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ANTIMICROBIAL ACTIVITY OF RESVERATROL AND GRAPE POMACE EXTRACT

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ABSTRACT

Resveratrol is commonly found in food and drinks, including red wine and grapes. Grape extracts have a potent antimicrobial activity *in vitro*. The antimicrobial activity of plant extracts is the base of their potential application in food preservation agents, pharmaceuticals, cosmetics, alternative drugs and natural therapies. The aim of our study was to evaluate the antimicrobial activity of resveratrol and Blue Frankish pomace extract against Grampositive and Gramnegative bacteria as well as yeasts from the genus *Candida*. Six bacterial strains (three Grampositive bacteria *Staphylococcus aureus* CCM 2461, *Enterococcus faecalis* CCM 4224 and *Listeria monocytogenes* CCM 4699; three Gramnegative bacteria *Escherichia coli* CCM 3988, *Pseudomonas aeruginosa CCM* 1959 and *Salmonella enteritidis subsp. enteritidis* CCM 4420) and three yeast strains (*Candida albicans* CCM 8186, *Candida krusei* CCM 8271 and *Candida tropicalis* CCM 8223) were evaluated using the antimicrobial assay. Pure resveratrol and grape pomace extracts of red variety Blue Frankish were used. Our results show that resveratrol and red grape pomace extract have a very good antimicrobial activity against Grampositive bacteria when compared with Gramnegative bacteria and yeasts.

Keywords: grape pomace extract; resveratrol; pathogenic bacteria and yeasts; antimicrobial activity

INTRODUCTION

Winemaking is currently one of the most relevant agro-industrial activities in the world. Undoubtedly, grapes are an abundant fruit crop worldwide, with *Vitis vinifera* being the species most frequently cultivated for wine production **(Pareja et al., 2015; Barba et al., 2016)**.

Resveratrol (3,5,4′-trihydroxystilbene) is a naturally occurring stilbenoid which has been gaining considerable attention in the medical field due to its diverse biological activities – it has been reported to exhibit antioxidant, cardioprotective, anti-diabetic, anticancer, and antiaging properties. Given that resveratrol is a phytoalexin, exhibiting an increased synthesis in response to infection by phytopathogens, there has been interest in exploring its antimicrobial activity **(Chan, 2002)**.

Although there is still very limited work on the antibacterial activity of resveratrol, it has been shown that resveratrol exhibits antibacterial activity against several Gram-positive and Gram-negative foodborne bacteria **(Chan, 2002; Tegos et al., 2002; Paulo et al., 2010; Paolillo, Carratelli and Rizzo, 2011; Alvarez, Moreira and Ponce, 2012; Alvarez, Ponce and Moreira, 2013; Kumar et al., 2012; Plumed-Ferrer et al., 2013; Augustine et al., 2014; Ferreira et al., 2014; Morán et al., 2014; Promgool, Pancharoen and Deachathai, 2014; Subramanian, Soundar and Mangoli, 2016; Duarte et al., 2015; Kim et al., 2014; Makwana et al., 2015; Ferreira and Domingues, 2016; Liu et al., 2016; Seukep et al., 2016; Silva et al., 2016; Surendran Nair et al., 2016; Klancnik et al., 2016; Lai, Chiu and Chiou, 2017; Lee and Lee, 2017; Oliveira, Domingues and Ferreira, 2017)**.

Based on the available literature, resveratrol has been demonstrated to exhibit different antibacterial activities against numerous strains of foodborne pathogens including *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, and Listeria monocytogenes, E. coli* O157:H7, *Salmonella* Typhimurium, *Vibrio cholera, Campylobacter jejuni, Campylobacter coli, Arcobacter butzleri*, and *Arcobacter cryaerophilus* **(Ma et al., 2018)**.

Grape pomace is a potential source of natural antioxidant and antimicrobial agents. The phenolic compounds in grape pomace extracts exhibit antioxidant, anticancer, and antidiabetic properties **(Ruberto et al., 2007; Hogan et al., 2009; Parry et al., 2011; Zhou and Raffoul, 2012; González-Centeno et al., 2013; Snopek et al., 2018)**, as well as antibacterial activity against *E. coli, L. monocytogenes*, and *S. aureus* **(Ozkan et al., 2004; Darra et al., 2012)**.

More recently, the impact of the gastrointestinal digestion step on the phytochemical content in food, and on their bioactivities (mainly antioxidant capacity), have attracted special attention, which is evidenced by a great number of publications dedicated to this theme

(Gumienna, Lasik and Czarnecki, 2011; Tavares et al., 2012; Correa-Betanzo et al., 2014).

The aim of this work was to demonstrate the antimicrobial properties of resveratrol and red grape pomace extract towards both Grampositive and Gramnegative bacteria as well as yeasts. The microorganisms' sensitivity to resveratrol and red grape pomace extract were determined using the disk diffusion method, while the broth microdilution method was selected to determine the minimal inhibitory concentration (MIC).

Scientific hypothesis

Antimicrobial activity of the resveratrol against bacteria and yeasts.

Antimicrobial activity of red grape pomace extracts against bacteria and yeasts.

To find the lowest minimal inhibition concentration of resveratrol and pomace extract.

To find the most sensitive and most resistant microorganisms to resveratrol and pomace extract.

MATERIAL AND METHODOLOGY

Grape

Ripe grapes from wine cultivars grown in Vrbové (48° 37′ 12″ N, 17° 43′ 25″ E) Slovakia were collected. For the antimicrobial activity, the red variety Blue Frankish grape pomace extracts were used (Figure 1).

Resveratrol

Pure reveratrol was obtained from Sigma Aldrich.

Pomace extract preparation

Pomace extracts were prepared from a single production lot. A portion of the pomace samples (50 g) was immediately freeze-dried after receiving. The samples were extracted with 96% ethanol at a 1:10 ratio (m/v) using overnight shaking. The extracts were filtered through Whatman No. 2 filter paper to remove unwanted residues. After evaporating the organic solvent, the filtrates were dissolved in dimethyl sulfoxide (DMSO) at 20 mg.mL^{-1} as the stock solution and stored at -20 °C for further investigation.

Figure 1 Grape Blue Frankish.

Microorganisms

Nine strains of microorganisms were tested in this study, including three Grampositive bacteria *Staphylococcus aureus* CCM 2461, *Enterococcus faecalis* CCM 4224 and *Listeria monocytogenes* CCM 4699; three Gramnegative bacteria *Escherichia coli* CCM 3988, *Pseudomonas aeruginosa* CCM 1959 *and Salmonella enteritidis subsp. enteritidis* and three yeast strains: *Candida albicans* CCM 8186, *Candida glabrata* CCM 8270, *Candida krusei* CCM 8271 and *Candida tropicalis* CCM 8223. All tested strains were collected from the Czech Collection of microorganisms (Brno, Czech republic). The bacterial suspensions were cultured in the Muller Hinton broth (MHB, Oxoid, Basingstoke, United Kingdom*)* at 37 °C for 24 h and yeasts were cultured in the Sabouraud dextrose broth (SDB, Oxoid, Basingstoke, United Kingdom*)* at 25 °C for 24 h.

Some aspects were considered for the selection of the microorganisms in this study: *S. aureus*, *E. faecalis*, *L. monocytogenes*, *S. enteritidis* and *E. coli* are known to cause foodborne diseases, *P. aeruginosa* is commonly resistant to multiple antibiotics **(Stover et al., 2000)** and *C. albicans*, *C. tropicalis* and *C. krusei* are the main fungi responsible for invasive bloodstream fungal infections, a significant cause of mortality in immunocompromised patients **(Selvarangan et al., 2003)**.

Disc diffusion method

The agar disc diffusion method was used for the determination of antimicrobial activity of the pomace extracts. Briefly, a suspension of the tested microorganism (0.1 ml of 10^5 cells mL^{-1}) was spread onto Mueller Hinton Agar (MHA, Oxoid, Basingstoke, United Kingdom) and Sabouraud dextrose agar (Oxoid, Basingstoke, United Kingdom) at 25 °C. Filter paper discs (6 mm in diameter) were impregnated with 15 µL of the pomace extract and placed on the inoculated plates. Tetracycline was used as a positive control to determine the sensitivity of the studied microorganisms. The plates were kept at 4 °C for 2 h and subsequently incubated aerobically at 37 °C for 24 h and 25 °C for 48 h for bacteria and yeasts, respectively. The diameters of the inhibition zones were measured in millimeters. All the tests were performed in triplicate.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) is the lowest concentration of the sample that will inhibit the visible growth of microorganisms. Pomace grape extracts were dissolved in DMSO (conc. 20 mg.mL^{-1}). MICs were determined by the microbroth dilution method according to the Clinical and Laboratory Standards Institute recommendation (**CLSI, 2019**) in Mueller Hinton broth (Oxoid) for bacteria and Sabouraud dextrose broth (Oxoid) for yeasts. Briefly, the DMSO solutions were prepared as serial two-fold dilutions to obtain a final concentration ranging from 3.9 to 2000 µg.mL^{-1}. The range of resveratrol concentrations tested was 2 – 512 µg.mL^{-1}, before the addition of the cells. Each well was then inoculated with the microbial suspension at the final density of 0.5 McFarland. After a 24 h incubation at 37 °C for

bacteria and 25 °C for yeasts, the inhibition of microbial growth was evaluated by measuring the well absorbance at 570 nm using an absorbance microplate reader Biotek EL808 with shaker (Biotek Instruments, USA). The 96 microwell plates were measured before and after the experiment. Wells without resveratrol and pomace extract were used as positive controls of growth. Pure DMSO was used as a negative control. This experiment was done in eight-replicates for a higher accuracy of the MICs of used pomace grape extracts. The results were expressed in $\mu g.mL^{-1}$.

Statistic analysis

All experiments were carried out in triplicate and the results are reported as means with standard deviations. The experimental data were subjected to analysis of variance (Duncan's test), at the confidence level of 0.05 using the XL STAT 2019 software.

RESULTS AND DISCUSSION

The disk diffusion method shoved that resveratrol exhibited a better antibacterial activity against all tested Grampositive bacteria (Table 1), when compared to Gramnegative bacteria. The lowest antimicrobial activity was found against yeasts which is consistent with previous studies using similar strains **(Paulo et al., 2010)**. The antimicrobial activity using the disc diffusion method ranged from 10.00 ±2.00 (*C. albicans*) to 22.67 ±2.08 (*E. faecalis*).

Similar results were found in case of the pomace extract. The best antimicrobial activity was found against *E. faecalis* while the lowest antibacterial activty was detected against *C. krusei*.

The high antimicrobial effect was observed in the variants with grapevine seeds against *E. coli* **(Jakubcová et al., 2015)**.

In study of **Chan (2002)** established that resveratrol conferred the antibacterial effect. DMSO did not reduce the growth of the tested bacteria, except for a slight decrease in the case of *E. faecalis* at 3.3%. When present at 171 $\mu g.mL^{-1}$ of resveratrol in 1.7% DMSO, the growth of *S. aureus* was inhibited by 80 – 90%. A similar degree of inhibition was observed in case of *E. faecalis* and *P. aeruginosa* at 342 $\mu g.mL^{-1}$ of resveratrol in 3.3% DMSO.

In our work, resveratrol concentrations ranging from 2 to 512 $\mu g.mL^{-1}$ have been tested, and its MIC for all Grampositive bacteria (S*taphylococcus aureus, Enterococcus faecalis and Listeria monocytogenes*), Gramnegative bacteria (*Escharichia coli, Pseudomonas aeroginosa* and *Salmonella enteritidis*) and yeasts (*Candida albicans, Candida krusei* and *Candida tropicalis*) were determined. The microorganism that presented the highest sensitivity towards resveratrol was *Enterococcus faecalis* (MIC 64 $\mu g.mL^{-1}$), followed by *Staphylococcus aureus* and *Listeria monocytogenes*, which have presented with a MIC of 128 $\mu g.mL^{-1}$ (Table 2). Similar results with similar bacteria were obtained in the study by **Paulo et al. (2010)**. In this work, resveratrol concentrations ranging from 3.125 to 400 $\mu g.mL^{-1}$ have been tested, and its MIC for all Grampositive bacteria (*Bacillus cereus*, *Staphylococcus aureus* and *Enterococcus faecalis*) was determined. The microorganism that presented the highest sensitivity towards resveratrol was *Bacillus cereus* ATCC 11778 (MIC 50 $\mu g.mL^{-1}$), followed by *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212, with a MIC of 100 $\mu g.mL^{-1}$.

It was not possible to obtain resveratrol concentrations higher than 400 $\mu g.mL^{-1}$ due to its poor solubility **(Jeandet et al., 2002)**, and therefore its MIC on Gram-negative bacteria was not determined. Consequently, the efficacy of inhibition (in terms of percentage) presented by different

Table 1 Screening of antimicrobial activity of resveratrol using the disk diffusion test in mm.

Bacterial strains	Resveratrol	Pomace extract
Staphylococcus aureus	22.33 ±1.15^{a}	16.66 ±1.53^{b}
Enterococcus faecalis	22.67 ±2.08^{a}	17.67 ±1.53^{a}
Listaria monocytogenes	19.00 ±1.00^{a}	16.67 ±1.53^{b}
Escherichia coli	14.33 ±2.08^{a}	9.00 ±1.00^{b}
Pseudomonas aeroginosa	15.67 ±1.15^{a}	13.00 ±1.73^{b}
Salmonella enteritidis	15.00 ±1.00^{a}	13.33 ±1.53^{b}
Candida albicans	10.00 ±2.00^{a}	6.00 ±1.00^{b}
Candida krusei	12.33 ±2.52^{a}	4.67 ±0.58^{b}
Candida tropicalis	11.33 ±1.53^{a}	5.33 ±0.58^{b}

Note: mean ± standard deviation; different letters in column denote mean values that statistically differ one from another.

Table 2 Screening of antimicrobial activity of resveratrol using the broth microdilution method in $\mu g.mL^{-1}$.

Bacterial strains	Resveratrol	Pomace extract
Staphylococcus aureus	128	500
Enterococcus faecalis	64	250
Listaria monocytogenes	128	250
Escherichia coli	512	1000
Pseudomonas aeroginosa	256	500
Salmonella enteritidis	256	500
Candida albicans	512	500
Candida krusei	512	1000
Candida tropicalis	256	1000

concentrations of resveratrol was used to evaluate its activity against these bacteria. At a concentration of 400 μg.mL^{-1}, the inhibition percentages observed for *Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae* were respectively, 81, 80 and 58%. In addition, it was not possible to determine the MBCs, since the maximum tested concentration was 400 μg.mL^{-1}.

Kačániová et al. (2018) tested four strains of bacteria (two Gram-positive bacteria *Staphylococcus aureus* CCM 2461, *Bacillus cereus* CCM 2010*;* two Gram-negative bacteria *Escherichia coli* CCM 3988, *Pseudomonas aeruginosa CCM 1959)* and four yeasts strains (*Candida albicans CCM 8186, Candida glabrata CCM 8270, Candida krusei CCM 8271 and Candida tropicalis CCM 8223*). For the detection of the antimicrobial activity, the grape pomace extracts of white variety Pálava and red variety Dornfelder were used. *Pálava pomace* extracts were less efficient against the microorganisms tested and Dornfelder extracts were more active against Grampositive bacteria and yeasts. The best antimicrobial activity of pomace extract Blue Frankish grape in our study was found to be similar to resveratrol against Grampositive bacterial strains. The antibacterial activity of four grape pomace extracts was evaluated in the study of **Xu et al. (2015)**. All extracts exhibited antibacterial activity against *L. monocytogenes* and *S. aureus*, but no antibacterial activity was detected against *E. coli* O157:H7 and *S. typhimurium*. Our results partially agree with previous studies on the antimicrobial activity of whole grapes or grape pomace extracts against both Gram-positive and Gram-negative bacteria, with the most pronounced effects against Gram-positive bacteria **(Darra et al., 2012)**.

CONCLUSION

The present study showed that resveratrol and red pomace extract of Blue Frankish grape showed better antibacterial activity against Grampositive *Staphylococcus aureus*, *Enterococcus faecalis and Listeria monocytogenes* when compared with Gramnegative bacteria and yeasts using both methods, the disc diffusion and the minimal inhibiotn concentration.

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