Total phenolic content, antiradical, antimicrobial and antibiofilm properties of grape and apple vinegar

ABSTRACT

Vinegar is a natural product- produced from alcoholic fermentation- that has shown strong antimicrobial activity. The aim of this study was to determine the total phenolic content and antiradical activity of the commercial grape (GV) and apple vinegar (AV) as well as to evaluate their antibiofilm and antimicrobial activities against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. GV showed higher total acidity and total phenolic content, and lower antiradical activity (DPPH activity) compared to AV. The populations of *S. aureus* and *P. aeruginosa* were significantly reduced by neat GV and AV samples. The antibacterial activity of GV was superior to AV ($p<0.05$). While AV and GV samples at 50% concentration did not form a visible zone of inhibition against *S. aureus*, they showed an inhibitory effect against *P. aeruginosa* (16.25 mm for GV and 16.5 for AV). The vinegar applied at the lowest concentration (25%) did not show any antibacterial effect on either bacteria. Solutions containing 25% to 6.25% vinegar samples prevented almost 100% biofilm formation in both bacteria. Taken together, commercial GV and AV significantly reduced the viability of *S. aureus* and *P. aeruginosa*, thereby decreasing biofilm formation.

Keywords: Antimicrobial activity, Antibiofilm activity, Antiradical activity, Food pathogens, Vinegar

INTRODUCTION

Microorganisms are able to grow on food matrixes, food industry equipment, surfaces and biofilm which is an extracellular matrix formed by many different bacteria, including *Bacillus* spp., *Listeria monocytogenes*, *Staphylococcus* spp., *Escherichia coli* and *Pseudomonas aeruginosa* in different environments (Giaouris et al., 2015). The presence of more than one bacterial species in a biofilm facilitates their attachment to surfaces (Galie et al., 2018). The extracellular matrix, which consists mainly of polysaccharides, is responsible for the strong endurance of these complexes (Flemming et al., 2016). Several pathogens, a major cause of foodborne diseases, related with bacterial biofilms on food matrixes or factory equipment may lead to intoxications or infections in humans. The formation of biofilm and spread of biofilm-related infections in food cause significant health risk for human and great economic problems in food industry (Camargo et al., 2017). *Staphylococcus aureus*, a non-spore-forming, non-motile spherical bacterium, *P. aeruginosa*, a heterotrophic, motile, rod-shaped bacterium, are commensal bacteria with carriage rate in humans and wide distribution in environment and can form biofilms on surfaces along the food production chain. Therefore, consumption of food contaminated with these bacteria may pose a threat to human health (Xu et al., 2019; Pometto and Demirci, 2015).

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Research Article

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Disinfection is defined as the treatment of surfaces to control foodborne pathogenic bacteria, using physical and chemical methods. Disinfection represents one of the most crucial processing steps affecting the quality and safety of a food product (Deng et al., 2019). Various chemicals are used to disinfect the surfaces and equipment and their use is the most efficient way of disinfection. Meanwhile, due to their toxic effects on environment and health, there are doubts about the use of these synthetic chemicals as disinfectants, especially in the food industry. However, the development of sanitizers that are not harmful to non-target organisms, animals and human, is necessary for use in the food industry (Ölmez and Kretzschmar, 2009).

Sanitization based on the application of organic acids includes one of the most important interventions in the food industry to control microbiological safety and quality (Loretz et al., 2010). Organic acids are an inexpensive and effective alternative to synthetic disinfectants that reduce both the population and the prevalence of pathogenic bacteria (Loretz et al., 2011a, 2011b). Vinegar, due to the presence of significant amounts of organic acids and other natural substances with antibacterial effect, has been proven to have some disinfectant properties (Chang and Fang, 2007; Sengum and Karapinar, 2004; Wu et al., 2000).

Vinegar is a natural fermented product containing various nutrients and bioactive compounds such as acetic acid, gallic acid, catechin, epicatechin, chlorogenic acid, caffeic acid, p-coumaric acid, and ferulic acid which have a wide variety of therapeutic properties including antioxidant, antibacterial, antiobesity, antihypertensive, and cholesterol-lowering (Budak et al., 2014; Kahraman et al., 2021). Its antibacterial activity is attributed to the presence of organic acids, polyphenols, and melanoidins. Polyphenols and melanoidins, which are produced from raw materials and fermentation processes, also contribute to the antioxidant properties in vinegars (Chen et al., 2016). It has been reported that both grain and fruit vinegar can improve antioxidant capacities and reduce oxidative damage in *in vitro* and *in vivo* experiments (Chou et al., 2015; Verzelloni and Tagliazucchi 2007; Coelho et al., 2017). Vinegar is produced by carbohydrates-rich foods such as grape, apple and other fruit juices. Rice, malt and beer can be also used as raw materials for producing vinegar. The materials used in the production of vinegar change the vinegar content, so their therapeutic effects (Budak et al., 2014; Samad et al., 2016). The aim of the present study was to assess the total phenolic content and antiradical activity of the commercial grape and apple vinegar as well as to evaluate their antibiofilm and antimicrobial activities against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

MATERIAL and METHOD

Sample preparation

The apple (AV) and grape (GV) vinegar samples used in the current study were commercially supplied from a local market. Samples were stored at +4 °C until use.

Physicochemical properties

The pH values of the vinegar samples were determined by a digital pH meter (704 pH Meter, Metrohm) at 25ºC ± 2ºC. Total acidity quantification of the vinegar samples was performed by titration method described by Kahraman et al. (2021).

Total phenolic compounds

In this study, the total phenolic content of vinegar samples was determined by using the Folin-Ciocalteu method based on the procedure of Pawar and Dasgupta (2018), and gallic acid (Sigma, USA) was used as a standard. The results were expressed as milligrams of gallic acid equivalents (GAE, mg gallic acid/g). Gallic acid was dissolved and diluted in ethanol

(Merck, Germany) for two-fold serial dilutions ranging between 3.12 and 200 µg/mL. After mixing 200 µL of filtered samples or standard solutions with 400 µL of distilled water in tubes, 200 µL of 10 % Folin–Ciocalteu's (F–C) phenolic reagent diluted in distilled water was added to the tubes. After 5 min incubation, 200 µL of 1 M sodium carbonate solution was added to the tubes. The mixtures (300 µL), after 30 min incubation in the dark at room temperature, were added into a 96-well plate. Ethanol was used as blank. Absorbance measurements were performed at 750 nm using a microplate spectrophotometer (Multiskan Go, Thermo Scientific).

Radical scavenging activity

Antioxidant activity (%) of each sample was assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay. The DPPH solution at the concentration of 200 µM was prepared in methanol. 50 µL of two concentrations (10% and Neat) from each vinegar sample (prepared in distilled water) and 150 µL of the DPPH solution were added to the well of the 96-well plate. After 30 min incubation in the dark at room temperature, the absorbance was measured at 517 nm by using a microplate reader (Multiskan Go, Thermo Scientific). Absolute methanol was used as blank. The percentage of the radical scavenging activity (RSA) was calculated based on the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{(\text{Ac} - \text{As})}{\text{Ac}} \times 100$$

Ac: Absorbance of control [DPPH + Methanol without sample]

As: Absorbance of sample [DPPH + Sample (vinegar)]

Bacterial strains and preparation of inoculum

Staphylococcus aureus (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) strains were obtained from the Laboratory of Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University stock culture collection. The bacterial strains were grown in Tryptic Soy Agar (BK047HA, BİOKAR) and incubated for 18-24 h at 37 °C. Each bacterial cell was transferred into 0.9% sterile saline buffer and adjusted to 0.5 McFarland scale (approximately 1.5 x 10^8 CFU/mL).

Agar well diffusion

The antibacterial activity of vinegar samples was determined by agar well diffusion method (Collins et al., 1995). The two-fold serial dilutions of samples (Neat, 50%, 25%, 12.5%, 6.75%, 3.12% and 1.56%) were prepared in sterile distilled water. Each strain of bacteria was adjusted to a 0.5 McFarland standard in 0.9% sterile saline buffer solution. Bacterial suspensions were streaked on Mueller Hinton Agar (BK048HA, BİOKAR) using sterile cotton swabs. Wells (4 mm height and 6 mm in diameter) were made using a sterile borer and filled with the different concentration of vinegar (100 µL/well). Enrofloxacin (64 µg/mL) and sterile distilled water were used as positive and negative controls, respectively. The plates were incubated at 37°C for 24 h. Following incubation, the diameter of inhibition zone was measured with digital caliper.

Biofilm formation

The effects of apple and grape vinegar on biofilm formation was determined as previously described by Čabarkapa et al. (2019) and Sudagıdan & Yemenicioğlu (2012) with slight modifications. Briefly, three wells of a sterile flat-bottomed 96-well polystyrene microtiter plates (TPP 92096, Switzerland) were filled with 145 µL TSB+1% sucrose and 55 µL vinegar dilutions prepared in 0.9% sterile saline solution (Neat, 50%, 25%, 12.5%, 6.75% and 3.12%). Afterwards, 20 µL of each bacterial suspension (adjusted to 0.5 McFarland standard) was inoculated into each microplate well. The final volume of each well was 220

µL. The following controls were used for each microplate; positive control: TSB+1% sucrose (145 µL), 0.9% sterile saline solution (55 µL) and bacterial suspension (20 µL); negative control I: TSB+1% sucrose (220 µL); negative control II: TSB+1% sucrose (145 µL) the appropriate vinegar concentration in sterile distilled water (55 µL) and 0.9% sterile saline solution (20 µL). The plates were incubated at 37°C for 24 h. After the incubation period, the nonadherent bacteria were removed and the microplate wells were gently washed three times with 250 µL sterile distilled water. The formed biofilm on the bottom of wells was fixed with 200 µL of methanol, then incubated for 15 min at room temperature. The wells were emptied and allowed to dry at 55 °C for 1 h, and stained with 200 µL of crystal violet (CV; 0.5%) for 10 min. The excess dye was washed under tap water. Glacial acetic acid (250 µL, 33%, v/v) was added into the wells to extract the absorbed CV from bacterial cells, and the absorbance of the eluted solution was measured at 600 nm using the microplate reader (Epoch, BioTek, USA). The effect of vinegar samples on biofilm formation was calculated based on the following equation (Čabarkapa et al. 2019):

$$\text{Reduction (\%)} = [1 - \frac{(A1 - A2)}{(\text{Apc} - \text{Anc})}] \times 100$$

A1: Absorbance of test wells,

A2: Absorbance of wells with negative control II,

Apc: Absorbance of positive control,

Anc: Absorbance of wells with negative control I (broth only).

Statistical analyses

All experiments were replicated three times. Homogeneity and normality test were applied on data. The biofilm reduction results of the vinegars were evaluated with one-way ANOVA followed by Duncan post hoc multiple comparison test and data of inhibition zone were analyzed with students t-test using statistical software SPSS 21.0. Results were expressed as the mean±standard deviation (SD) and values of $P<0.05$ were considered significant.

RESULTS

Physico-chemical properties of vinegar samples are given in Table 1. The pH values of AV and GV were 3.03±0.16 and 2.94±0.09 respectively. The total acidity for GV (4.08±0.10 g/100 ml) was superior to AV (3.99±0.21 g/100 ml) (Table 1). Total phenolic content was 58.68 ± 2.06 mg gallic acid/g in GV and 96.11 ± 1.14 mg gallic acid/g in AV. Apple vinegar exhibited higher DPPH radical scavenging activity than GV. DPPH activity (%) of AV and GV was 90.39 ± 0.004 and 88.34 ± 0.002, respectively.

Table 1. Physico-chemical properties of vinegar samples.

	Grape vinegar	Apple vinegar
pH	2.94±0.09	3.03±0.16
Total acidity (g/100 ml)	4.08±0.10	3.99±0.21
Total phenolic (ug Gallic acid/mg)	96.11±1.14	58.68±2.06
DPPH 10%	22.88±0.01	17.01±0.009
DPPH 100%	88.34±0.002	90.39±0.004

Antimicrobial activities of apple and grape vinegar against selected pathogens are given in Table 2. Agar well diffusion method showed that both AV and GV samples had antibacterial activity against *S. aureus* and *P. aeruginosa* (Figure 1). The antibacterial activity of GV was superior to AV ($p<0.05$; Table 2). The populations of *S. aureus* and *P. aeruginosa* were significantly reduced by neat GV and AV samples. While AV and GV samples at 50% concentration did not form a visible zone of inhibition against *S. aureus*, they showed an

inhibitory effect against *P. aeruginosa* (16.25 mm for GV and 16.50 for AV). The vinegar applied at the lowest concentration (25%) did not show any antibacterial effect on either bacteria. Overall, both neat vinegar samples showed strong inhibition against the bacteria tested (Table 2).

Table 2. Antimicrobial activity of apple and grape vinegar against *S. aureus* and *P. aeruginosa* strains using agar well diffusion method

	Zone of inhibition (mm)			
	***S. aureus* ATCC 25923**		***P. aeruginosa* ATCC 27853**	
Concentration (%)	**GV**	**AV**	**GV**	**AV**
Neat	22.75±0.21[a]	21.75±0.07[b]	22.0±0.98	21.55±0.63
50	ND	ND	16.25±0.07	16.5±0.14
25	ND	ND	ND	ND
Enrofloxacin (64 µg/mL)	31.9±0.14	31.65±0.21	15.85±0.21	17.5±0.35

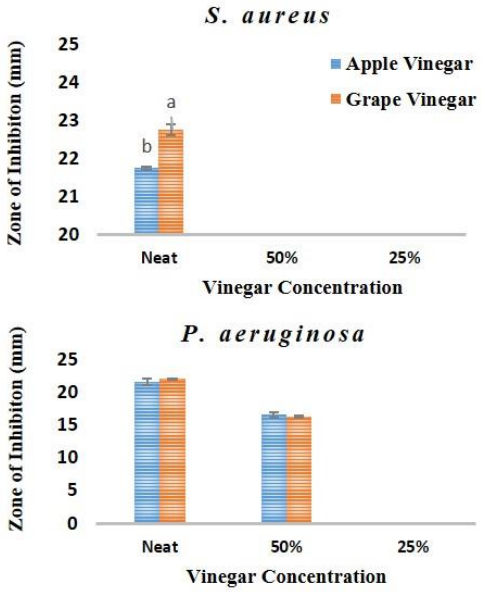

Figure 1. Inhibition zone of *S. aureus* and *P. aeruginosa* strains against apple and grape vinegars. Different superscripts (a,b) indicate that the means are significantly different from each other ($p<0.05$).

Effectiveness of grape and apple vinegar on the biofilm forming ability of selected pathogens is given in Table 3. The solutions containing 50% to 6.25% vinegar samples (both AV and GV) prevented almost 100% biofilm formation in both bacteria. However, solutions containing lower amounts of vinegar inhibited stronger biofilm formation by *P. aeruginosa*.

DISCUSSION

Vinegar is a fermented plant-based product and its content shows differences depending on raw material and techniques used in production process. In this study, the pH values of AV and GV were in line with previous studies (Akbaş et al., 2010; Kahraman et al., 2021). Besides, the vinegar samples complied with regulatory limits for total acidity (total acidity ≥40g/L) which is an important indicator for assessing the quality of vinegar (TSE, 2016).

Previous studies have shown that vinegar samples have high radical scavenging activity and high phenolic content. Aydın and Gökışık (2019) showed that the DPPH activity of the vinegar obtained from Vitis vinifera samples varied between 83.66% and 95.81% and the total phenolic content was 160.23 ± 0.007 µg GAE/ml. In another study, it has been reported that the antioxidant activity of GV and AV samples was 0.119 ± 0.023 and 0.147 ± 0.003 µg TE / mL, respectively. The total phenolic content of both vinegar samples was shown to be 1.025 ± 2.828 and 988 ± 2.828 mg GAE/L (Sengun et al, 2019). In the current study, total phenolic content and antiradical activity of vinegar samples were determined according to spectrophotometric methods. Interestingly,

although high total phenolic content was expected in GV (58.68 ± 2.06 mg gallic acid/g), high phenolic content was obtained from AV (96.11 ± 1.14 mg gallic acid/g). Although GV had a higher phenolic content than AV (Table 1), it had low antiradical activity, which may be due to the presence of various bioactive compounds such as flavonoids that provide antiradical activity (Chen et al., 2016).

Table 3. Effectiveness of different concentration of grape and apple vinegar on the biofilm forming ability of *S. aureus* and *P. aeruginosa*

Vinegar Concentration (%)	Biofilm forming reduction (%)			
	***S. aureus* ATCC 25923**		***P. aeruginosa* ATCC 27853**	
	GV	**AV**	**GV**	**AV**
25.00	98.33±0.40[a]	99.73±1.80[a]	100.75±0.30[a]	100.15±0.52[a]
12.50	98.31±1.29[a]	97.79±0.62[a]	99.40±0.48[a]	99.70±1.04[a]
6.25	97.64±1.52[a]	97.51±2.63[a]	99.40±0.52[a]	98.65±0.92[a]
3.13	49.41±1.82[b]	48.07±1.87[b]	74.66±1.56[b]	65.32±3.82[b]
1.56	28.16±2.84[c]	32.25±2.02[c]	70.59±2.36[b]	63.06±3.09[b]
0.78	17.76±3.31[c]	12.32±3.57[c]	63.65±1.80[b]	62.01±3.12[b]

Each value represents the mean ± standard deviation (SD). Different superscripts within a column (a,b,c) indicate that the means are significantly ($p<0.05$) different from each other. GV: grape vinegar, AV: apple vinegar

Currently, vinegar has gained popularity as an all-natural cleaner due to its contents including organic acids and other compounds with antibacterial activity. Organic acids, such as acetic acid, demonstrate antibacterial activity and their antibacterial activity seems to be associated with altering proton and associated anion concentration in the cytoplasm, resulting in disruption of purine bases and essential enzymes and decrease in bacterial viability (Gómez-García et al., 2019; Lingham et al., 2012). Several studies have reported that vinegar inactivates several bacteria such as *L. monocytogenes*, *Salmonella* Enteritidis, *S. sonnei*, *S. aureus*, *E. coli* and *Enterococcus faecalis*, thus inhibiting growth of and killing most foodborne pathogens (Medina et al., 2007; Chang and Fang., 2007; Zhang et al., 2018; Mohanty et al., 2017). The results obtained in this study are similar to the results obtained from the studies (Baldas and Altuner, 2018; Ousaaid et al., 2021; Yagnik et al., 2018, 2021; Gaber et al., 2020; Bakir et al., 2017; Janchovska et al., 2015). In the current study, generally, we also found that *P. aeruginosa* was more susceptible to vinegars than *S. aureus*, which confirms that of previous study reported that gram negatives are more sensitive than positives (Halstead et al., 2015).

Bacterial biofilms formed by a range of pathogenic microorganisms are a notable challenge in food safety and human health. Inhibiting or preventing biofilm formation has long been an important issue and the most effective way against biofilm formation is to inhibit bacterial growth by using antibacterial agents (Roy et al., 2018). Studies examining the effects of vinegar on biofilm formation are limited. Generally, studies have examined the effects of acetic acid, which is abundant in vinegar, on biofilm formation. Acetic acid is formed during the fermentation of vinegar and present in a 3-5% concentration. 70% apple cider vinegar significantly reduced the biofilm formed by *S. aureus* (Pedroso et al., 2018). Halstead et al. (2015) reported that acetic acid at a concentration of 0.31% statistically significantly inhibited the biofilm formation by *P. aeruginosa*. Tsang et al. (2018) demonstrated that 5% and 3% acetic acid eradicated 96.1% and 85.9% of biofilm-associated methicillin-sensitive *Staphylococcus aureus* (MSSA), respectively. In the present study, both of vinegar solutions (50% to 6.25%) prevented

almost 100% biofilm formation in both bacteria. A positive relation was found between the biofilm reducing ability of vinegar samples and their antibacterial activity in this study.

CONCLUSION

Commercial grape and apple vinegar significantly reduced the viability of *S. aureus* and *P. aeruginosa*, thereby decreasing biofilm formation. Treatment with the vinegar might offer a useful method to decrease the risk of *S. aureus* and *P. aeruginosa* infection either in public spaces or at home.

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