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Effectiveness of Grape (*Vitis Vinifera* L.) Seed Extracts on Fungi and Bacteria Management

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ABSRACT

Grape (*Vitis vinifera* L.) seeds from 3 grape varieties were powdered and the fatty material was extracted. These extracts were tested for grape storage restriction fungi *Botrytis cinerea, Alternaria alternata, Aspregillus niger* and *Penicillium expansum* and antibacteria activity for Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumonia,* and Gram-positive *Enterococcus faecalis*, *Streptococcus pneumonia* and *Staphylococcus aureus* by pour plate method. The grape seed extracts (GSE) were tested against periimplantitis microflora. Suspension of microorganisms was made in sterile normal saline and adjusted to 0.5 Macfarland standard (10^8 Cfu mL^{-1}). From the stock of 65536 mg mL^{-1} GSEs, serial dilutions were made up to 4 mg mL^{-1}. It was found that, no effective restriction and/or inhibition for tested fungi and Gram-negative *Escherichia coli* 35218, *Klebsiella pneumonia* 700603, *Pseudomonas aeruginosa* 27853, and Gram-positive *Enterococcus feacalis* 51299 bacteria while Gram-positive *Staphylococus aureu* 44300 was inhibited at 32768 µg mL^{-1} GSE of 'Müşküle' variety and GSE 65536 µg mL^{-1} GSE of 'Öküzgözü' and *Streptococcus pneumonia* 49619 bacteria were inhibited at 2048 µg mL^{-1} 4096 µg mL^{-1} and 32768 µg mL^{-1} concentration of 'Kara Dimrit', 'Öküzgözü' and 'Müşküle' GSEs. The results of the study showed that GSEs has potential antimicrobial effects which can be further studied.

1. Introduction

Decays due to *Botrytis cinerea, Aspergillus niger, Cladosporium herbarum, Penicillium expansum,* and *Rhizopus stolonifer* are the main factors restricting the production and commercialization of table grapes (*Vitis vinifera* L.) in the grape producer countries (Franck et al., 2005). Important economic losses usually occur during harvest, cold storage, and transportation of table grapes to markets (Latorre et al., 2002; Franck et al., 2005; Donoso and Latorre, 2006).

Grape (*Vitis vinifera* L.) seeds are considered a rich source of polyphenolic compounds that show antioxidant and antimicrobial effects. GSEs were tested for antibacterial activity by minimum inhibitory concentrations (MIC) method, finding that the inhibitory effect of phenolic compounds from seeds extracts are more potent against Gram-positive bacteria than Gram-negative (Monagas et al., 2003; Arnous and Meyer, 2008; Nirmal and Narendhirakannan, 2011; Adámez, et al., 2012). Grape seed oils (GSO) having antimicrobial activities (Shrestha et al., 2012). Kara et al., (2012) showed that antifungal properties of GSO against phytopathogenic fungi, and potential use of GSO as a preservative agent for table grape preservation in storage period that were comparable by SO_2 generating pads treatment.

Botrytis cinerea Pers. is the most important pathogen affecting table grape production and responsible for significant economic damage in vineyards worldwide (Elmer and Reglinski, 2006), presents high variability in biological traits which can be explained by the high degree of genotypic diversity among isolates (Cotoras et al., 2009). Pre-and post-harvest decay caused by *Aspergillus niger* Tiegh, *Cladosporium herbarum* (Pers.) Link, *Penicillium expansum* Link and *Rhizopus stolonifer* (Ehrenb.) Vuill, has been reported (Zahavi et al., 2000; Latorre et al., 2002).

Chemical control and use of fungicides are the most effective way of preventing the occurrence of *Botrytis* disease (Leroux, 1996). However, following an increased public health concern and fast development of

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resistance to novel fungicides by fungi, biocontrol has become an interesting alternative to conventional methods (Raspor et al., 2010). There are very few options for pathogen suppression, and disease control is dependent upon cultivars with inherent resistance (Topfer and Eibach, 2002). The main principles of biocontrol are defined as the use of living organisms, their products or the use of a biological process to control pest populations (Droby et al., 2009).

GSE against *Alicyclobacillus acidoterrestris* vegetative cells and spores leading to leakage of cellular constituents and may prevent the development of spores into vegetative cells that highlights the potential use as natural antimicrobials to inhibit the growth of *A. acidoterrestris* (Shrestha et al., 2012).

In recent times, there have been increases in antibiotic resistant strains of clinically important pathogens which have led to the emergence of new bacterial strains that are multi-resistant (Aibinu et al., 2004). The non-availability and high cost of new generation antibiotics with limited effective span have resulted in increase in morbidity and mortality (Williams, 2000). Therefore, there is a need to look for substances from other sources with proven antimicrobial activity. Consequently, this has led to the search for more effective antimicrobial agents among materials of plant origin, with the aim of discovering potentially useful active ingredients that can serve as source and template for the synthesis of new antimicrobial drugs (Pretorius et al., 2003; Brown et al., 2009; Moreillion and Que, 2014).

GSE showed high antioxidant and antimicrobial activity which revealed the medicinal properties which positive inhibitory effects with *S. aureus* at minimum inhibitory concentrations (MIC) of 0.625 mg mL^{-1} and minimum cidal concentrations (MCC) of 1.25 mg mL^{-1} respectively. However, the extracts showed minimal or no reactivity against strains of *E. coli*, *K. pneumonia*, *C. parapsilosis* and *C. albicans* (Shrestha et al., 2012). Muscadine grape skin extracts possessed the strongest activity to overall anti- *Helicobacter pylori* efficacy (Brown et al., 2009).

The *Campylobacter* genus comprises 17 species, 14 of which have been associated with human illnesses, and of these, *C. jejuni* and *C. coli* causes more than 95% of the infections attributed to this genus (Park, 2002). *Campylobacter* species are the leading cause of bacterial food-borne diarrheal illness worldwide (Ganan et al., 2012). In the range from 5.08 to 6.97 log CFU mL^{-1}, demonstrating the strong capacity of the GSE to inhibit *Campylobacter* growth (Silván et al., 2013).

Porphyromonas gingivalis and *Fusobacterium nucleatum* bacteria responsible for both periodontitis and bad breath inhibited by GSE (97% polyphenols) (Furiga et al., 2009). GSEs were tested for antibacterial activity by pour plate method against *Bacillus cereus, Bacillus coagulans, Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*, Gram-positive bacteria were completely inhibited at 850-1000 ppm, while Gram-negative bacteria were inhibited at 1250-1500 ppm concentration (Jayaprakasha et al., 2003). Baydar et al., (2006) were examined antibacterial activities of GSE of three different grapes against 15 bacteria at 1%, 2.5%, 5% and 10% concentrations by using the agar diffusion method against some pathogenic and spoilage bacteria including *Aeromonas hydrophila, Bacillus cereus, Enterobacter aerogenes, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Mycobacterium smegmatis, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Salmonella enteritidis, Salmonella typhimurium, Staphylococcus aureus* and *Yersinia enterocolitica*. All tested bacteria were inhibited by GSE using agar well diffusion method. GSEs at 4% concentration were inactive against *A. hydrophila, B. amyloliquefaciens, B. megaterium* and *B. subtilis*, while the acetone: water: acetic acid (90:9.5:0.5) extract at 4% was effective against most of the 15 test bacteria. GSEs at 4% and 20% concentrations may be useful as antibacterial agents to prevent the deterioration of food products (Baydar et al., 2006).

According to Mirkarimi et al., (2013) the antimicrobial activity of the GSE (*Vitis vinifera* L.) was for MIC and minimal bactericidal concentration (MBC) for *Aggregatibacter actinomycetemcomitans* was 3.84 mg mL^{-1} and 7.68 mg mL^{-1} respectively. The GSE has inhibitory and bactericidal effects against *Aggregatibacter actinomycetemcomitans*. There were not any bactericidal, bacteriostatic, and inhibitory effects against *Streptococcus mutans.*

The aim of this work was to investigate the effect of GSE from 3 grape varieties 'Müşküle' (white), 'Öküzgözü' and 'Kara Dimrit' (blue-black) for testing antifungal activity on grape storage restriction fungus *Botrytis cinerea, Alternaria alternata, Aspregillus niger* and *Penicillium expansum* and for antibacterial activity on Gram-negative *Escherichia coli* 35218, *Klebsiella pneumonia* 700603, *Pseudomonas aeruginosa* 27853, and Gram-positive *Enterococcus feacalis* 51299, *Staphylococus aureus* 44300, *Streptococcus pneumonia* 49619 bacteria.

2. Material and Method

2.1. Plant materials

In this context, the grape seeds extract of 3 grape varieties of Müşküle, 'Öküzgözü' and 'Kara Dimrit' extracts have gained considerable attention as antifungal and antibacterial have tested in vitro. *V. vinifera* L. varieties are 'Müşküle' widely grown in Bursa Province, Öküzgözü, widely grown in Elazığ and Malatya Provinces and 'Kara Dimrit', widely grown in Nevşehir province of Turkey were collected at optimal maturity from the commercial vineyard of the original production ecologies (İznik-Bursa, Hoşköy- Elazığ,

and Gülşehir-Nevşehir Turkey respectively) in 2013. 'Müşküle' was purchased local market and crushed and seeds were taken out. Grape seeds of 'Öküzgözü' were collected from Dimes which is alcoholic beverage-processing industry at Tokat and 'Kara Dimrit' seeds were taken out from crushed fruits an industrial unit at Nevşehir.

2.2. Extraction

Dried grape seeds were powdered and the fatty material was extracted in a Soxhlet extractor with petroleum ether (60–80 °C) for 6 h at Selçuk University Faculty of Agriculture Department of Horticulture. The defatted powder was extracted with acetone: water: acetic acid (90:9.5:0.5) for 8 h each separately by the method of Jayaprakasha et al., (2003). All solvents/chemicals used were of analytical grade and obtained from Merck, Istanbul, Turkey.

2.3. Sources and maintenance of organisms

The fungi (*Botrytis cinerea, Alternaria alternata, Aspregillus niger* and *Penicillium expansum*) were obtained and confirmed at the research laboratory of the Department of Food Science of Okan University Istanbul Turkey. They were maintained on Mueller-Hinton *Agar medium* (Sigma, TR). Twenty-four-hour old pure cultures were prepared for use each time.

Gram-positive bacteria (*Enterococcus faecalis* 51299, *Streptococcus pneumonia* 49619 and *Staphylococcus aureus* 44300) and Gram-negative bacteria (*Escherichia coli* 35218, *Pseudomonas aeruginosa* 27853, and *Klebsiella pneumonia* 700603) were obtained and confirmed at the research laboratory of the Department of Medical Microbiology of Necmettin Erbakan University Konya Turkey.

2.4. Culture media

Mueller-Hinton Agar (Sigma, TR) was prepared according to the manufacturer's instruction, autoclaved and dispensed at 20 mL per plate in 12 x 12 cm petri dishes. Set plates were incubated overnight to ensure sterility before use.

2.5. Antimicrobial bioassay

Suspension of micro-organisms was made in sterile normal saline and adjusted to 0.5 MacFarland standard (10^8 Cfu mL^{-1}) (NCCLS, 2000). From the stock of 65536 mg mL^{-1} extract, serial dilutions were made to 65536, 32768, 16384, 8192, 4096, 2048, 1024, 512, 256, 128, 64, 32, 16, 8, 4 mg mL^{-1} (Anonym, 2000). Each labelled medium plate was uniformly inoculated with a test organism by using a sterile cotton swab rolled in the suspension to streak the plate surface in a form that lawn growth can be observed. A sterile cork borer of 5 mm diameter was used to make wells on the medium. 0.1 mL of the various extract concentrations were dropped into each, appropriate labelled well (Atata et al., 2003; Bonjar, 2004). The inoculated plates were kept in the refrigerator for 1 hour to allow the extracts to diffuse into the agar (Atata et al., 2003). The Mueller Hinton Agar plates were incubated at 37 °C for 24 hours. Each determination was carried out in triplicate.

2.6. Determination of MBC

Equal volume of the various concentration of each extract and Mueller Hinton broth (Sigma, TR) were mixed in micro-tubes to make up 0.5 mL of solution. 0.5 mL of MacFarland standard of the organism suspension was added to each tube (Bonjar, 2004). The tubes were incubated aerobically at 37 °C for 24 h. 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 MBC was determined by sub culturing the test dilution on Mueller Hinton Agar and further incubated for 24 h. The highest dilution that yielded no single bacterial colony was taken as the MBC (Akinyemi et al., 2005).

2.7. Determination of MIC

To measure the MIC values, various concentrations of the stock, 65536, 32768, 16384, 8192, 4096, 2048, 1024, 512, 256, 128, 64, 32, 16, 8, 4 mg mL^{-1} were assayed against the test bacteria. The MIC was defined as the lowest concentration.

3. Results

3.1. Antifungal activities

GSEs from Müşküle, 'Öküzgözü' and 'Kara Dimrit' grape varieties, 50 µL inserted to disks fulfilled potato dextrose agar, and sown in *Penicillium expansum, Aspergillus niger, Botrytis cinerea* and *Altenaria alternata* that were tested antifungal activity by 0.5 µg mL^{-1}, 1 µg mL^{-1}, 2048 µg mL^{-1}, 65536 µg mL^{-1}, and pure GSE dosages. There was no inhibition zone on the disks (Figure 1).

Groll et al. (1996) and Ghouila et al. (2017) showed that *Vitis vinifera* L. grape seed extracts have antioxidant, antimicrobial and antifungal effects. GSE was submitted to the antifungal tests against *Aspergillus niger* which is considered as the main cause of the majority of fungal infections (Groll et al., 1996). An inhibition zone of 15.00 ± 0.81 mm was obtained against this fungus, indicating that the sensitivity of *Aspergillus niger* to GSE at 1000 µg mL^{-1} may be considered as positively important. GSE showed a significant resistance against this agent by developing an inhibition zone around the mycelium of 18.00 ± 0.82 mm for a concentration of 1000 µg mL^{-1} (Djerbi et al., 1985). Ghouila et al. (2017), reported that the GSE have antioxidant, antimicrobial and antifungal effects. This simiar result shows an important sensitivity of these bacterial species to GSE at the concentration of 1000 µg mL^{-1}. The same results were obtained by Radovanovic et al. (2009).

3.2. Antibacterial activities

The all extracts at all concentrations were no bactericidal, bacteriostatic, and inhibitory activities against *P. aeruginosa, K. neumoniae, E. faecalis, E. coli* at the end of 48 h. The most sensitive of the bacteria was *S. pneumonia* in all GSE applications of 'Müşküle', 'Öküzgözü' and 'Kara Dimrit' varieties at 2048 µg mL^{-1} and *S. aureus* inhibited by 'Müşküle' 32768 GSE and 'Öküzgözü' 65536 µg mL^{-1} concentration (Table 1).

Antibacterial and antimicrobial effect of GSEs have been reported by Jayaprakasha et al., (2003); Baydar et al., (2006); Brown et al., (2009); Furiga et al., (2009), Nirmal and Narendhirakannan, (2011); Adámez et al., (2012); Shrestha et al., (2012); Mirkarimi et al., (2013); Silván et al., (2013); Molva and Baysal, (2015); that were differed by genus and strains. On the other hand, GSEs' compositions also differ by cultivars Baydar et al., (2006) and Sabir et al., (2012).

Reagor et al. (2002), showed that the grape seed extract was consistently antibacterial against 67 distinct biotypes tested with susceptibility zone diameters equal to or greater than 15 mm in each case. According to Mohammed et al. (2016) alcoholic grape seed extracts have antibacterial activity against four bacterial isolates (*Escherichia coli, Proteus* sp., *Bacillus* sp., *Staphylococcus aureus*), and Kandasamy et al. (2016), GSE produced moderate zone of inhibition ranging between 11-15 mm among the 35-test common clinical isolates namely *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp. and *Pseudomonas aeruginosa*.

According to Butkhup et al., (2016) Shiraz (*Vitis vinifera* L.) red grape cultivar methanolic extract from the seed and skin were active against all Gram-positive bacteria, but these exerted less of an inhibiting effect on the growth of the tested Gram-negative bacteria. The results obtained from the grape extracts were very promising, especially the activity of the methanolic extract of the seeds (GSD), which was effective against *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633 and *S. faecalis* TISTR 459 (MIC = 16 µg mL^{-1}). The highest MIC value of 512 µg mL^{-1} for GSK was estimated for *E. coli* ATCC 29214. The activity of the GSD and GSK against both Gram-positive and Gram-negative bacteria may be indicative of the presence of broad-spectrum antibiotic compounds, which are distributed mainly in the seeds and skins of grapes.

4. Discussion

Grape seeds are proposed to have antimicrobial activity, antioxidant effect and various other benefits to mankind. Many studies were done to assess the antifungal and antibacterial effect of grape seed extract against common clinical isolates and drug resistant pathogenic strains (Djerbi et al., 1985; Reagor et al., 2002; Radovanovic et al., 2009; Shrestha, 2012; Su et al., 2012; Butkhup et al., 2016; Kandasamy et al., 2016; Mohammed et al., 2016; Ghouila et al., 2017). The bactericidal effect of grape seed extract is accounted for the presence of Stigmasterol, a sterol molecule which cause degradation of bacterial components by surface interaction and pore formation in the bacterial cell wall. It might also be related to the presence of tannins which has the ability to inactive microbial adhesions, enzymes and cell envelope transport proteins, their complexity with polysaccharide and their ability to modify the morphology of microorganisms. Therefore, this observation is suggestive of the antibacterial effect of grape seed extract (Kandasamy et al., 2016). According to Shrestha (2012), the structure-activity correlation assays showed that the hydroxyl group of the phenolic compound was found to be effective against *E. coli* and the benzene ring was effective against *S. aureus*. Adámez, et al., (2012) and Butkhup et al., (2016) also indicated the GSE more effective on Gram-positive bacteria. The results provide evidence that the grape seed extract could be a potential antibacterial agent and this effect can further be made evident with improved methodologies (Kandasamy et al., 2016). These strong sensitivities of the bacteria to GSE may be related to the inhibition of the hydrolytic enzymes (proteases and carbohydrolases) or other interactions capable of inactivating microbial adhesins, transport proteins and cell envelope due to the composition of extract in procyanidines, as stated by Cowan (1999).

5. Conclusion

GSEs have no effects on test fungus that are *Botrytis cinerea, Penicillium expansum, Aspergillus niger* and *Altenaria alternate* which can be further studied by different varieties GSEs, and fungal genus and strains. Our results suggest that the use of GSE is a feasible alternative as antibacterial agents to prevent *Staphylococcus aureus* and *Streptococcus pneumoniae*. The results of the study showed that grape seed extract has potential antimicrobial effects which can be further studied. These findings establish a basis for a possible usage of these native varieties as an alternative to synthetic products.

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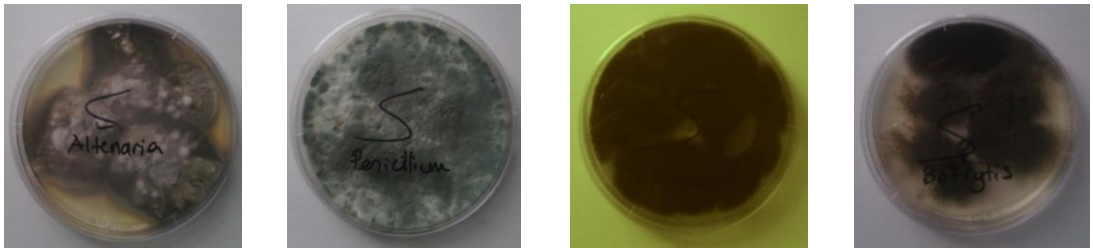
Table 1. Effectiveness on bacteria of the GSEs*

Dosage (μg mL^{-1})	Ec-KD	Ec-Ö	Ec-M	Kp-KD	Kp-Ö	Kp-M	Pa-KD	Pa-Ö	Pa-M	Ef-KD	Ef-Ö	Ef-M	Sa-KD	Sa-Ö	Sa-M	Sp-KD	Sp-Ö	Sp-M
Pure bacteria																		
Pure oil																		
4																		
8																		
16																		
32																		
64																		
128																		
256																		
512																		
1024																		
2048																+	+	+
4096																+	+	+
8192																		
16384																		
32768															+	+	+	+
65536														+				

*Ec-KD: *Escherichia coli* 35218-Kara Dimrit, Ec-Ö: *Escherichia coli* 35218-Öküzgözü, Ec-M: *Escherichia coli* 35218-Müşküle; Kp-KD: *Klebsiella pneumonia* 700603-Kara Dimrit, Kp-Ö: *Klebsiella pneumonia* 700603, Kp-M: *Klebsiella pneumonia* 700603-Müşküle; Pa-KD: *Pseudomonas aeruginosa* 27853 - K Dimrit; PA-Ö: *Pseudomonas aeruginosa* 27853, Pa-M: *Pseudomonas aeruginosa* 27853-Müşküle; Ef-KD: *Enterococcus feacalis* 51299-Kara Dimrit, EfÖ: *Enterococcus feacalis* 51299-Öküzgözü, Ef-M: *Enterococcus feacalis* 51299-Müşküle; Sa-KD: *Staphylococus aureus* 44300-Kara Dimrit, Sa-Ö: *Staphylococus aureus* 44300-Öküzgözü, Sa-M: *Staphylococus aureus* 44300-Müşküle; Sp-KD: *Streptococcus pneumonia* 49619-Kara Dimrit, SpÖ: *Streptococcus pneumonia* 49619-Öküzgözü, Sp-M: *Streptococcus pneumonia* 49619-Müşküle.



Alternaria alternata *Penicillium expansum* *Aspregillus niger* *Botrytis cinerea*

Figure 1. Effectiveness on fungi of the GSEs