Journal of Food Biosciences and Technology,
Islamic Azad University, Science and Research Branch, Vol. 10, No. 2, 39-48, 2020

Antimicrobial Effects of Grape and Pomegranate Waste Extracts against two Foodborne Pathogens

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Received: 26 July 2019 Accepted: 26 April 2020

ABSTRACT: In recent years, increasing in the level of public health consciousness has led to a drastic decrease in the use of synthetic food preservatives. Therefore, there has been a growing demand for novel antimicrobials. Apart from that, finding low cost sources has been always an important issue for food marketers. The present study was designed to investigate in-vitro antibacterial activities of grape and pomegranate waste separately and in combination with broth dilution methods against two bacteria (*Staphylococcus aureus* and *Escherichia coli*). The utilized solvent, as an extractant, was hot water. The total phenolic contents of grape and pomegranate wastes were also evaluated. Results revealed that the hot-water extract of the mixed wastes was the most potent, with the MIC / MBC of 52 / 286 mg/ml against *E. coli* and 75/98 mg/ml against *S. aureus*. It was also observed that the pomegranate waste contained higher level of phenolic compounds (439±0.82 mg GAE/L) as compared to the grape waste (333.6±1.25 mg GAE/L). It also exhibited higher antibacterial activity (lower MIC and MBC against tested bacteria) than the extract of grape waste. These results suggest that by-products of grape and pomegranate that are inexpensive and available can be employed as a potential source of antibacterial compounds.

Keywords: *Escherichia coli, Grape, Natural Antimicrobials, Pomegranate, Staphylococcus aureus, Waste.*

Introduction

In recent years, in response to consumers' concerns around synthetic preservatives, considerable effort has been made to find novel antimicrobials derived from a variety of natural sources. In general terms, food preservative refers to artificial or natural additives are employed to inhibit bacterial and fungal growth in order to improve the quality and shelf life of food products. Natural antimicrobials can be obtained from different sources including plants, animals, bacteria, algae and fungi. Consequently, there is an ongoing research to find natural reservoir of phytochemical compounds that could be used as an alternative to antibiotics and synthetic chemical, which could cause serious side effect. Whilst, the cost and availability are always the main issues for manufacturers, byproducts of processed foods have received great attention as potential sources of low-cost antibacterial compounds.

Oftentimes large amount of by-products in food industry, including fruit pomace, seeds, peels, pulps, unused flesh, and husks have been considered as waste while, recent studies revealed that they are promising sources of bioactive agents and valuable components with several functionalities

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(Gaber *et al.,* 2015; Mamma *et al.,* 2008; Nitschke & Costa, 2007). It has been also confirmed that these unusable parts have a similar or even higher proportion of bioactive compounds than the usable parts, for example, the total phenolics in peels of lemons, oranges and grapefruits have been found to be 15% higher than the level found in the peeled fruits (Ramadan *et al.,* 2015). Accordingly, they also have a wide range of antimicrobial properties due to the total contents of phenolic compounds (polyphenols, tannins, and flavonoids) (Kumar and Vijayalakshmi, 2013).

Pomegranate (*Punica granatum* L.) is the fruit of a deciduous shrub that is native of Mediterranean and Southern Asia. Iran is one of the largest producers of pomegranate by producing around 670,000 tones in a year under the local name of Anar (Anonymous, 2005). This fruit has a long history in traditional medicine, for centuries; different parts of pomegranate like bark, leaves, immature fruits and fruit rind have been used for traditional treatment of diseases like dysentery diarrhea, helminthiasis, and respiratory pathologies (Jayaprakasha *et al.,* 2006; Choi *et al.,* 2011; Sanchez-Lamar *et al.,* 2008; Braga *et al.,* 2005). Over the past decades, scholars have shown great interest in investigating the medicinal and nutritional benefits of pomegranate. According to these researches, pomegranate has a wide range of therapeutic properties from treatment and prevention of obesity, diabetes, arthritis, dental conditions cancer, and cardiovascular disease to protection from ultraviolet radiation (Gaber *et al.,* 2015; Hamza *et al.,* 2015; Neelam & Singh, 2012). Beside therapeutic benefits, other biological properties such as antioxidant, antibacterial, antiprotozoal and antifungal have been also well reported (Basu & Penugonda, 2009; Meerts *et al.,* 2009; Guo *et al.,* 2008).

Grape (Vitis spp.) is one of the fruit cultivated almost in all regions of the world (Gruenwald *et al.,* 2004). It is a common commodity in forms of grape juice, jams and raisins in the food market all over the world. Numerous studies focused on the health-promoting and antioxidant effects of grapes (Adamez *et al.,* 2012; Baydar *et al.,* 2006; Jayaprakasha *et al.,* 2003). These studies confirmed various biological capacities such as antioxidant and antibacterial for this plant. Grapes is (Vitis vinifera) also well known for the high level of polyphenols content, which is a novel antimicrobial agent; in this way grape seeds extract has been proved as an antimicrobial against *Escherichia coli* O157:H7 therefore it has a great potential for being a food preservative (Abtahi *et al.,* 2010; Rhodes *et al.,* 2006).

As byproducts of fruits and vegetables are documented as a rich sources of minerals, and bioactive agents, this study was designed to evaluate the in vitro antibacterial activities of grape and pomegranate peels and seeds extracts. Whereas previous studies mostly explored the antimicrobial activity of pomegranate and grape based on extracts from different parts of these fruits separately (e.g. Al-Zoreky, 2009; Gould *et al.,* 2009; Opara *et al.,* 2003; Machado *et al.,* 2003; Adamez *et al.,* 2012), this study investigated the mixture of seeds and peels extract of pomegranate waste along with grape waste (50/50) by using hot water as an extractant. Simultaneously, it was attempted to assess the composition and antibacterial activity of the mixed peels and seeds extract (50/50) of these two fruits' waste separately. In addition, this study focused on one gram-positive bacterium (*Staphylococcus aureus)* and one gram- negative bacterium (*Escherichia coli).* Both of these bacteria have been identified as main public health problems therefore many food incidences recorded in hospitals were related to them, hence they are still in center of attention for identifying antimicrobial compounds against them.

Materials and Methods

- *Preparation of plant materials*

Pomegranate fruits were purchased from the local market in Saveh-Iran. Fruits were washed and the *arils* (with seeds*) were manually separated* from the *peels*. The arils were then pressed in order to yield juice and seed. The collected peels and seeds were dried at room temperature for about 5 days. In next steps, *the dried* samples were *ground* separately with an electric grinder *into powder* and then sieved through fine mesh metallic sieve and stored in refrigerator until required.

Red Grape pomace, composed of seeds and skins, were *kindly* provided by Sunich Alifard Company. The samples were manually separated, and then dried, powdered, sieved the same as above mentioned methods (Figure 1).

- *Preparation of the extracts*

20g of fine powders (10 g of peels+10 g of seeds) from *pomegranate* and 20g of fine powders of grape were weighed. These two samples (20 g) were separately dissolved in 200 mL sterile distilled water and then heated at 89±1˚C in a water bath (Memmert, Germany) for 30 min. The obtained extracts were filtered through double-layer gauze. Subsequently, the filtered extracts were centrifuged at 5000 rpm for 15 min. All obtained supernatants were poured into disposable sterile plates to make thin layers and were dried in a vacuum oven (Gallenkamp, England) at 45˚C for 18 hours (Figure 2).

(a) (b)

Fig. 1. Dried pomegranate (a) and grape (b) waste

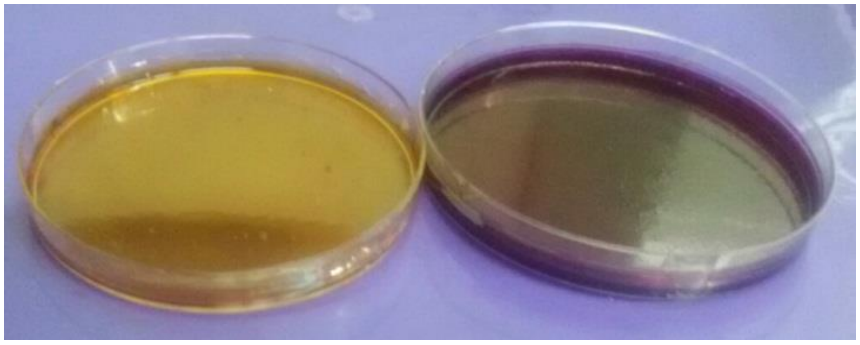


Fig. 2. Dried extracts of pomegranate and grape waste

- *Phytochemical screening*

The extracts were subjected to phytochemical analysis to ascertain the presence of metabolites such as alkaloids, tannins, flavonoids and saponins.

- Test for tannins

One ml of aqueous extract (1 ml) was mixed with 10 ml of distilled water and filtered. Ferric chloride reagent (3 drops) was added to the filtrate. A blue-black or green precipitate confirmed the presence of gallic tannins (Jaffer *et al.*, 1988).

- Flavonoids analysis

5ml of aqueous extract was mixed with mixture containing 10ml of 1:1solution (50% ethanol plus 10ml of 50% NaOH). A yellow precipitate in the solution confirmed the presence of flavonoids (Jaffer *et al.*, 1988).

- Alkaloids analysis

Dragendroff's reagent test was conducted for detection of alkaloids. 0.5 g of peel extract was dissolved in 5 ml of 1% HCl and then the mixture was kept in water bath for 2 minutes. 1 ml of filtrate is treated with dragendroff's reagent. Turbidity or precipitation is a clear indication of the alkaloids presence (Hajoori *et al.*, 2014).

- Saponin analysis

The tested solution was mixed with water using test tube and shaken properly. Arising foam suggests the presence of saponin (Hajoori *et al.*, 2014)**.**

3-5 Test for Phenols**:** One milliliter of the extract was added to 1 mL of 10% $FeCl_2$ and mixed together. The presence of blue precipitate confirmed the presence of phenols (Nwokocha *et al.*, 2011).

- *Determination of Total Phenolic Content*

The Folin-Ciocalteu reagent method was applied to estimate the *total phenolic content*. Stock solution of gallic acid was prepared by dissolving 0.500 g of dry gallic acid in 10 mL of ethanol and diluted to 100 mL volumetric flask with distilled water. In order to prepare a calibration curve, 0, 1, 2, 3, 5, and 10 mL of the above Gallic acid stock solution was added into 100 mL volumetric flasks, and then diluted to volume with water. These solutions will have phenol concentrations of 0, 50, 100, 150, 250, and 500 mg/L gallic acid, the effective range of the assay (Sherikar & Mahanthes, 2015).

60 µL of an extract sample was mixed with 2 mL of Na2CO3 (7.5%), and 2.5 mL of 10-fold diluted Folin-Ciocalteu reagent thoroughly using a vortex mixer (Assistant Reamix 2789, Heppenheim, Germany). The mixed solution was allowed to stand for 30 minutes at room temperature and then its absorbance was measured at 760 nm using a spectrophotometer (Perkin Elmer Lambda 25 UV/VIS, the US). Blank was concomitantly prepared, containing 60 µL distilled water, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of NaHCO3 (Pan *et al.*, 2011). The samples were prepared in triplicate order for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and then the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was read from the calibration line; then the content of phenolics in extracts was expressed as mg of gallic acid equivalents per liter of sample. In this study, Folin-Ciocalteu reagent method was used to estimate the *total phenolic content.* The data were analyzed using SPSS 19 software. Quantitative variables were compared by independent ***t***-test between the two groups. Data are expressed as (mean ± standard deviation) and $P < 0.05$ was considered as statistically significant.

- *Determination of antibacterial activity*

- Microorganisms and culture

The two bacteria were kindly provided by

the *Center of Pomegranate Research of Ferdowsi, University of Mashhad*. They were one gram positive bacterium (*S. aureus* PTCC 1399) and one gram-negative bacterium (*E. coli* PTCC 1431).vThey were stored in 50% glycerol (v⁄v) *at* -20°C for *24 hours before starting* the *experiment*, the *frozen bacteria were grown* overnight in Mueller Hinton broth (25 mL) at 37 °C. After spending 24 h, *the Overnight cultures of E. coli* and S. *aureus were* centrifuged (5300 rpm for 15 min) and then the supernatant was *discarded***.** *Bacterial pellets* were re-suspended in sterile normal saline and adjusted to 0.5 Mac-farland standard (1.5×10^8 CFU/ml), prepared by adding 0.05ml of Barium chloride ($BaCl_2$) (1.17% BaCl2.2H2O) to 9.95ml of H_2SO_4 (1%) with constant stirring (NCCLS, 2000).

- Determination of MIC

The bacterial suspensions were diluted further with sterile normal saline to a level of 1.5×10^6 CFU/ml. The broth-dilution method was adopted to determine the minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts. Different concentrations of the extracts were made by sterile distilled water. 500 µL of extracts at different concentrations were added to sterile glass tubes containing 500 µL Mueller Hinton broth and 1000 µL of bacterial suspension (1.5×10^6 CFU/ml). Two control tubes were maintained for each test batch. These include tube-containing extract without inoculum and the tube containing the growth medium and inoculum. The tubes were incubated at 37 °C for 18–24 h. Because of the turbidity and dark color of the extracts, 100 µL of the mixture in the tubes were spread onto the surface of Mueller Hinton agar. Plates were incubated at 37 °C for 18–24 h. The MIC was defined as the lowest concentration (mg/ml) of the extract that the number of colonies was less than others. MBC value determined from the lowest concentration of extract, which exhibited no bacteria colonies growing. The test was conducted twice.

Results and Discussion

Major groups of compounds, that are responsible for antimicrobial activity include phenolics, phenolic acids, quinones, saponins, flavonoids, tannins, coumarins, terpenoids, and alkaloids (Gyawali & Ibrahim, 2014).

In this study phytochemical screening of five secondary metabolites (alkaloids, tannins, saponins, flavonoids and phenols) from aqueous extract of pomegranate and grape waste were tested. The results are shown in (Table 1).

Table 1. Phytochemical constituents of Grape and pomegranate seed and skin extracts

Phytochemical	**AEP***	**AEG****
Tannins	++	+
Flavonoids	++	++
Saponins	+	-
Alkaloids	+	+
Phenols	++	+
Total phenolic content (mg GAE/l)	440.9±1.82 [a]	334.5±1.20 [b]

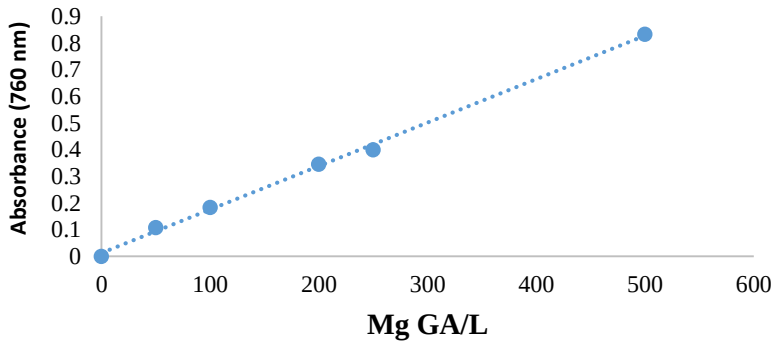
+: Present, ++: Deeply present,-: Absent
*AEP = Aqueous extract of pomegranate waste
**AEG= Aqueous extract of grape waste

- Determination and comparison of total phenolic content

In this study despite similar conditions at all stages of preparing extracts, the result for total phenolic in grape waste extract at various temperatures and times with water as solvent were 288-852 mg GAE/L (Figure 3). Factors influencing the content of phenolic compounds in plant tissues are genetic factors, quality of solar UV radiation, soil conditions, environmental and climatical conditions. The degree of maturity at harvest time, postharvest handling and storage conditions are other factors (Mameshloo *et al.*, 2011).

- Determination of MIC

The MIC (mg/ml) of all extracts is presented in Table 2. It appeared that the aqueous extract of mixed wastes has shown highest antimicrobial activity compared to the other extracts. It is possible due to the synergistic effect. Moreover, aqueous extract of pomegranate wastes possessed higher antimicrobial activity than aqueous extract of grape wastes. The possible reason could be relating to the total phenolic content which was higher in the aqueous extract of pomegranate wastes. The MIC values for test bacteria seemed to correlate with the total phenolic content found in the extracts.



Y= 0.0016+0.0126

R^2= 0.9979

Fig. 3. Gallic acid standard curve

Table 2. Antimicrobial activities (MICs and MBCs) of grape and pomegranate waste extracts against selected bacteria

Microorganisms	Extracts	MIC (mg/ml)	MBC (mg/ml)
S. aureus PTCC 1399	AEP	165	238
	AEG	462	492
	AEM	75	98
E. coli PTCC 1431	AEP	165	520
	AEG	480	--
	AEM	50	286

AEP = aqueous extract of pomegranate wastes.
AEG = aqueous extract of grape wastes.
AEM= aqueous extract of Mixed wastes

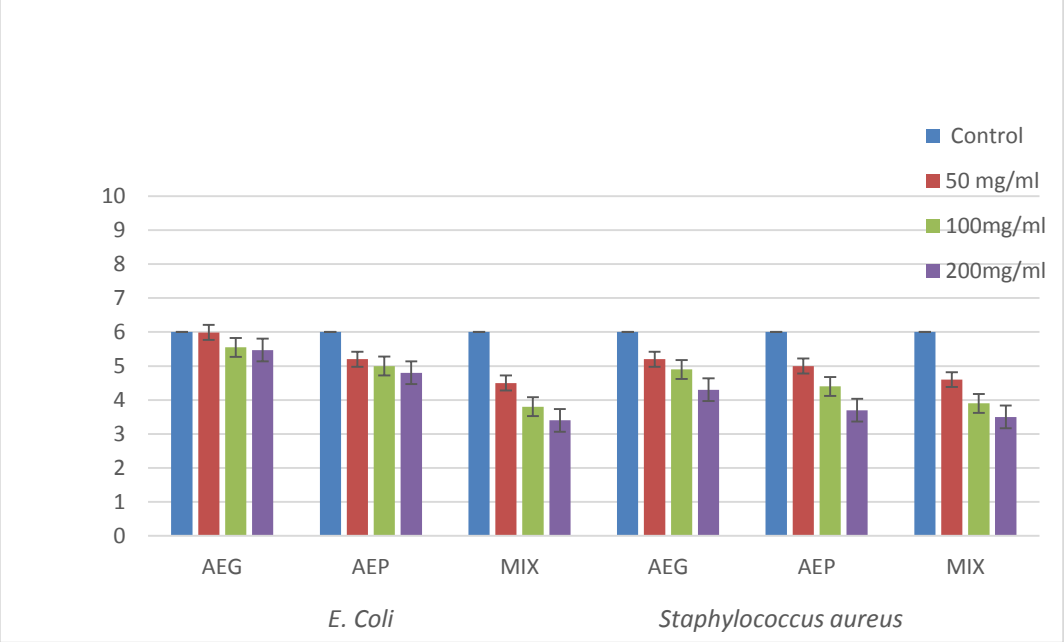


Fig. 4. Antimicrobial effect of pomegranate and grape waste extract on *E. Coli* and *Staphylococcus aureus*

Voravuthikunchai *et al.* (2004) found that the aqueous extract of pomegranate peel was active against *E. coli* O157:H7, with MIC/MBC 0.19/0.39 mg/ml. Eswar *et al.* (2013) stated that aqueous pericarp extract of *punica granatum* was active against *E. coli* and *Staphylococcus aureus* with MIC 10 µg/ml. In this study the MICs of aqueous extracts of pomegranate wastes against *S. aureus* and *E. coli* (165/165 mg/ml) were comparable to the study of Nuamsetti *et al.* (2012). According to this report, the aqueous extract of pomegranate peel was active against *E. coli* and *Staphylococcus aureus* with MICs 207 and 104 mg/ml, respectively.

Al-Nimer *et al*. found that the MIC of grape seed extract was ranged from 1.152 to >150 µg mL-1. Al-Nimer *et al.*, 2012). Adamez *et al.* (2012) also reported that the MIC of aqueous extract of grape seed were between 50 to >100 µL/ml; this result shows a significant difference with the results of this study. Possible justifications for this difference could be different extraction method, strain sensitivity, or antimicrobial procedures used in the test (Nuamsetti *et al.*, 2012).

Conclusion

In recent years, suspicion has been growing continuously over the use of synthetic preservatives due to serious side effects that have been recognized. Under the present situation, food marketers are demanding more for novel preservatives with natural roots. Accordingly, scholars have been motivated to investigate natural sources to identify new and also easy to access components that could adjust as antibacterial agents in food preservatives.

Moreover, cost is always an important issue in any market. In this way, waste of food has been noticeably considered as a potential and reasonable source for more exploring in this field. Based on these explanations, present study aimed to investigate in vitro antibacterial activities of by-products left from grape and pomegranate.

According to the results of this study, the mixture of grape and pomegranate waste

extracts (seeds and peels) are valuable sources of antibacterial compounds that might be suggested to employed as low-cost and natural antimicrobial agents.

Acknowledgement

This work was supported by the Iran National Science Foundation grant number 96013862.

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