μl inoculums of the microorganism suspension. Plates were incubated at 37 °C for 24h (bacteria), 28 °C for 48h (yeasts), and 28 °C for 72–96h (fungi). Microorganism growth was detected by optical density (OD) (ELISA reader, ELX800, Biotek Instruments). The MIC was defined as the lower concentration of the extract that inhibited the microorganism growth, after the incubation period.

J. Experimental Design

A series of experiments at different ethanol/water ratios (80:20, 60:40, 40:60), temperature (25, 40, and 55 °C) and extraction time (20, 24, and 28 h) were carried out. All experiments were performed in triplicate. Analysis of variance (ANOVA) was performed by SPSS for Windows, version 21.0.0. ANOVA was followed by the Duncan posthoc test, and the level of significance was set at $P < 0.05$.

III. Results and Discussion

Some preliminary experiments carried out during the first stage of our work allowed us to identify more important parameters of the solvent extraction of pomegranate peel (data not show).

A. Extraction Yield, TPCs, TFs and Anthocyanins

The results in Fig. 1 show that the presence of 40% ethanol in extraction solvent could significantly enhance extraction yield and the amount of TPCs, TFs, and anthocyanins. Ethanol accelerates the extraction of phenolic compounds, probably because it denatures cellular membrane [14]. The use of ethanol can increase the polarity of the solvent [15]. According to the findings of [16], 30-50% ethanol in solvent is helpful to improve the phenolic extraction.

As shown in Fig. 1, increasing the extraction temperature from 25 to 55 ˚C, in different time and different ethanol percentage in solvent, significantly decreased TPCs, TFs, and anthocyanins. In high temperature, some of phenolic compounds have been destroyed [17].

High amount of TPCs, TFs, and anthocyanins belong to the extracts that were obtained by ethanol: water ratios of 60: 40, at 25 ˚C for 24h extraction time.

B. Antibacterial and Antifungal Properties of PPEs

Antibacterial and antifungal properties of PPEs were evaluated by agar diffusion and the MIC methods. The results are presented in Table I and II. A comparison of the antimicrobial potency for all extracts showed that the most effective antimicrobial properties belong to the extract that was obtained by ethanol: water ratios of 60: 40, at 25 ˚C for 24h extraction time. This is in a good correlation with the TPCs, TFs, and anthocyanins content of the extracts (Fig. 1). Among the tested bacteria, *S. aureus* showed the highest sensitivity followed by *L. monocytogenes*. *E. coli* showed the lowest sensitivity. Gram positive bacteria are more sensitive to plant extracts [6]. The antibacterial activity of the extracts of pomegranate peels against *S. aureus* (13- 16 mm inhibition zone, Table I) and *L. monocytogenes* (10-13 mm inhibition zone, Table I) was comparable to [6]. They have reported that the inhibition zone of PPE against *S. aureus* and *L. monocytogenes* was 17 and 11-14 mm, respectively [6]. According to Table I, PPEs also have antifungal properties. Inhibition zone of PPE for *A. niger* and *S. cerevisiae* was 6.33-13.66 and 5.33-10.66 mm, respectively that is comparable with the others [18]. Quantitative evaluation of antimicrobial

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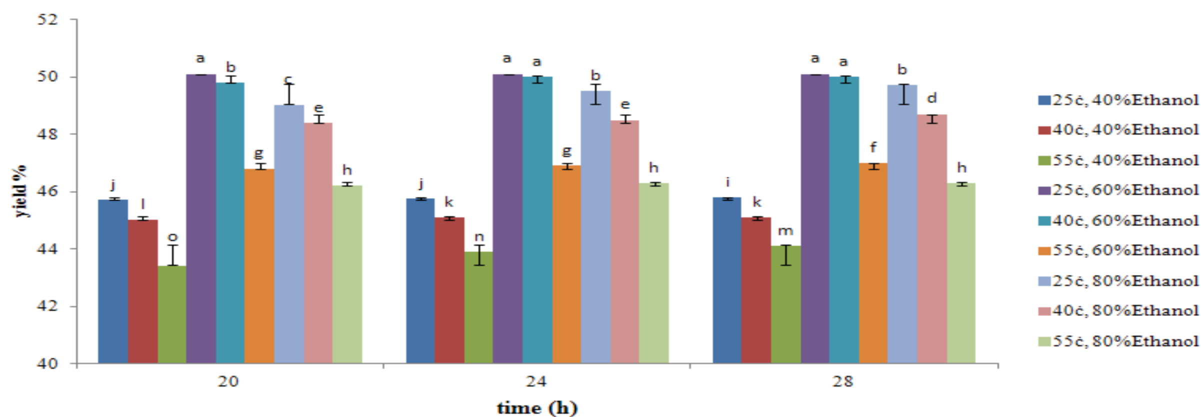
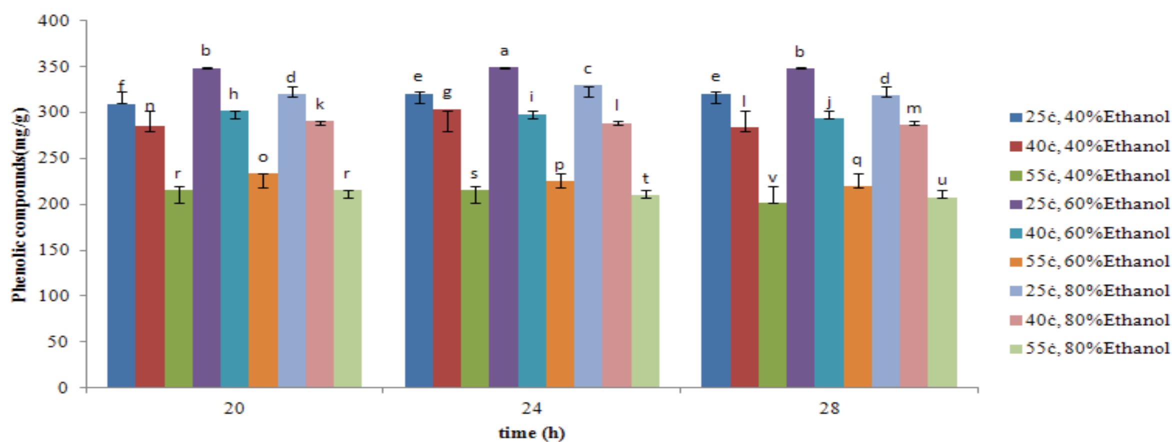
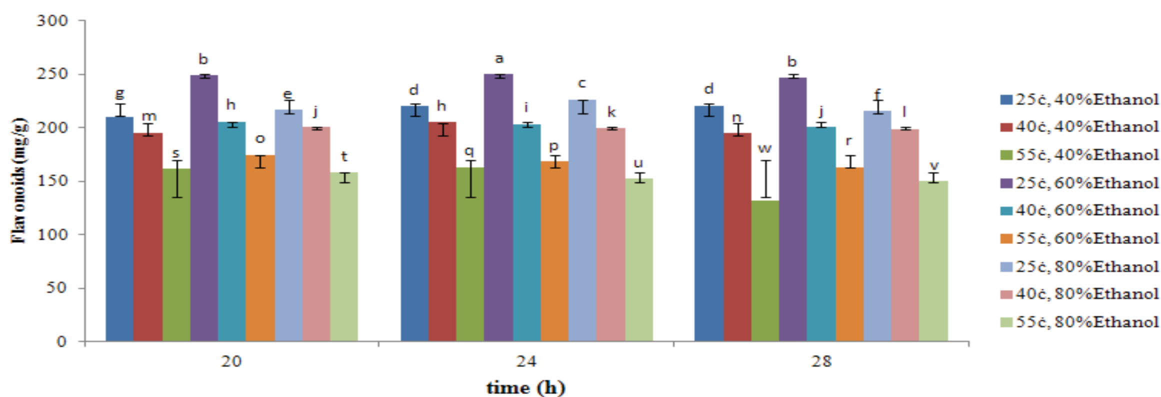
activity of all extracts was performed against test microorganisms by broth dilution techniques. The MIC, in mg/ml, of all extracts is presented in Table II. It appeared that the MIC range for tested microorganisms was 0.2 to 2000 mg/ml of PPEs. Plant materials can be classified as antimicrobial agents based on the MIC values of its extracts. Strong inhibitors have MIC value below 500 µg/ml, moderate inhibitors have MIC value between 600 and 1500 µg/ml, while weak inhibitors have MIC above 1600 µg/ml [19], [20]. It has been reported that the MIC value of PPE against *S. aureus* and *L. monocytogenes* were 2 and 0.5 mg/ml, respectively [21]. Gullon et al. [7] recorded MICs values of 50 mg/ml for 80% methanol PPE against *S. aureus*, *L. monocytogenes*, *E. coli*, and *Salmonella* spp.



(a)



(b)



(c)

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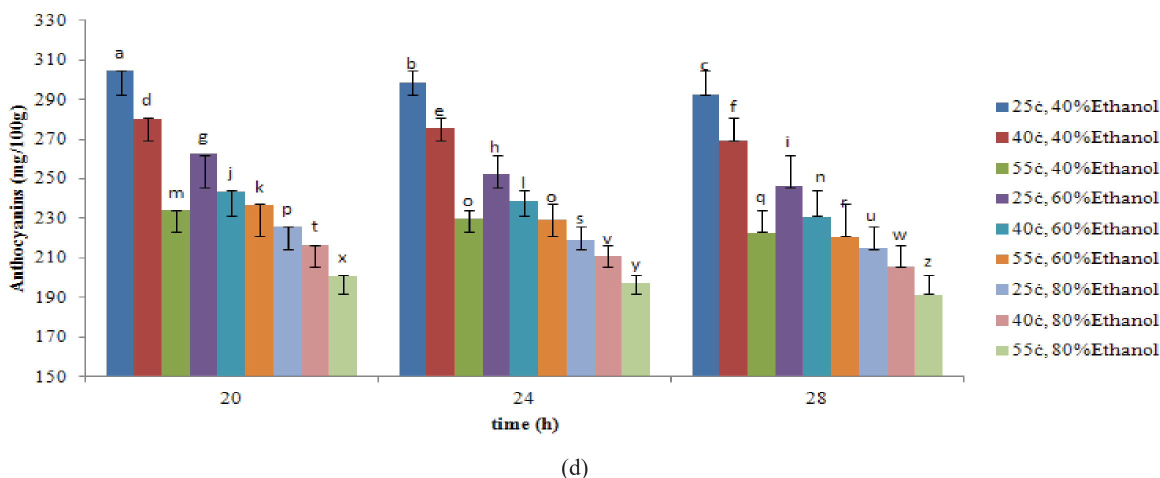

(d)

Fig. 1 Effect of different solvent extraction parameters on extraction yields (a), TPCs (b), TFs (c) and anthocayanins (d) of PPEs

The antimicrobial properties of PPEs are attributed to the polyphenolic compounds which include the gallic acid, ellagic acid, punicalagin A, and punicalagin B; three polyphenolics unique for pomegranate peel [22]. Flavonoids and anthocyanins also have antimicrobial properties. Silvan et al. [1] reported that antibacterial activity of phenolic compounds followed the sequence: phenolic acids > catechins and proanthocyanins > flavonols. According to the study of Hayrapetyan [6], antimicrobial activity of pomegranate red peel (high anthocyanin) was higher than pomegranate pink peel (low anthocyanin).

TABLE I
INHIBITION ZONE (MM) OF PPEs AGAINST TESTED MICROORGANISMS

treatments			Microorganisms (10^6 cfu/ml)					
Temp (˚C)	Time (h)	Ethanol:water	*S. enteritidis*	*E. coli*	*L. monocytogenes*	*S. aureus*	*A. niger*	*S. cerevisiae*
25	20	40:60	11.33±0.577	11.33±0.577	12.33±0.577	15.33±0.577	12.33±0.577	8.66±0.577
25	20	60:40	13.33±0.577	11.33±0.577	12.33±0.577	18.33±0.577	15.33±0.577	12±1
25	20	80:20	11.33±0.577	10.33±0.577	11.33±0.577	14±1	10.666±1.527	8.66±0.577
25	24	40:60	12.33±0.577	11.66±0.577	12.66 ±0.577	16.66±0.577	13.66±0.577	9.67±0.577
25	24	60:40	13.33±0.577	12.33±0.577	13.67±0.577	16.33±0.577	13.67±1.528	10.33±1.528
25	24	80:20	11.33±0.577	10.33±0.577	10.33±0.577	13.33±0.577	10.33±1.528	8.33±1.527
25	28	40:60	12±1	12±1	12.33±1.528	15.66±0.577	13.66±0.577	10.66±0.577
25	28	60:40	12.66±0.577	11.33±0.577	12.33±0.577	16.67±0.577	13.33±1.528	10.33±0.577
25	28	80:20	10.33±0.577	10±1	9.67±0.577	12.33±0.577	9.33±0.577	7.33±0577
40	20	40:60	8.66±1.528	9.33±0.577	11.66±0.577	13.67±1.528	11.33±0.577	8.33±0.577
40	20	60:40	10.33±0.577	11.66±0.577	11.33±0.577	13.33±0.577	9.67±2.08	8.33±1.528
40	20	80:20	9.33±0.577	10.33±0.577	9.33±0.577	11.33±0.577	8.33±1.527	6.33±0.577
40	24	40:60	11±1	9.66±0.577	11.66±0.577	14.33±0.577	13±1	8.66±1.528
40	24	60:40	10.66±0.577	10.33±0.577	9.33±0.577	11.66±1.528	9.33±1.528	7.66±0.577
40	24	80:20	9.33±0.577	9.66±0.577	8.33±0.577	10.33±0.577	8.66±2.08	5.33±0.577
40	28	40:60	9.66±0.577	10.33±0.577	9.66±1.528	14.66±0.577	10.66±1.527	8.33±1.528
40	28	60:40	9.33±0.577	10.66±0.577	9.33±0.577	12.33±0.577	8.33±1.527	8.33±1.528
40	28	80:20	7.66±1.527	9.33±0.577	8.33±0.577	10.33±0.577	8.33±0.577	5.33±0.577
55	20	40:60	8.33±0.577	9.66±0.577	9±1	11.66±1.528	9.33±0.577	6.66±0.577
55	20	60:40	8.33±0.577	9.66±0.577	9.33±0.577	11±1	6.66±0.577	6.33±0.577
55	20	80:20	7.33±0.577	6.33±0.577	8.33±0.577	10±1	7.33±1.527	6.33±1.528
55	24	40:60	7±1	9.33±0.577	10±1	10.66±1.527	8.66±2.08	6.33±0.577
55	24	60:40	8.33±0.577	10.33±0.577	8.33±0.577	11.33±0.577	7.33±1.528	6.33±0.577
55	24	80:20	7.33±0.577	8.33±0.577	8.66±0.577	9±1	7.33±1.528	6.66±0.577
55	28	40:60	7.33±0.577	9.33±0.577	9.33±0.577	12.33±0.577	10.33±1.528	7.33±0.577
55	28	60:40	7±1	9.33±0.577	9±1	10.33±0.577	7.33±1.528	7.33±0.577
55	28	80:20	7.33±0.577	6.66±1.528	8.33±0.577	9.33±0.577	7.33±1.527	6.33±0.577

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TABLE II
MIC (MG/ML) FOR DIFFERENT PPEs AGAINST TESTED MICROORGANISMS

Treatments			Microorganisms (10^6 cfu/ml)					
Temp (˚C)	Time (h)	Ethanol: Water	*S. enteritidis*	*E. coli*	*L. monocytogenes*	*S. aureus*	*A. niger*	*S. cerevisiae*
25	20	40:60	200	200	200	2	200	2000
25	20	60:40	2000	2000	2000	0.2	2000	2000
25	20	80:20	200	200	200	2	200	2000
25	24	40:60	200	200	200	2	200	2000
25	24	60:40	2000	2000	2000	0.2	2000	2000
25	24	80:20	200	200	200	2	200	2000
25	28	40:60	200	200	200	2	200	2000
25	28	60:40	20	20	20	0.2	20	200
25	28	80:20	200	200	200	2	200	2000
40	20	40:60	200	200	200	2	200	2000
40	20	60:40	200	200	200	2	200	2000
40	20	80:20	200	200	200	2	200	2000
40	24	40:60	200	200	200	2	200	2000
40	24	60:40	200	200	200	2	200	2000
40	24	80:20	200	200	200	2	200	2000
40	28	40:60	200	200	200	2	200	2000
40	28	60:40	200	200	200	2	200	2000
40	28	80:20	200	200	200	2	200	2000
55	20	40:60	200	200	200	2	200	2000
55	20	60:40	2000	2000	2000	20	2000	2000
55	20	80:20	2000	2000	2000	20	2000	2000
55	24	40:60	2000	2000	2000	20	2000	2000
55	24	60:40	2000	2000	2000	20	2000	2000
55	24	80:20	2000	2000	2000	20	2000	2000
55	28	40:60	2000	2000	2000	20	2000	2000
55	28	60:40	2000	2000	2000	20	2000	2000
55	28	80:20	2000	2000	2000	20	2000	2000

IV. CONCLUSION

Maximum amount of phenolic compounds, flavonoids, anthocyanins, and the strongest antimicrobial activity were obtained by ethanol: water ratios 60:40 at 25 °C for 24 h extraction time. All PPEs showed antimicrobial properties against tested microorganisms. *S. aureus* and *S. cerevisiae* showed the highest and lowest sensitivity, respectively.

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