



Antibacterial and Antibiofilm Activity of Grape Seed Extract Against Carbapenem Resistant and Biofilm Producer Enterobacteriaceae

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Abstract

Background and objectives: Carbapenem-resistant and biofilm producing Enterobacteriaceae are a major health problem. This study was carried to determine the antibacterial and antibiofilm activity of grape seed extract (GSE) against carbapenem-resistant and biofilm producing Enterobacteriaceae isolates. **Methods:** Antibiotics susceptibility patterns were detected by the disk diffusion method. carbapenem-resistant Enterobacteriaceae (CRE) isolates were screened by carbapenems disks and imipenem minimum inhibitory concentrations (MIC). The biofilm formation was detected by the microplate method. The carbapenemase genes were detected by PCR. The total polyphenolic content of GSE was determinate by Folin Ciocalteu technique. The antibacterial and antibiofilm effects of GSE were tested by the MIC and biofilm inhibitory concentration (BIC), respectively. **Results:** In this study, total phenolic content of extracted 1 gram of GSE was equivalent to 700 mg gallic acid. Eighteen non-duplicated CRE isolates were selected. All isolates were fosfomycin susceptible. Variable frequency of resistance to the other tested antibiotics was observed. The *bla*OXA-48 was the most common carbapenemase type. Among 18 isolates, 13 were biofilm producer while GSE inhibited CRE growth at 1024 µg/mL for 15 isolates and 2048 µg/mL for three isolates. Biofilm production was inhibited by GSE in 2000 µg/mL, 4000 µg/mL and 8000 µg/mL after 72 h incubation. **Conclusion:** The significant antibacterial and antibiofilm effects of GSE suggested GSE as a promising candidate for treatment of infections caused by these organisms.

Keyword: biofilm; carbapenem-resistant; Enterobacteriaceae; grape seed extract

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Introduction

Most Enterobacteriaceae are human microbiota; however, Enterobacteriaceae is one of the most common opportunist pathogens of human and may cause infections such as urinary tract infections, sepsis, hospital, respiratory tract and intra-abdominal infections [1]. The increased level of resistance to antibiotics among Enterobacteriaceae has been stated as challenges in empiric therapy, especially when multidrug-resistant (MDR) Enterobacteriaceae caused infections are suspected or endemic [2].

Recently, in response to broad-spectrum resistance, the carbapenems have been appointed as the agents of last resort in a treat of these infections [3]. As a result, carbapenem-resistant Enterobacteriaceae (CRE) have emerged and widespread outbreaks of CRE have been increasingly reported [4]. CRE differ from most other MDR pathogens in that there is no reliable option for their treatments. The increasing frequency of CRE has potentially adverse effects on global public health and should be appointed

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as of emergency by the international medical community [5]. Available alternatives for CRE treatment have been limited by pharmacologic properties, side effects and administration issues. Surveys to the introduction of new drugs with targeted anti-CRE effects should be a topic priority for the pharmaceutical companies, funding agencies, and governments worldwide [6].

Biofilm formation is a major cause of implant failure and often limits the lifetimes of many indwelling medical devices. Once in the biofilm, extracellular polymeric substances shield bacteria from opsonization and phagocytosis. In addition, in vitro experiments have demonstrated that the bacteria in biofilms are considerably less susceptible to antibiotics than their planktonic counterparts. Treatment of the established biofilm infection is frequently futile with current remedies [7,8]. The growing antibiotic resistance is considered as a serious global health problem. There is a pressing need to discover new classes of antibacterial agents particularly from plant sources. Plants have different protective compounds against pathogens; therefore, phytochemicals can be a good source for the preparation of new antimicrobial agents [9]. Some studies have described the antibacterial effects of grape seed extracts (GSE) against bacterial pathogens [10,11]. In this study, we intended to examine the antimicrobial and antibiofilm effects of GSE obtained from Azerbaijan, Iran on CRE and biofilm producing Enterobacteriaceae.

Material and Methods

Ethical considerations

The present study was approved by the Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran (IR. TBZMED. REC.1395.969).

Preparation of grape seed extract

Grapes were purchased from the local market and were identified as *Vitis vinifera* var. *Shani* by Dr. Abbas Delazar; School of Pharmacy and Drug Applied Research Centre, Tabriz University of Medical Sciences, Tabriz, Iran. Their seeds were removed and washed with distilled water. They were dried at 25 °C and were ground. Then they were extracted successively by using ethanol in an extractor for 20 h. The extracts were dried in a

condition of decreased pressure and certain temperature (40- 50 °C) in a rotary evaporator [12].

Bacterial strains

This study was performed on CRE isolated from hospitals of Tabriz, Iran during 2015. The standard biochemical methods were performed for identification, and carbapenem disks were used for screening of CRE. Bacterial isolates included 16 isolates of *Klebsiella pneumoniae*, one isolate of *Enterobacter aerogenes* and one isolate of *Escherichia coli*.

Antibiotics susceptibility testing

Disk diffusion method

Antibiotics susceptibility patterns were performed by the disk diffusion method. The antibiotic disks (Mast, England) included ceftriaxone, ceftazidime, ertapenem, ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, fosfomycin, cefazolin, cefuroxime, cefepime, aztreonam, imipenem, meropenem, streptomycin, gentamicin, tobramycin, kanamycin, amikacin, tetracycline, nalidixic acid, ciprofloxacin, nitrofurantoin and trimethoprim-sulfamethoxazole [13].

Imipenem MIC

The micro broth dilution method was used to determine the imipenem Minimum Inhibitory Concentration (MIC) according to the CLSI guidelines. The imipenem concentrations used ranged from 0.25 to 32 µg/mL. Cation-adjusted Mueller-Hinton broth (CAMHB) (Merck, Germany) containing *E. coli* ATCC 25922 without imipenem was applied as the positive control of bacterial growth [13,14].

Modified Hodge test

One-tenth dilution of 0.5 McFarland equivalent *E. coli* ATCC 25922 suspensions were inoculated on the surface of Mueller-Hinton agar (MHA). A meropenem disk (10 µg) was placed in the center of the test plate after inoculation. Carbapenemase-producing *Klebsiella pneumoniae* ATCC 1705 (as the positive control), *K. pneumoniae* ATCC 1706 (as a negative control) and tested isolates were streaked in a straight line from the edge of the disk to the edge of the plate. The plate was incubated overnight at 35±2 °C in ambient air for

16-24 hours. Following incubation, the MHA plate was examined for enhanced growth around the test or quality control organism streak at the intersection of the streak and the zone of inhibition. Enhanced growth of background cultured *E. coli* around the test organism streak was considered as a positive for carbapenemase-producing Enterobacteriaceae [15].

DNA extraction

DNA extraction was done according to the tissue buffer boiling method. First, 20 μL of tissue buffer (0.25% SDS + 0.05 M NaOH) was mixed with a single colony of a bacterial isolate and the mixture was incubated for 10 min at 95 °C. The mixture was centrifuged for 1 min at 13000 g and finally, 180 μL of Milli-Q water was added and the extracted DNA was frozen at -20 °C for long time storage.

Detection of carbapenemase genes

All 18 carbapenem-non-susceptible isolates were further evaluated for the presence of carbapenemase genes by PCR and sequencing of related encoding genes including blaIMP, blaVIM, blaGIM, blaSIM, blaSPM, blaKPC, blaNDM, blaSME, blaIMI, blaOXA and blaNMC-A.

In the present study, the DNA concentrations of supernatants were adjusted to 50 ng/ μL , and 1 μL was added to the multiplex PCR in a 25 μL reaction mixture. Amplification was performed using conditions previously described by Dallenne et al. [16].

Amplification products were visualized on a 1% agarose gel containing ethidium bromide. In the current study, strains for quality control of each gene were provided by the microbiology department of the Tabriz Institute for Pharmaceutical Research, Tabriz, Iran. Finally, the PCR products were purified using the QIAquick PCR Purification kit (Qiagen Company) and bidirectional sequencing was performed using an ABI 3730XL DNA analyzer. Each sequence was evaluated against already known carbapenemase gene sequences using the nucleotide BLAST [17] and the Lahey Clinic database [18].

Detection of biofilm

The microtitre plate assay was used for determination of biofilm formation. One to three

colonies were suspended in five mL of TSB and incubated for 18 h at 35 °C. After incubation, the culture was vortexed and thereafter diluted in the ratio of 1: 100 in TSB supplemented with 0.25% glucose, and 200 μL of this suspension was transferred and incubated in 96-well plates for 18 h at 35 °C. The plates were carefully washed with water and air-dried then stained with 200 μL of 0.9% crystal violet solution for 15 min. After removing the dye solution and washing with water, the attached dye was solubilized with 95% ethanol and the optical density of the adherent biofilm was determined twice by microtitre plate reader at wavelength 630 nm. TSB supplemented 0.25% glucose without organism was considered as the negative control. The ability of biofilm formation was classified into three classifications based on OD value: $\text{OD} \leq \text{ODc}$ = non-biofilm producer (-), $\text{ODc} \leq 2\text{ODc}$ = weak biofilm producer (+), $2\text{ODc} < \text{OD} \leq 4\text{ODc}$ = moderate biofilm (++) producer, $4\text{ODc} < \text{OD}$ = strong biofilm (+++) producer [19].

Determination of total phenolics content of GSE

The total phenolics content of the GSE was detected by the Folin Ciocalteu technique. One mL of the GSE in acetone/water (6/4) was mixed carefully with 0.2 mL of Folin-Ciocalteu reagent for three min. Then, one mL of 2% (w/v) sodium carbonate was added and agitated with a vortex mixer. Afterwards, they were kept in dark for 30 min. The absorbance of the GSE was detected at 750 nm using a spectrophotometer. The assessment was compared to the standard curve of the prepared gallic acid solution and expressed as grams of gallic acid equivalents (GAE) per 100 grams of the extract.

Antibacterial effects of GSE

The MIC of GSE was determined by the microbroth dilution method at concentrations of 32, 64, 128, 256, 512, 1024, 2048, 4069 $\mu\text{g/mL}$. MIC was determined as the lowest concentration which inhibited visible bacterial growth.

Antibiofilm effects of GSE

For evaluation of antibiofilm effects of the GSE, biofilm inhibitory concentration (BIC) was determined by the previously described method [20]. One hundred μL of bacterial suspension equivalent to 0.5 McFarland in broth medium

was transferred to the wells of a flat-bottom 96-well microtiter plate. Bacterial biofilms were formed by immersing the pegs of a modified polystyrene microtiter lid into this biofilm growth plate and incubation at 37 °C for 20 h. Peg lids were rinsed three times in sterile water, placed onto flat-bottom microtiter plates containing serial concentrations of GSE in CAMHB per well, and incubated for 18 to 20 h at 37 °C. The peg lids were washed in sterile water and placed into extract-free CAMHB in a flat-bottom microtiter plate. For transferring biofilms from pegs to wells, each plate was centrifuged at 805 g for 20 min. The peg lid was discarded and replaced by a standard lid. The optical density was measured at 650 nm in a microtiter plate colorimeter before and after incubation at 37°C for 6 h. The biofilm inhibitory concentration (BIC) was determined as the lowest concentration of GSE that inhibited the biofilm formation [21].

Data analysis

The data were analyzed by the descriptive statistics in SPSS software for Windows (version 19 SPSS Inc., Chicago, IL, USA).

Results and Discussion

In the present study, 18 non-duplicated clinical CRE isolates were screened by ertapenem, imipenem and meropenem disks. These isolates were collected from burn infection (seven isolates), urine (five isolates), blood (four isolates), wound (one isolate) and trachea (one isolate). All of these bacteria were resistant to ceftriaxone, ceftazidime, ertapenem, ampicillin, amoxicillin-clavulanic acid and piperacillin-tazobactam. The isolates were fosfomycin susceptible. The frequency of resistance to cefazolin, cefuroxime, cefepime, aztreonam, imipenem, meropenem, streptomycin, gentamicin, tobramycin, kanamycin, amikacin, tetracycline, nalidixic acid, ciprofloxacin, nitrofurantoin and trimethoprim-sulfamethoxazole has been presented in figure 1. According to the broth dilution assay, all initially screened isolates were confirmed as CRE. The range of imipenem MICs was from 4 to 16 µg/mL. MICs of imipenem were 4 µg/mL in four isolates, 8 µg/mL in ten isolates, and 16 µg/mL in four isolates (table 1).

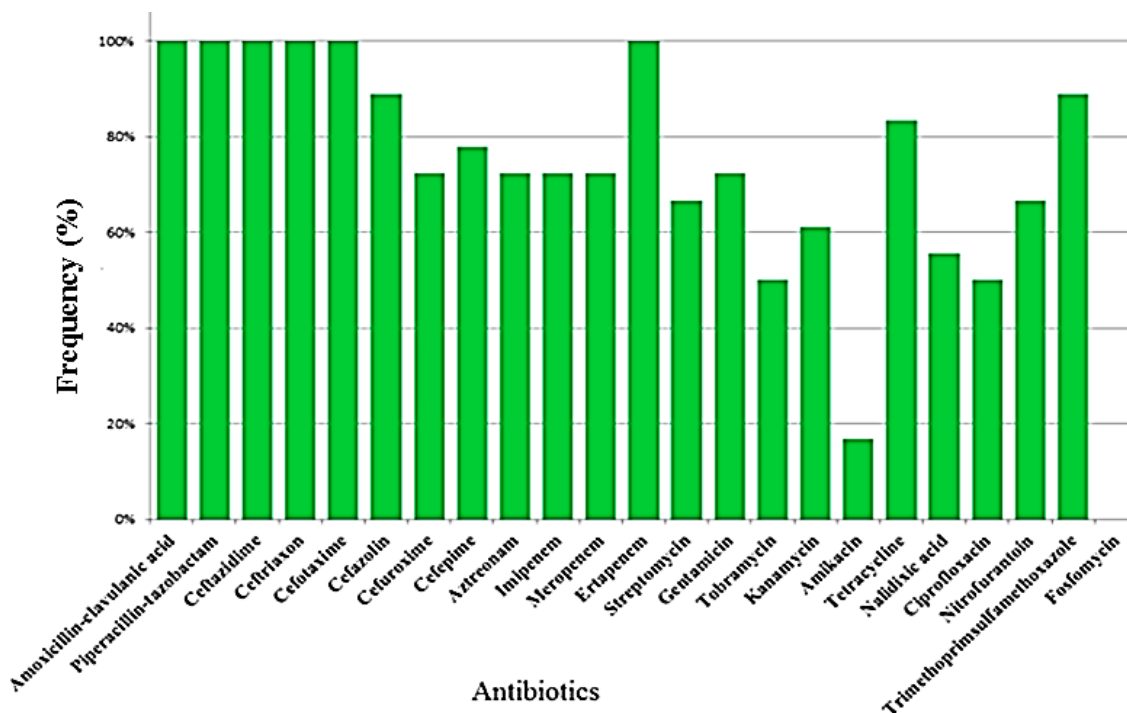


Figure 1. Antibiotics susceptibility patterns of bacterial isolates in this study

Table 1. Antimicrobial and antibiofilm effects of grape seed extract

Bacterial isolates	IMI MIC, $\mu\text{g/mL}$	GSE MIC, $\mu\text{g/mL}$	Biofilm formation	GSE BIC, $\mu\text{g/mL}$
<i>K. pneumonia</i>	4	1024	-	NT
<i>K. pneumonia</i>	8	1024	+	4000
<i>K. pneumonia</i>	8	1024	+	8000
<i>K. pneumonia</i>	8	1024	++	8000
<i>K. pneumonia</i>	4	1024	+	8000
<i>E. aerogenes</i>	8	1024	++	4000
<i>K. pneumonia</i>	8	2048	++	8000
<i>K. pneumonia</i>	16	2048	+++	4000
<i>K. pneumonia</i>	8	1024	+	2000
<i>E. coli</i>	4	1024	-	NT
<i>K. pneumonia</i>	8	1024	++	4000
<i>K. pneumonia</i>	16	1024	++	8000
<i>K. pneumonia</i>	16	1024	+++	4000
<i>K. pneumonia</i>	8	1024	-	NT
<i>K. pneumonia</i>	8	2048	++	4000
<i>K. pneumonia</i>	8	1024	-	NT
<i>K. pneumonia</i>	4	1024	-	NT
<i>K. pneumonia</i>	16	1024	+	8000

Abbreviation: BIC: biofilm inhibitory concentration, IMI: imipenem, GSE: grape seed extract, MIC: minimum inhibitory concentration, NT: not-tested, -: negative biofilm producing, +: weak biofilm producing, ++: moderate biofilm producing, -: negative biofilm producing, +: weak biofilm producing, ++: moderate biofilm producing, +++: strong biofilm producing

Among 18 CRE isolates, the Modified Hodge Test was positive for 17 isolates. The detected carbapenemase genes included *blaOXA-48*, *blaNDM-1*, *blaKPC-2* and *blaKPC-3* genes in eleven, seven, three and one cases, respectively. Carbapenems are the antibiotics of choice for treatment of infections caused by ESBL-producing Gram-negative bacteria due to fewer defeat rates and better results [22]. Another study from Iran reported OXA-48 as a frequent carbapenemase gene among Enterobacteriaceae isolates [23]. The carbapenemase genotypes may vary according to the geographical regions, previous exposure to antibiotics, patient's characteristics and the social factors.

However, the emergence of β -lactamases with direct carbapenemase activity has associated with an increased frequency of CRE. CRE are significantly problematic because of the high mortality associated with their infections and the potential for extensive transmission of carbapenem-resistance genes via mobile determinants [4]. Currently, there are few choices for the treatment of infections caused by CRE. Physicians have been enforced to assess the use of agents such as colistin and fosfomycin, which have been rarely administrated due to serious side effects. Additional treatment options for CRE include optimization of the treatment regimens

and combination therapy [24].

The growing incidence of antibiotic resistance is a serious public health problem, and thus there is an urgent need to discover new classes of antibacterial agents, especially from plant-derived sources. Plants are known to produce enormous varieties of compounds to protect themselves from being attacked by plant pathogens [9]. Overall, inhibition of CRE isolates was observed at concentrations of 1024 $\mu\text{g/mL}$ for 15 isolates and 2048 $\mu\text{g/mL}$ for three isolates by GSE (table 1). GSE is a rich source of monomeric phenolic compounds [11]. As naturally occurring antioxidants, phenolic compounds have been reported to possess diverse beneficial bioactivities, including antifungal, antiviral, anti-inflammatory and anti-mutagenic properties [25]. GSE was also reported for its potential of being a food preservative due to its antimicrobial activity. It has been exhibited as a promising source for the manufacture of new generations of antibacterial agents [26].

In the present study, total phenolics content of 1 gram of GSE was equivalent to 700 mg gallic acid. Han has reported that the GSE had 94% of polyphenol [27]. Two separate studies reported that the GSE had 94% and 97% of polyphenols [26,28]. Jayaprakasha reported the percentage of phenolic contents $46\pm 1.6\%$ and $38\pm 1.4\%$ in GSE

extracted by acetone and methanol, respectively [11]. The total phenolics content of grape product varies with soil composition, environment, geographic conditions, and agriculture practices or experience to diseases [28,29].

The antibacterial effects of GSE have been reported against a broad range of bacteria in vitro. Al-Habibi reported all methicillin-resistant *S. aureus* (MRSA) to be sensitive to GSE equivalent to 20 µg/mL flavonoid content [30]. Jaypraksha reported Gram-positive bacteria such as *S. aureus* and *Bacillus* spp. and Gram-negative bacteria such as *E. coli* and *Pseudomonas aeruginosa* were inhibited at 850-1000 ppm and 1250-1500 ppm concentration of GSE, respectively. These results show that GSE was more effective against Gram-positive than Gram-negative bacteria [11]. Bayder has reported antibacterial effect of GSE against tested bacteria including *Aeromonas hydrophila*, *Bacillus cereus*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *E. coli*, *E. coli* O157: H7, *Klebsiella pneumoniae*, *Mycobacterium smegmatis*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Yersinia enterocolitica* [10]. Other researchers have reported antibacterial effects of GSE against *Helicobacter pylori*, *Aggregatibacter actinomycetemcomitans* and oral anaerobe bacteria [26,31,32].

In the present study, inhibition of CRE isolates was observed at concentrations of 1024 µg/mL (for 15 isolates) and 2048 µg/mL (for three isolates) of GSE (table 1). Diverse MIC of GSE for the same species has been commonly observed. It could be due to an intrinsic diversity of different strains [30]. In addition, it indicated that there was an association between the biochemical properties of the most abundant compounds in the tested extract and the antibacterial effects [10].

The antibacterial mechanism of polyphenols may be related to inhibition of the hydrolytic enzymes, microbial adhesions, proteins transport and nonspecific interactions with carbohydrates. Phenolic components may oxidize sulfhydryl groups [32].

According to the microtitre plate assay, 13 isolates were biofilm producing and screened for determination of antibiofilm effects of GSE. The inhibitory effects of GSE on biofilm were

observed at a concentration of 2000 µg/mL (one isolate), 4000 µg/mL (six isolates) and 8000 µg/mL (six isolates). GSE did not show detectable anti-biofilm effects after 12 h and 24 h. Table 1 has shown the characteristics of CRE and GSE antibacterial and BIC for each isolate. Only a few studies have tested the antibiofilm effect of GSE. Furiga has reported antibiofilm effects of GSE at the concentration of 2000 µg/mL against biofilm of oral anaerobes in a dose-dependent method [26]. Zhao reported that biofilm formation of *Streptococcus mutans* was inhibited by GSE at 4 mg/mL [33].

The potential toxicity of some grape components has been studied. Ugartondo et al. have detected the toxicity of epicatechin conjugates obtained from grape on fibroblast and keratinocyte cell lines. The cytotoxicity of the epicatechin derivatives was similar in the two cell lines; the cytotoxic doses of these components were reported at concentration 3-7 fold higher than their antioxidant amounts after exposure for 24-72 h [34,35].

In conclusion, the current study has shown that GSE could be a potential option for CRE and biofilm producing Enterobacteriaceae. It is the first report for the antibacterial and anti-biofilm activity of GSE against CRE. GSE may be an alternative antimicrobial agent to the treatment of infections caused by CRE isolates. It is suggested that GSE be further studied to evaluate its antibacterial and antibiofilm potential and mechanism of action.

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Author contributions

Reza Ghotaslou: conception and design of study, acquisition of data(laboratory), analysis of data drafting of article and/or critical revision; Farzaneh Farshkhahi: acquisition of data: laboratory or clinical and drafting of article; Abbas Delazar: acquisition of data: laboratory, and critical revision; Mohammad Yousef Memar: acquisition of data and analysis of data

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

GSE: Grape seed extract; CRE: carbapenem-resistant Enterobacteriaceae; MIC: minimum inhibitory concentrations; PCR: polymerase chain reaction; BIC: biofilm inhibitory concentration; MDR: Multidrug-resistant