



ANTIOXIDANT, ANTIMICROBIAL ACTIVITY OF POMEGRANATE PEEL WASTES EXTRACTED IN DIFFERENT SOLVENTS AND IDENTIFICATION OF PHENOLIC COMPOUNDS WITH HPLC-DAD

Sukru Karatas¹✉, Elif Cakir¹, Munise Ustun¹

¹Department of Food Engineering, Istanbul Aydin University, PO Box 38, 34295 Küçükçekmece, Istanbul, Turkey

✉karatassukru@gmail.com

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ABSTRACT

The aim of this research was to investigate the extract of antioxidant and antimicrobial properties of pomegranate peel (PP) wastes and their effect on the *E. coli*, *S. aureus*, *S. mutans* at different concentrations of 10-100%. Total phenolic compound substance and antioxidant modification were inspected using Folin Ciocalteu and ABTS methods respectively. The antimicrobial activity was tested on the microorganisms using diffusion method and the scanning of the phenolic compounds were analyzed using HPLC-DAD and the most abundant compound was estimated to be the punicalin, gallic acid, ellagic acid and quercetin. The most common phenolic compound was detected to be punicalin and the highest antioxidant activity was about 821.72 mmol trolox/mg with (60%) acetone extract and the effect on the phenol increased up to 445.04 GAEq (mg GA/g) when PP extracted with ethanol, methanol and (60%) acetone. The results were evaluated and statistically analyzed according to antioxidant and antimicrobial effect of each extract on the *E. coli*, *S. aureus* and *S. mutans* which were found to be the most abundant when PP extracted with (10%-60%) acetone

1. Introduction

Punicagranatum, a member of the Punicaceae family, has been known as a healing food since ancient times, and it is grown mostly in Asia, from Iran to Himaya in the Mediterranean region (Das et al., 1999; Jafri, Aslam, Javed, & Singh, 2000; Meerts et al., 2009; Vidal et al., 2003; Viuda-Martos, Fernández-López, & Pérez-Álvarez, 2010).

Pomegranate has positive effects on health due to anti-cancer, anti-obesity, anti-diabetic and anti-ulcerogenic properties and attracts attention (Alexandre et al., 2019; Zhu & Liu, 2013) due to enhancing nutritional features in the human diet (Alexandre et al., 2019). Pomegranates may be consumed fresh and may be processed as industrial products like fruit juice, jelly, jam, and vinegar and forms

significant amounts of by-product (Alexandre et al., 2019; Sood & Gupta, 2015). The by-products appear to be a source of valuable compounds (Sood & Gupta, 2015) as pomegranate peel contains high amounts of bioactive compounds and is even a source of natural antioxidants with strong biological activity content (Akhtar, Ismail, Fraternal, & Sestili, 2015). The flavonoids in pomegranate peel were shown to be rich in hydroxybenzoic acids and hydroxycinnamic acids. Pomegranate proteins with antimicrobial properties form a complex with sulfhydryl groups and were determined to show inhibitory effects against bacterial cell (Cristani et al., 2007; Goel, Puniya, Aguilar, & Singh, 2005). Synthetic and natural preservatives are used to inhibit pathogenic

organisms causing problems with food safety and to increase shelf life in the world in general. However, interest in natural preservatives has increased as synthetic preservatives cause problems in terms of health. Studies have determined that compounds in the peel sections of fruit with antimicrobial activity may prevent food degradation. Studies have shown that the extraction of active compounds in pomegranate from a variety of sections of the fruit displays variations according to the type of extraction and form of phenolic extraction (Al-Zoreky, 2009; Hegde et al., 2012; Pradeep, Manojbabu, & Palaniswamy, 2008; Tanveer et al., 2015). Many studies have been performed about pomegranate to date; however, it is important to determine the amount and type of solvent pomegranate peel wastes to provide the highest antioxidant activity amounts and best antibacterial effect.

This study determined the total phenolic compounds, antioxidant variation with Folin Ciocalteu and ABTS methods, and antioxidant compounds with phenolic compound screening using HPLC-DAD for extracts obtained using different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%) of methanol, ethanol and acetone solvents. Additionally, the aim was to determine the antibacterial effects of extracts obtained with different solvents from peel wastes on food pathogens like *E. coli*, *S. aureus* and *S. mutans* with the disk diffusion method.

2. Materials and methods

2.1. Materials

2.2.1. Pomegranate Samples

Pomegranate samples were purchased from a local market and constituted as research material. The pomegranates were separated from peels and membrane and granulated; the grains were pressed by a juicer and kept in a deep freezer at -18°C until the analysis. Peel pomegranate wastes were dried in vacuum oven at 55°C and dried pomegranate peels were grinded by blender and kept in a refrigerator at -40°C until used. In order to be able to evaluate the research results statistically was analyzed by using of Kruskal Wallis method (Hegde et al., 2012).

2.2.2. Methods

The extraction methods was carried out on the 30 samples of dried pomegranate peels were taken into an Erlenmeyer and extracted using of Methanol, Ethanol and Acetone (HPLC grade Merck) with dilution of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% solutions (Batta & Rangaswami, 1973). Each solvent (v:v) was prepared from one gram of sample and mixed with 20 ml of solvent then mixtures were replaced on the shaker for 24 hours at the end of that time the Erlenmeyer were removed and extracts replaced into the centrifugal tubes then centrifugated at 4000 rpm for 5 mins. At the end of this duration, filtrations were performed through filter paper then 1 to 1.5 ml of filtrates were taken into rotary evaporater in order to remove the solvents from each sample then replaced into the Eppendorf tubes to indicate antimicrobial activity of the pomegranate peel wastes extract and also to determine phenolic compounds by HPLC-DAD analysis (Fang, Zhang, & Wang, 2007).

2.2.3. Estimation total phenolic content (TPC)

The content of total phenolics in the extracts was determined by spectrophotometric method at 750 nm according to the Folin-Ciocalteu procedure and calculated as mg/g of gallic acid equivalents (Sellappan, Akoh, & Krewer, 2002). Principle of the method is based on a redox reactions, in which Folin-Ciocalteu reagent of phenolic components are came down in an alkaline environment and convert into an oxidized form then total phenolic substance.

2.2.4. Antioxidant activity

The method recommended by Miller and Rice-Evans (Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993; Miller & Rice-Evans, 1997; Rice-Evans, Miller, & Paganga, 1996) was used. This method is based on the principle (ABTS⁺ (2,2'-azinobis-(3-ethylbenzotiazoline-6-sulphonic acid))), ensuring nominal measurement is compared to antioxidative substance amount, kept by radical cation with standard amounts of Trolox, which is a synthetic antioxidant (water-soluble vitamin E analog). The samples, separated depending on estimated antioxidant contents from all samples

for antioxidant determination, were diluted with pure water at certain rates. ABTS and 4.9 mM solvent with 14 mM concentration and $K_2S_2O_8$ (potassium persulphate) solvent were combined at the same ratio and kept waiting in a dark place and at room temperature not less than 12-16 hours. Prepared stock solution was diluted with ethanol. For each solvents in different concentrations at the 20 μ l, 30 μ l, 40 μ l values, antioxidant activities were determined for 3 minutes at 30 seconds intervals. Calculations were made taking account of dilution factors.

2.2.4. Determination of phenols with HPLC-DAD

After extraction for phenol analysis, blowing away solvents from separated samples by rotary evaporator analysis sample were obtained. Zorbax C Column was used. Mobile phase 2.5%, Acetic Acid/97.5% Acetonitrile, flow rate 1mL/0.5mL⁻¹, Lichrospher Column: C18 Nucleosil column (150x4,6mm) were adjusted as particle size is 5 μ m and injection volume 20 μ L. A scan was made at 280nm and 360nm wavelengths. Preparing 50, 60, 80,100,120, and 150ppm, standard from stock Gallic acid standard by methanol, calibration curve was drawn. For quercetin, 40, 50, 60, and 100ppm standards of stock solvent were prepared by methanol and calibration curve was drawn. For catechin 100, 120, 150, 200, and 250ppm standards were prepared by water and calibration curve was drawn.

2.2.5. Antimicrobial activity determination

After extraction for antimicrobial activity determination, separated samples were used. 1 loop was received from the stock culture, stored at -80 °C and put into the tube, containing 9ml nutrient broth. It was left for incubation at 37°C for 24 hours. At the end of the duration, 100 μ l of culture, called subculture was put into the tube, containing nutrient broth and left for incubation for 14-15 hours. *Escherichia coli*, *Streptococcus mutans*, *Staphylococcus aureus* were used in the study (Dehkordi, Basti, Gandomi, Misaghi, & Rahimi, 2019). EMB and PCA were used as medium. For *S. mutans* and *S. aureus* PCA, for *E. coli* EMB, was used as a medium. Well diffusion method was used in the studies. Pour plate and spread plate methods were used. Since pour plate did not give result for *E. coli*, spread plate method was used. Each petri was divided into 4 equal pieces. And a well was opened into each divided part. Separated 50 μ l sample solvent was put into each well and left for incubation

3. Results and discussions

3.1. Quantification of bioactive compounds

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3.1.1. Total amount of phenolic substance

Total phenol content for ethanol, methanol and acetone in different concentrations are shown in Fig. 1A.

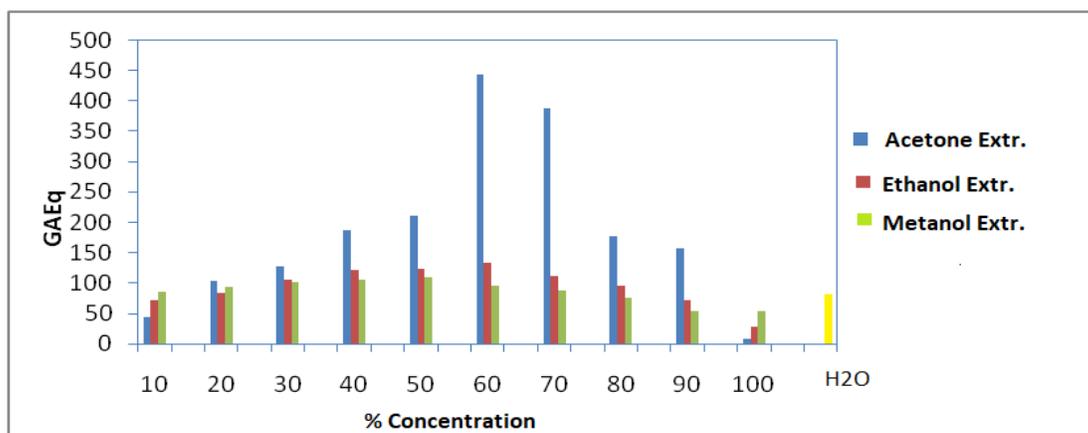


Figure1(A) Total phenol content GAEq:mg/g of gallic acid equivalent

When the results obtained from spectrometric measurements are examined, it is seen that acetone has the highest effect, making available total phenol content in all extractions and solvent types. The data from total phenol content of pomegranate applied Kruskal-Wallis test (Asymp.Sig. < 0,05) is examined, it is determined that the highest effect is on acetone and respectively on the extracts obtained from ethanol and methanol extractions. In view of the

findings, which we obtained at the end of the study, while especially 60% acetone solvent 445.012GAEq (mg/g of gallic acid equivalent) affects the total phenol highly, it is determined that this is lower for ethanol and methanol. Kinetic measurements of ethanol, methanol and acetone in certain time periods are shown in Fig. 1B.

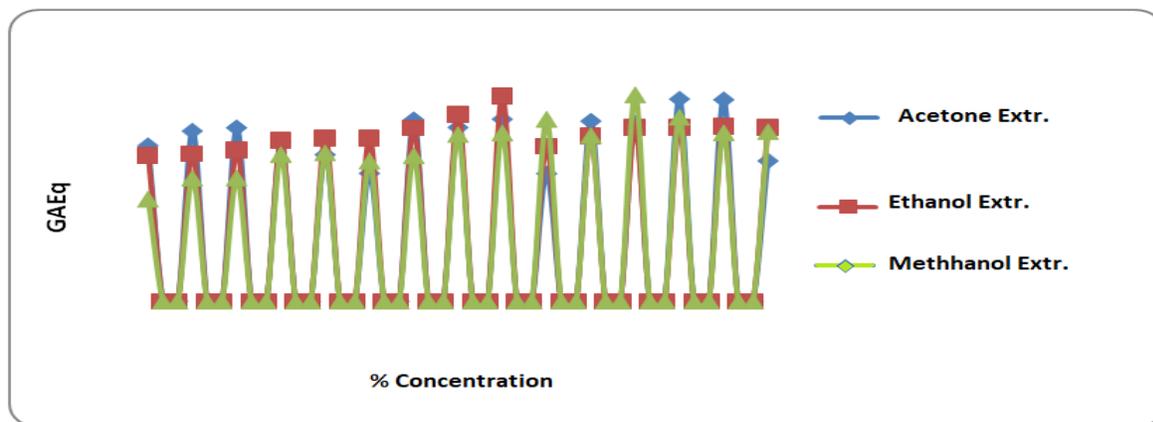


Figure 1(B). Kinetic measurements for total phenol

When the results obtained from spectrophotometric measurements are examined, it is seen that the kinetic measurement results for 3 solvents are close. In the extracts obtained from extraction against time, when total phenol effect is observed, it is seen that the extraction time does not have a significant effect on the efficiency.

3.2. Antioxidant activity

Antioxidant activity effects in ethanol, acetone and methanol extractions are shown by Figures 2A, 2B, and 2C, respectively. The results of spectrophotometric measurements demonstrated that antioxidant activities in all solvents are very high. The highest effect is measured 254228 aox in the extract obtained from 60% ethanol extraction and 143154 aox in the extract obtained from 40% methanol extraction. Meanwhile, antioxidant activities in ethanol, methanol and acetone in different concentrations are shown in Figure 3. The results demonstrated that antioxidant activity of

acetone in all extractions and solvent types are the highest one. When the results obtained from the ethanol, methanol, acetone activities were considered it was seen that the aox activity given was high. In addition, it was estimated that aox activities were reduced depending on the duration of aox activity. When this data is applied to the Kruskal-Wallis test (Asymp. Sig. < 0,05) and examined, it was determined that the highest effect is on the acetone, ethanol and methanol extracts respectively. The highest efficiency is estimated as 821.72 mmol trolox/mg in the 60% acetone extraction. These results indicated that the antioxidant level of pomegranate was high and they were similar with the previous studies (Mertens-Talcott, Jilma-Stohlwetz, Rios, Hingorani, & Derendorf, 2006). In their study, Singh et al. (Singh, Chidambara Murthy, & Jayaprakasha, 2002) determined that in their extraction using ethyl acetate, methanol and water from pomegranate peels and seeds, the peels showed

83% and 81% antioxidant activity at 50 ppm with methanol extraction.

The average syneresis values of probiotic yoghurt samples were given in Table 1. On the 30th minute of the measurements, the highest syneresis rate was determined in P2 sample on the 1st day (18.50 mL), while the lowest syneresis rate was in K sample (12 mL) on the 14th and 21st days. As a result of the analysis of variance, the difference between the storage days were significant ($p < 0.05$). In the samples containing rice milk, the syneresis rate was lower in P3, compared to the two other samples. Among all the results, difference between P1 sample and K sample was not significant ($p > 0.05$). On the 60th minute of the measurements, the highest syneresis rate was in P2 sample on the 1st

3.3. Antimicrobial activity determination

Antimicrobial effects for extractions of different acetone concentrations are shown in Table 1. Statistically significant different zone diameters were determined on the *E.coli*, *S.aureus* and *S.mutans* according to the type of extraction with different concentrations of ethanol, methanol and acetone. When antimicrobial effects for extractions of different ethanol concentrations are examined, it is determined that *E.coli* created a zone having an average 21.5 mm diameter, acetone average 20,5mm and methanol 13,5mm as a

result of the first measurement in 10% ethanol extraction. While the antibacterial effect of acetone on *E.coli* was higher than 10-40%, ethanol and methanol were found to be statistically similar at these rates. Zone diameter

14mm at 50% extraction while it was measured as 10mm at 100% extraction. When the table is examined, it is observed that while ethanol concentration increases, zone diameters gradually decrease. The zone diameter decreased due to the increase in acetone concentration as well. In methanol extraction, the zone diameter was found to be statistically similar up to 70%, and the highest zone diameter was determined at 50%. The 90% and 100% methanol extraction zone diameter was found to be higher than ethanol and acetone extraction at the same rates, and it was statistically significant. When the zone diameters were compared on the *S. aureus* pathogen bacteria, as the amount of ethanol, acetone, and methanol increased, the zone diameters decreased. The highest effect in ethanol was determined at 10-30%, in acetone 70-80% and in methanol at 10-40%. The highest antibacterial effect on *S. mutans* was determined in acetone extraction, and the lowest effect was seen in ethanol. There was no antimicrobial effect against *S. mutans* above 50% methanol extraction. In a study on the antibacterial effect of pomegranate peel on methanol extraction, it was reported that it exhibited antibacterial activity against *L. monocytogenes*, *Y. enterocolitica*, *E. coli* and *S. aureus* (Prashanth, Asha, & Amit, 2001). It has been observed that 80% methanol extraction is an active inhibitor against pathogenic bacteria such as *L. monocytogenes*, *E. coli*, *S. aureus* and *Y. enterocolitica* in the process of extraction with water, methanol and ethyl alcohol by traditional methods (Al-Zoreky, 2009).

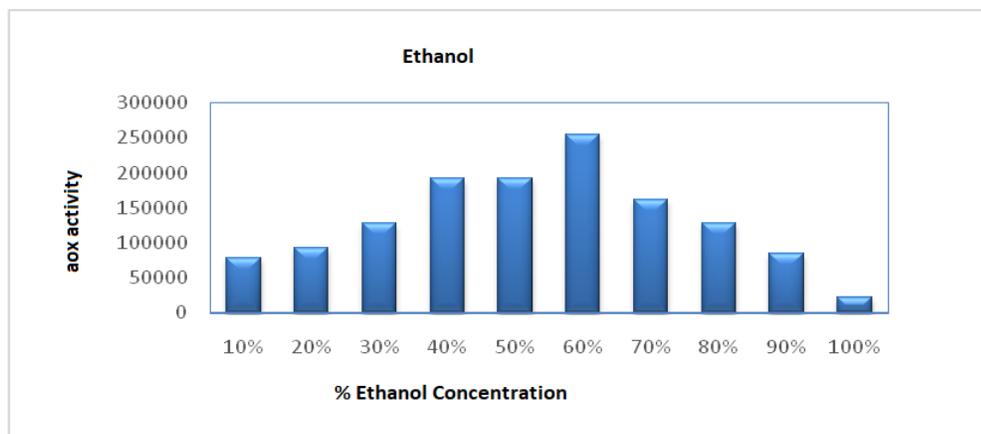


Figure 2A. Aox activity in ethanol extraction in relation to the Trolox TM standard concentration

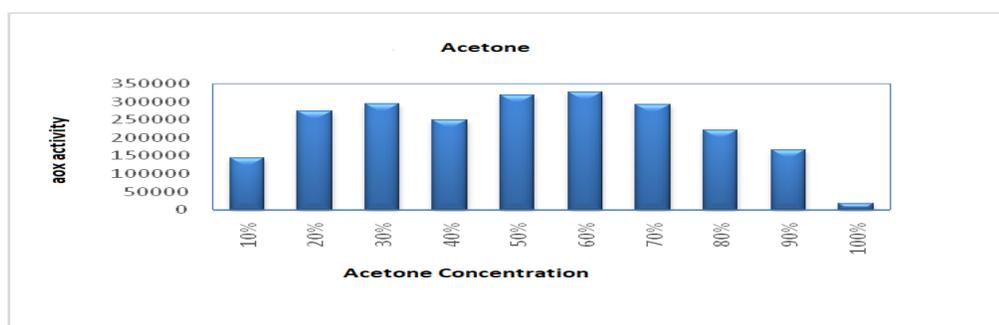


Figure 2B. Aox activity in acetone extraction in relation to the Trolox TM standard concentration

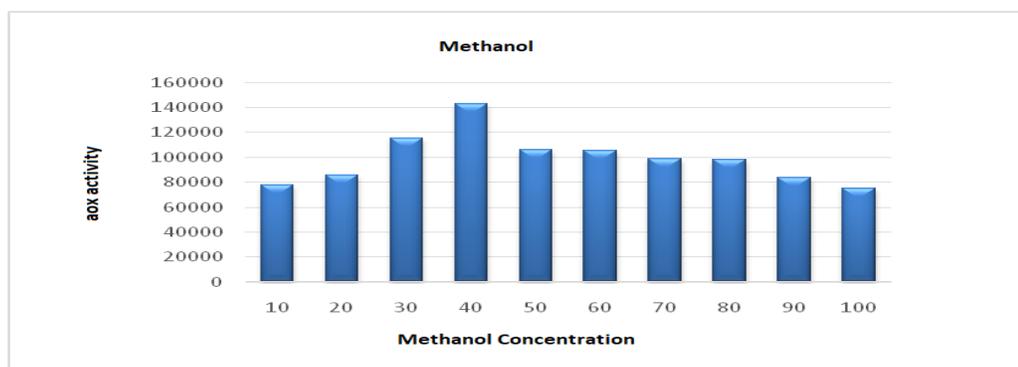


Figure 2C. Aox activity in methanol extraction in relation to the Trolox TM standard concentration

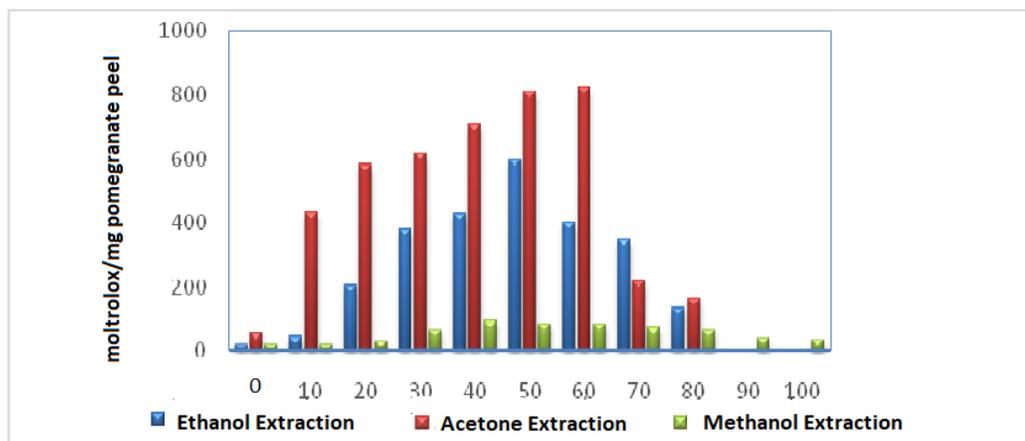


Figure 3. Aox activity in ethanol, acetone, and methanol extraction in relation to the Trolox TM standard concentration

3.4. Determination of phenols with HPLC-DAD

As shown on the Fig. 3 at 280 nm performed by us according to the results obtained from HPLC chromatogram in 50% acetone extraction, components, which cover the largest area are Punicalagin derivatives. In addition to

this, gallic acid, punicalin-A and Ellagic acid derivatives follow this. Similarly the study of Nasr et.al (Nasr, Ayed, & Metche, 1996) quantitatively determined ellagic tannins and gallic and elagic acids in the peels of Tunisian pomegranate.

Table 1. Antimicrobial Effects of omegranate Peel Against Some Foodborne Bacteria in Extracts of Different Solvents (mm zone diameter)

%	<i>E.coli</i>			<i>S.aureus</i>			<i>S.mutans</i>		
	Etanol	Aseton	Methanol	Etanol	Aseton	Methanol	Etanol	Aseton	Methanol
10	21.5±0.7 ^{Aa}	20.5±0.7 ^{Ca}	13.5±0.7 ^{Bb}	18.5±0.7 ^{Ba}	14±0.0 ^{Bb}	12.5±2.1 ^{ABCb}	1±0.0 ^{Bc}	17.5±0.5 ^{Aa}	12.5±2.1 ^{Ab}
20	19±2.8 ^{Aab}	24±0.0 ^{Aa}	16.5±0.7 ^{ABb}	22.5±0.7 ^{Aa}	14±0.0 ^{Bb}	15±0.0 ^{Ab}	nd	18.5±0.5 ^{Aa}	15±0.0 ^{Ab}
30	19±2.8 ^{Aab}	22±0.0 ^{BCa}	14.5±0.7 ^{ABb}	13.5±0.7 ^{Ca}	14±0.0 ^{Ba}	13.5±0.7 ^{ABCa}	nd	15±3 ^{ABCa}	13.5±0.7 ^{Aa}
40	15±0.0 ^{Bb}	23±1.4 ^{ABa}	16±1.4 ^{ABb}	10±0.0 ^{DEa}	14±1.4 ^{Ba}	12.5±3.5 ^{ABCa}	nd	10±2 ^{CDa}	13.5±2.1 ^{Aa}
50	18.5±0.7 ^{Aa}	15±0.0 ^{Db}	17.5±0.7 ^{Aa}	12±2.8 ^{CDa}	9.5±0.7 ^{Da}	11±1.4 ^{BCa}	nd	9.5±1.5 ^D	nd
60	11±0.0 ^{Cb}	16±0.0 ^{Da}	16.5±0.7 ^{ABa}	10±0.0 ^{DEb}	11±0.0 ^{Cb}	14±1.4 ^{ABa}	nd	17.5±0.5 ^A	nd
70	13±1.4 ^{BCc}	14±0.0 ^{Ab}	16.5±0.7 ^{ABa}	10.5±0.7 ^{Db}	16±0.0 ^{Aa}	11±1.4 ^{BCb}	nd	17.5±0.5 ^A	nd
80	6.5±0.7 ^{Dc}	14±0.0 ^{Aa}	14±1.4 ^{Bb}	7.5±0.7 ^{EFc}	17±0.0 ^{Aa}	10.5±0.7 ^{BCb}	nd	13.5±1.5 ^{ABCD}	nd
90	4.5±0.7 ^{Db}	8±1.4 ^{Eab}	13.5±3.5 ^{Ba}	7±1.4 ^{Fa}	10±1.4 ^{CDa}	11.5±2.1 ^{ABCa}	10±0.0 ^A	16±3 ^{AB}	nd
100	5±0.0 ^{Dc}	9±1.4 ^{Eb}	14±1.4 ^{Ba}	6.5±0.7 ^{Fa}	2±0.0 ^{Eb}	10±0.0 ^{Ca}	10±0.0 ^A	11.5±0.5 ^{BCD}	nd

A-E: Values with different letters in the same column differ statistically significantly (P<0.05). a-c: Values with different letters in the same row differ statistically significantly (P <0.05), nd: not detected

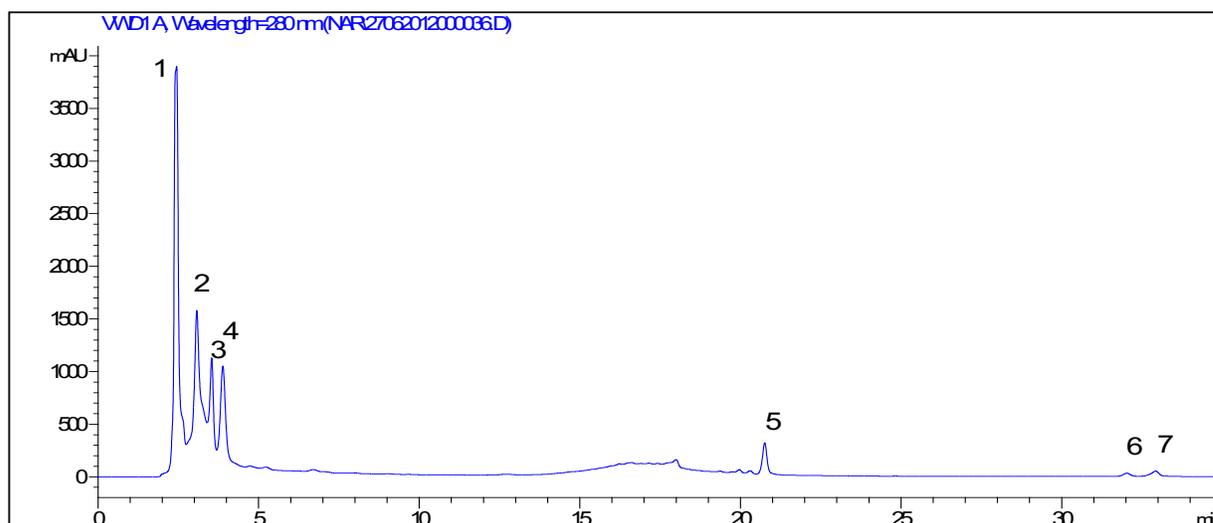


Figure 4. HPLC chromatogram of phenolic components in pomegranate peel with adding 400ppm standard gallic acid in 50% acetone extraction (1-2 Punicalagin derivatives, 3- Gallic acid, 4- Punicalin-A, 5-Ellagic acid derivatives, 6-Punicalagin-B).

4. Conclusions

This research studied the total phenolic content, antioxidant activity, antimicrobial activity and phenolic compounds from pomegranate peel using solvent extractions methods by methanol, ethanol and acetone with concentrations of 10-100% (v/v). Total phenolic contents were determined by Folin Ciocalteu method and results showed that maximum total phenol content was 445.012GAEq (mg/g of gallic acid equivalent) at 60% acetone solvent. Antioxidant activities were calculated by ABTS method and results demonstrated that highest antioxidant activity was about 821.72 mmol trolox/mg with (60%) acetone extract. Also, HPLC-DAD analysis showed that the most abundant phenolic compounds were the punicalagin, gallic acid, ellagic acid and quercetin. The highest zone diameters against *E. coli* and *S. mutans* pathogen bacteria were determined in acetone extraction, and the highest zone diameter against *S. aureus* was determined in ethanol extraction. The antimicrobial effects of pomegranate peel extract on the *E. coli*, *S. aureus*, *S. mutans* showed that the antimicrobial effect is seen on the *E. coli* from the acetone solvent. Thus, acetone can be considered as a useful solvent for extraction of phenolic compound from pomegranate peel to approach antioxidant and

antimicrobial effect of them as a preventive and therapeutic in human health.

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