Original Article

Bactericidal effect of grape seed extract on methicillin-resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT — This study was conducted to measure the antibacterial activity of grape (*Vitis vinifera* L; Vitaceae) seed extract against methicillin-resistant *Staphylococcus aureus* (MRSA). Grape seed and skin extracts were tested for antibacterial activity against forty-three strains of MRSA by gel diffusion, growth and respirometric studies. All MRSA strains were found to be sensitive to grape seed extract. Complete inhibition of all bacterial strains tested was observed at a concentration of 3 mg/ml crude grape seed proanthocyanidins extract (GPSE), equivalent of 20.7 µg/ml flavonoid content. Antibacterial activity was bactericidal as shown by a disruption of the bacterial cell wall in scanning and transmission electron microscopy. Grape seed extract is known to be rich in potent antioxidant polyphenolics that could show antibacterial activity. Phenolic compounds in the grape seed extract were assayed by Folin-Ciocalteu's reagent. The considerable antibacterial activity of commonly available grape seed extract could signify a major advancement in the treatment of MRSA diseases.

Key words: *Vitis vinifera*, MRSA

INTRODUCTION

Grapes (*Vitis vinifera*) are consumed worldwide and their seeds are a rich source of phenolic compounds. Around 60-70% of the total grape phenolics are concentrated in their seeds (Zhao *et al.*, 1999). Phenolics are known to have therapeutic activities, acting as immunomodulators, antioxidants, antimutagens, antibacterials and hormone analogs (Halpern *et al.*, 1998). Grape phenolics are simple molecules, such as hydroquinone, pyrocatechol, caffeic acid, ferulic acid, p-coumaric acid, gallic acid, ellagic acid, and resveratrol (Siemann and Creasy, 1992). Furthermore, grape seeds extract is a rich source of diverse bioflavonoids, collectively known as grape seed proanthocyanidins extract (GPSE) (Jayaprakasha *et al.*, 2001). Bioflavonoids are present both in the outer skin of grapes as well as in their seeds, while anthocyanidins are present predominantly in grape skin (Sato *et al.*, 2001). The therapeutic role of grape GPSE is thought to be due to antioxidant activity, which may protect tissues against oxygen free radicals, mutagenesis and lipid peroxidation (Hertog *et al.*, 1995).

A variety of pharmaceutical preparations, which are

applied in the management of the non-infectious diseases, have shown *in vitro* some antibacterial activity. These drugs are called "non-antibiotics" Antibacterial agents can act therapeutically as antibiotics or as nonantibiotic antibacterial compounds. Mechanism of action of antibacterial agents includes inhibition of cell wall synthesis, membrane disruption, inhibition of nucleic acid or protein synthesis, and interference with folate syntheses (Axelsen, 2002). Phenolic compounds are known for their ability to damage microbial cells by altering the selective permeability of the plasma membrane, leading to leakage of vital intracellular substances (Cowan, 1999).

Methicillin-resistant *Staphylococcus aureus* (MRSA), is one of the most significant Gram-positive bacterial pathogens, causing both nosocomial and community-acquired infections worldwide (Mylotte and Tayara 2000). *Staphylococcus aureus*, including MRSA aggregate into grape-like clusters, and cause many diseases, ranging from mild skin infections to more serious invasive infections such as pneumonia, septicemia, and deep-seated abscesses (Holmes *et al.*, 2005). MRSA strains, first described in 1961, have become multidrug-resistant, endemic pathogens (Barber, 1961).

Currently, all MRSA therapies are harsh regimens with severe side effects. Simple therapies including natural products, such as flavonoids in *Scutellaria barbata* D. Don (Lamiaceae) have been reported (Sato *et al.*, 2000) to carry bacterial activities against Gram-positive bacteria, including methicillin-sensitive *Staphylococcus aureus* (MSSA) and MRSA. Many other reports have appeared describing antibacterial activity of polyphenols against *S. aureus* (Jayaprakasha *et al.*, 2003; Myer *et al.*, 2008; Rhodes *et al.*, 2006). Peng *et al.* (2008) have demonstrated antibacterial activity of wild grape root extract against Gram-positive pathogens like MSRA. The purpose of the present investigation was to study simple therapeutics such as phenols to inhibit MRSA. Thus we have examined proanthocyanidins GPSE-rich grape seed extract for the management of MRSA.

MATERIALS AND METHODS

Materials

Folin-Ciocalteu's reagent, potassium persulfate and pyrogallol were purchased from Fluka Chemika, Buchs, Switzerland. Gallic acid 1-hydrate, methanol and nutrient agar were purchased from E. Merck, Darmstadt, Germany. Noble agar was purchased from Difco laboratories, Detroit, MI, USA.

Methods

Preparation of GPSE

Grapes were purchased from the local market and their seeds were removed, washed thoroughly with distilled water, dried under shade at 25°C till constant weight and powdered with Waring blender. The seed powder thus obtained (150 g) was exhaustively extracted with light petroleum ether for 3 hr. The defatted material was three times extracted with redistilled methanol, 200 ml each. Extraction was carried out by stirring the powdered seeds with methanol for 1 hr at ambient temperature followed by suction filtration. The methanolic extracts were pooled and the solvent was removed under reduced pressure at 40°C using a rotary evaporator to give GPSE. The grape pulp, with its skin, was freeze dried and the material thus obtained was stored at 4°C in a tight screw-capped bottle. The freeze-dried material was re-dissolved in distilled water, filtered through Whatman filter paper #2 and used for further analyses.

Evaluation of total phenolics

Total phenolics in GSPE and the pulp were measured by Folin-Ciocalteu method (Singleton *et al.*, 1999) and

calculated as gallic acid and pyrogallol equivalents. Folin reagent measures the total reducing capacity of the sample. Bioflavonoids and monomeric anthocyanins were measured by two methods, as described in the literature (Wada and Ou, 2002; Bahorun *et al.*, 2004) and were calculated as quercetin equivalents (QE).

Bacterial strains

Forty-three strains of MRSA were isolated from clinical specimens collected from various hospital microbiology laboratories in the State of Kuwait. Strains were identified using Viteck II bioMérieux, France and were tested for resistance to methicillin.

Antibacterial assays

Antibacterial activity of GPSE was measured by gel diffusion (Ezeifeke *et al.*, 2004), minimum inhibitory concentration (MIC) (Jackson *et al.*, 1977) and bacterial respiration using a micro-oxymax respirometer (Al-Saleh and Obuekwe, 2005). A standard antibacterial assay, as described by Motti *et al.* (2007) was performed as described below.

The MRSA assay was performed in a peptone yeast extract medium (peptone 10 g l⁻¹, yeast extract 5 g l⁻¹, NaCl 5 g l⁻¹). Inoculation medium (20 ml) was incubated overnight at 37°C with shaking (150 rpm) and diluted 1,000-fold with fresh medium. Culture (198 µl) was transferred into a 96-well microtiter plate. Serial two-fold dilutions of the GPSE (final concentration ranging from 100 to 0.2 µg ml⁻¹) in DMSO (final concentration, 1% [vol/vol]) were added in duplicate to the 96-well microtiter plate. Two control wells were used, one with only solvent and one with 500 µg ml⁻¹ ampicillin. An initial absorbance reading was taken at λ595 nm, and the plates were incubated overnight at 37°C. Final absorbance readings were taken after 24 hr, and the level of growth inhibition was determined by comparing results with the solvent controls after subtraction of background.

Effect of grape seed extract on the bacterial ultra-structure

Bacteria were streaked on nutrient agar media and a loopful was transferred to 200 ml of sterile nutrient broth contained in a flask. The flask was incubated at 37°C for 18-24 hr. After incubation, the bacterial culture was centrifuged (5,000 rpm for 20 min) and pellet was washed three times with saline (0.85% NaCl) and optical density of the final pellet was adjusted to 0.6 (at λ 600 nm) using fresh sterile nutrient broth. A bacterial suspension (0.9 ml), as prepared above, was mixed with 100 µl of 2% nutrient broth and 1 ml of alcohol-free grape seed extract

re-dissolved in 50 mM phosphate buffer (pH 6.75), and these growth vials were left at room temperature (25°C) for 3 hr. Controls contained a bacterial suspension with nutrient broth in a phosphate buffer without grape seed extract. Sample preparation for microscopy was carried out as described by Gerhardt *et al.* (1994) depending on whether the results were to be visualized with scanning or transmission electron microscopy.

Statistical analysis

All experiments were carried out in triplicate and values were expressed as mean $\pm$ standard deviations (S.D.) using Microsoft Excel. Significant differences between groups were analyzed by one-way ANOVA, and Post Hoc Testing was used for inter-group comparisons using the least significant difference (LSD). Statistical significance was set at P-value < 0.05 using SPSS (Version 10.0).

RESULTS

Antibacterial activity

Gel diffusion method

All MRSA strains showed sensitivity, with varying level of inhibition, when tested against grape seed extract. MRSA strains could be divided into three groups based on the diameter of the inhibition zone: weakly sensitive (16 clones), moderately sensitive (15 clones) and highly sensitive (11 clones) (Table 1).

Bacterial growth inhibition, minimum inhibitory concentration (MIC) and respirometric studies

Five strains were selected for determination of MIC according to the method described by Jackson *et al.* (1977). Three strains (10, 11, and 17) were selected from the low sensitivity against MRSA group, while two strains (1, 28) were selected from the high sensitivity against MRSA group. In general, killing of all MRSA strains was observed at a concentration of 20.67 μ g of the flavonoid content (method 1) of the crude GPSE. In addition, using sub-inhibitory concentrations of the extract (0.7, 2.0, 4 μ g/ml flavonoid content), all tested strains demonstrated a strong correlation (correlation coefficient $R^2 \geq 0.9$)

between the extract concentration and percent inhibition that increased with increasing extract concentration (Fig. 1). These results were also supported by a respirometric assay.

Bacterial ultra structure

Bacterial ultra-structural changes were assessed by scanning electron microscopy. Five untreated MRSA strains appeared as round-to-oval cocci with smooth surfaces, while bacterial cells treated with grape seed extract appeared larger, rougher, and partially irregular (Fig. 2). Furthermore, the effect of grape seed extract on cell surfaces and components was studied by transmission electron microscopy. MRSA cells treated with grape seed extract were enlarged, with reduced intracellular contents, as compared with controls (Fig. 3).

Evaluation of total phenols and flavonoids

Total phenolic compounds in grape seed extract were present at 209.9 mg/g gallic acid equivalents (GE), whereas total phenols in lyophilized grape pulp were present at 0.9306 mg/g (GE). Therefore, GPSE was 209-fold richer in phenolic content than PPE along with its pericarp. In both samples, phenolic content, when calculated as pyrogallol equivalents (PE), was lower, at 184.8 mg/g and 0.8192 mg/g, respectively.

Total flavonoids and monomeric anthocyanins were measured by two methods as described by Bahorun *et al.* (2004) and were calculated as QE. GPSE did not show presence of anthocyanin while it was rich in flavonoids. On the other hand, grape pulp showed presence of both anthocyanins and flavonoids but their presence was very poor in these extracts (Table 1).

DISCUSSION

Many researchers have reported variable phenolic content in GPSE (Karadeniz *et al.*, 2005), possibly due to differences in grape varieties and/or differences in extraction methods. Baydar *et al.* (2004) have reported that grape seeds, when extracted in an acetone:water:acetic acid mixture (90: 9.5: 0.5 v/v), yielded a total phenolic content of 667.87 mg/g, while Lachman *et al.* (2004) have report-

Table 1. Effect of grape seed extract on the growth of MRSA strains as determined by gel diffusion method

Strain #	Diameter of inhibition zone (cm)	Strain sensitivity
2, 7, 9, 10, 11, 12, 13, 14, 17, 24, 25, 29, 34, 36, 42, 43	1.1-1.3	Weak sensitivity
4, 8, 19, 20, 21, 22, 30, 31, 32, 33, 35, 37, 38, 39, 41	1.4-1.5	Moderate sensitivity
1, 3, 5, 6, 15, 16, 23, 26, 27, 28, 40	1.6-1.7	High sensitivity

Fig. 1. Effect of grape seed extract on the growth of MRSA strains 1, 10, 11, 17, and 28. Number of grown colonies of the treated group was subtracted from untreated samples (control). Percentage inhibition was calculated by dividing it by the number of grown colonies of control and multiplying it by 100. Experiments were done in triplicate and SEM was calculated.

Fig. 2. Scanning electron micrographs of MRSA strain 17. Untreated MRSA strain 17 (control): bacterial cells appeared round to oval cocci with smooth surfaces (A and B), magnification, $\times 10,000$ and $\times 22,000$ respectively. Treated MRSA strain 17 with grape seed extract: bacterial cells appeared large and irregular shape and partially disintegrated (C and D), magnification, $\times 10,000$ and $\times 22,000$ respectively.

Bacterial effect of grape seed extract

Fig. 3. Transmission electron micrographs of MRSA strain 17. Untreated MRSA strain 17 (control): bacterial cells have thick dense cell wall with smooth surfaces (A and B), magnification, $\times 10,000$ and $\times 20,000$ respectively. Treated MRSA strain 17 with grape seed extract: bacterial cells show reduced residual cellular contents with partial disintegration of the bacterial cell surfaces (C, D and E), magnification, $\times 10,000$ and $\times 20,000$ respectively.

Table 2. Phenolic, flavonoid and monomeric anthocyanin content of grape Seeds and pulp measured as pyrogallol and gallic acid equivalents

Grape extract	Total phenols	Total flavonoids	Total monomeric anthocyanin
Grape seeds *	184.8 $\pm$ 7.1 mg/g PE	(Method I) 6.89 $\pm$ 0.13 mg/g QE	No anthocyanin
	209.9 $\pm$ 8 mg/g GE	(Method II) 17.68 $\pm$ 1.8 mg/g QE	
Grape pulp ^o	0.81 $\pm$ 0.017 mg/g PE	(Method I) 0.40 $\pm$ 0.01 mg/g QE	0.29 μ g/mg
	0.93 $\pm$ 0.02 mg/g GE	(Method II) 0.81 $\pm$ 0.05mg/g QE	

* Grape seeds extracted in methanol.

^o Grape pulp lyophilized and re-dissolved in water.

PE = pyrogallol equivalent; GE = gallic acid equivalent; QE = quercetin equivalent.

Values are mean $\pm$ S.D., n = 3.

ed a much lower phenolic content of 98.75 mg/g in grape seeds of the blue variety when extracted with 80% ethanol.

In our studies, total bioflavonoid content of grape seeds, as measured by using two established methods, was found to be 6.892 mg/g or 17.68 mg/g quercetin equivalents QE. This reflects a three-fold difference in the flavonoid content as measured by these two meth-

ods, showing method variability for the measurement of polyphenols in herbal extracts. Grape pulp, along with its pericarp (GPE), showed only negligible quantities of flavonoids by these assays (0.4026 mg/g and 0.81225 mg/g QE). We examined colorless grape pulp without its pericarp and found no presence of flavonoids, indicating that flavonoids were present only in the pericarp, which was

only a fraction of the grape total pulp by weight. The reason, then, for the low level of flavonoids in grape pulp (GPE) is likely that the weight of the pericarp as compared to the total grape pulp is negligible. Jayaprakasha *et al.* (2001) reported that the measurement of flavonoids in GPSE greatly depended upon the method of extraction. When grape seeds were extracted with ethyl acetate:water (17:3 v/v), it yielded the highest amount of flavonoids (540 mg/g, measured as catechin equivalents (CE)). However, when grape seeds were extracted with methanol, it yielded only 160 mg/g (CE) of flavonoids. The latter amount was ten times higher than the yield we obtained (QE) by extracting powdered grape seeds in methanol. This wide variation in results may reflect different extraction procedures using different solvents. In contrast, Karadeniz *et al.* (2005) reported total flavonoid contents of 1.069 mg/g and 0.452 mg/g (CE) for Muskule and seedless grape varieties. Our results are somewhat consistent with these values of polyphenols in grape seeds.

Proanthocyanidins, another class of polyphenols that comprise a significant component of the grape pericarp, are responsible for the color of many fruits and vegetables. From the UV absorption pattern of the extract, it was concluded that proanthocyanidins were not present in GPSE. However grape pulp and pericarp showed only a small amount of proanthocyanidins, 0.294 µg/mg (0.294 mg/l), measured as cyanidin-3-glucoside. This small amount of proanthocyanidins could be due to the minute contribution made by the grape pericarp, which is rich in these polyphenols. Negro *et al.* (2003) reported an absence of proanthocyanidins in grape seeds, and only 0.64 g l⁻¹ of proanthocyanidins in grape pericarp.

In the present study, all MRSA strains showed variable sensitivities to GPSE based on the gel diffusion method. The diameter of inhibition zones varied from 1.1 cm to 1.7 cm (Table 2). No inhibition zones were detected with grape GPE. The magnitude of inhibition (diameter of the zones) of the MRSA strains used in this study was comparable to that demonstrated by some known antibiotics (Fiebelkorn *et al.*, 2003).

The MIC assay was used to determine the lowest concentration of antibacterial compounds that would inhibit the growth of the tested MRSA strains. MIC is considered to be the gold standard for determining the susceptibility of microorganisms to antibacterials (Bala *et al.*, 2005). In the present study, MIC was determined to be 20.7 µg/ml of GPSE flavonoid content for all MRSA strains. This MIC value is higher than that determined by Jayaprakasha *et al.* (2003), who reported a value of 1,000 ppm for inhibition of *Staphylococcus aureus* by GPSE. Differences in MIC values for the same bacterial species have been com-

monly reported (Taguri *et al.*, 2004) and could be due to intrinsic differences between different strains of the same species.

Analyses of the inhibitory effects of GPSE on the growth and respiration of selected MRSA strains (1, 10, 11, 17 and 28) showed significant ($p < 0.05$) inhibition at different concentrations (0.1 mg/ml, 0.3 mg/ml, 0.6 mg/ml, 3 mg/ml, 6 mg/ml, 9 mg/ml, and 12 mg/ml) of GPSE. In addition, a significant ($p < 0.05$) difference between the growth rate of the treated and non-treated MRSA strains was observed. The potential inhibitory effect of GPSE has been demonstrated by other researches. Baydar *et al.* (2004) reported that Gram-positive bacteria such as *S. aureus*, *Bacillus cereus* and *Bacillus subtilis* were more-susceptible to GPSE than Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Escherichia coli*. This inhibitory effect of grape seed extract may be due to powerful antioxidant activities of the polyphenols, which are predominant in GPSE (Moniharapon and Hashinga 2004; Wang *et al.*, 2007) and exhibit inhibitory effects on bacterial growth (Taguri *et al.*, 2004). Resveratrol, a major component of grapes, inhibits *Neisseria gonorrhoeae* and *Neisseria meningitis* (Docherty *et al.*, 2001).

Antibacterial agents inhibit bacterial growth through a variety of complex mechanisms, including inhibition of cell wall synthesis, disruption of cell membranes, inhibition of nucleic acid synthesis and protein synthesis, and inhibition of nucleic acid metabolism (Oyaizu *et al.*, 2003). In the present study, electron microscopy results have shown that GPSE probably affects the cell wall and/or cell membrane (Figs. 2 and 3). Similar results have been reported by Sieradzki *et al.* (1999) who demonstrated uneven-walled morphology after treatment of *S. aureus* with vancomycin. This effect of vancomycin on the cell wall of MRSA has also been reported by other researches (Palazzo *et al.*, 2005). Additionally, antibiotic treatment is known to induce other cellular changes, such as reduced cellular contents (Lehrer *et al.*, 2003). Small natural product molecules also have the potential to impact bacterial cellular structures. For example, natural products such as flavonoids and polyphenolic phytochemicals have the ability to inhibit bacterial growth by disrupting membrane stability (Tsuchiya, 2001). The present study suggests that polyphenols in GPSE have bactericidal effects on MRSA strains. This may be due to the ability of GPSE to disrupt cell wall and/or cell membrane, along with cell enlargement, as indicated by electron microscopy and growth studies. Further studies to pinpoint the nature of the phenolic component responsible for this significant activity against MSRA are in progress.

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