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Antifungal activity and mechanism of action of carvacrol and thymol against vineyard and wine spoilage yeasts

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ABSTRACT

Antimicrobial activity of carvacrol and thymol against natural yeast flora of the grapes and yeasts known to cause wine spoilage was examined. Carvacrol and thymol exhibited comparable or better antifungal activity than potassium metabisulphite, commercially used wine preservative, against the natural yeast flora and spoilage yeasts. The antifungal activity for both the compounds was better at pH 3.5 than pH 6.5. Addition of carvacrol and thymol (64 μg/mL) in red wine resulted in inhibition of growth of the spoilage yeasts. Carvacrol and thymol exerted their antimicrobial action through membrane damage, leakage of cytoplasmic content and ergosterol depletion. In conclusion, carvacrol and thymol holds promise as a potential natural preservative for the control of wine spoilage.

1. Introduction

Vinification is critically influenced by different steps from growing, harvesting vines in the vineyard to fermentation, aging and storage in the winery. Inadequate precautions or poor practice during any of these steps leads to growth of wine spoilage organisms and consequent wine fault. Acetic acid bacteria (AAB) from genera *Acetobacter, Gluconobacter* and lactic acid bacteria (LAB) from *Leuconostoc, Lactobacilli* and *Pediococcus* genera are major contributors to wine spoilage (Bartowsky & Henschke, 2008). Whereas, the yeasts involved in wine spoilage mainly are from genera *Brettanomyces, Candida, Pichia, Metschnikowia, Debaryomyces, Schizosaccharomyces*, *Zygosaccharomyces, Kluyveromyces, Torulaspora* and *Hansenula* (Loureiro & Malfeito-Ferreira, 2003). Bacterial wine spoilage imparts mousy taint, bitterness, geranium notes, volatile acidity, oily and slimy-texture, and overt buttery characters to the wine (Bartowsky & Henschke, 2008). Whereas, the common spoilage effects due to yeasts are off odours, off-tastes, film formation, cloudiness or haziness, sediments and gas production in bottled wines (Loureiro & Malfeito-Ferreira, 2003; Toit & Pretorius, 2000).

Traditionally, sulphur dioxide (as potassium metabisulphite), sorbic acid, fumaric acid and dimethyldicarbonate (DMDC) are used for preservation of different wines in various countries. The added sulphite exists as molecular SO_2 at low pH values, as bisulphite ions at intermediate pH values, and as sulphite ions at high pH values (Romano & Suzzi, 1993). Molecular SO_2, the antimicrobial/active form from the three, is only around 5–10% in wines with pH 3, which further decreases with increase in pH and becomes negligible at pH 4. Though effective against LAB, some yeasts and AAB have been reported to proliferate at the maximum concentrations of SO_2 added. Health risks like allergic reactions in some individuals those with asthma and organoleptic changes are other problems associated with the use of SO_2. Sorbic acid, fumaric acid and DMDC are less commonly used due to their drawbacks like – sorbic acid is not effective in controlling LAB, AAB and yeasts *Brettannomyces, Zygosaccharomyces,* etc., fumaric acid gets inactivated by fumarase from LAB and yeasts, whereas though highly effective as antimicrobial, DMDC gets hydrolysed to CO_2 and methanol with no lasting activity in the bottled product. Due to these drawbacks and growing consumer bias against chemical preservatives, research efforts are directed towards use of different physical methods and exploitation of natural antimicrobial compounds obtained from plants, animals and microorganisms for wine preservation (Toit & Pretorius, 2000). Pulsed electric fields technology (Marselles-Fontanet, Puig, Olmos, Mínguez-Sanz, & Martín-Belloso, 2009; Puertolas, Lopez, Condon, Raso, & Alvarez, 2009), high power ultrasonics (Jiranek, Grbin, Yap, Barnes, & Bates, 2008), thermal inactivation (Couto, Neves, Campos, & Hogg, 2005) have also been suggested to prevent the growth of wine spoilage yeasts. Many studies have demonstrated the potential of natural products such as hydroxycinnamates and organic acids (Stead, 1993), chitosan

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(Gómez-Rivas et al., 2004), nisin (Rojo-Bezares, 2007), lysozyme (Gerbaux, Villa, Monamy, & Bertrand, 1997), antimicrobial peptides (Bom, Klis, de Nobel, & Brul, 2001; Enrique et al., 2007), killer toxins (Comitini, Pietro, Zacchi, Mannazzu, & Ciani, 2004; Santos, Mauro, Bravo, & Marquina, 2009), natamycin (Thomas et al., 2005), β-glucanases (Enrique et al., 2010), bovine lactoferrin-derived peptides (Enrique et al., 2008) and Vitamin K_5 (Miranda, Jorge, Dominguez, Cepeda, & Franco, 2011) for the control of wine spoilage yeasts and bacteria.

The use of essential oils for the prevention of food spoilage has been largely documented in the scientific literature, but their potential for wine preservation has not been explored. Carvacrol and thymol, mainly obtained from oregano, are two such essential oils. In the present study, we evaluated antifungal potential and mode of action of carvacrol and thymol against natural yeast flora of grapes and wine spoilage yeasts.

2. Materials and methods

2.1. Microorganisms and culture conditions

Natural yeast flora of grapes used in the present study was isolated previously from six different wine grape varieties (Chavan et al., 2009) and included – *Candida azyma, Candida quercitrusa, Debaryomyces hansenii, Hanseniaspora guilliermondii*, *Hanseniaspora uvarum, Issatchenkia orientalis, Issatchenkia terricola, Pichia membranifaciens, Saccharomyces cerevisiae, Zygoascus steatolyticus*. Yeasts that can cause wine spoilage were procured from National Collection of Industrial Microorganisms (NCIM), Pune, India and included *Dekkera bruxellensis* NCIM 3534, *D. hansenii* NCIM 3146, *Metchnikowia pulcherrima* NCIM 3109, *Schizosaccharomyces pombe* NCIM 3457, *Torulaspora delbrueckii* NCIM 3295 and *Zygosaccharomyces rouxii* NCIM 3460. The yeasts were maintained on slopes of MGYP (Malt Extract 0.3%, Glucose 1%, Yeast extract 0.3%, Peptone 0.5%) agar and sub-cultured every 15 days.

2.2. Antimicrobial activity assay

Pure carvacrol (Sigma, Sigma–Aldrich, Madrid) and thymol (A. G. Schering, Berlin) were evaluated for antifungal susceptibility testing by micro broth dilution method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2008) with a modification that the assay was performed using YPG medium at pH 6.5 and 3.5. Appropriate amount of compounds were dissolved in dimethyl sulfoxide to get 100× final strength. The stock is then diluted 1:50 in YPG (Glucose 1%, Yeast extract 0.3%, Peptone 0.5%) medium and 200 μL from this is added to the first row of a 96-well microtiter plate. The compound is serially diluted two fold in successive wells to get a range of 2–256 μg/mL. The yeast cells ($\sim 1 \times 10^3$ CFU/mL), freshly grown in YPG broth in logarithmic phase, were suspended in the medium and inoculated (100 μL) in the wells of the plate. The microtiter plate was incubated for 24–48 h, and growth was checked visibly and measuring absorbance at 600 nm using microtiter plate reader (Bio-Rad, India). The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration exhibiting >90% inhibition of visible growth compared to growth of the control.

2.3. Antimicrobial effect of carvacrol and thymol in wine

Effect of carvacrol and thymol on the growth of spoilage yeasts in red wine (Shiraz; pH 3.5; ethanol 12.0%) was checked. The experiments were carried out in 24 well plates containing 2 mL wine/well. Overnight grown yeast cultures (1×10^5 CFU/mL) were inoculated in the wine along with 64 μg/mL of thymol or carvacrol. Plates were incubated at 20 °C for 16 days without shaking. The tests were run in triplicate and appropriate controls were maintained. Growth was determined by measuring optical density (OD) at 600 nm every 24 h using a microtiter plate reader.

2.4. Propidium iodide staining

Damage or permeabilization of fungal membrane after carvacrol and thymol treatment was checked using a membrane impermeable dye, propidium iodide. For this, actively growing *D. hansenii* ($\sim 1 \times 10^6$ CFU/mL) were suspended in YPG medium containing carvacrol or thymol (64, 128 and 256 μg/mL) and PI (3 μM). After incubation for 6 h at 28 °C with constant shaking (180 rpm), cells were harvested by centrifugation and suspended in phosphate buffer saline (PBS, pH 7.2). The cells were then visualized by epi-fluorescence microscope (Leitz Laborlux S, Germany) equipped with a 50 W mercury lamp and a filter set (N 2.1 filter block with excitation filter BP 515-560, and an emission filter LP580). The digital images were acquired with a Canon Powershot S80 camera and ZoomBrowser EX 5.5 software for image acquisition and management.

2.5. Release of cellular contents

To check the leakage of cytoplasmic contents, *D. hansenii* (1×10^7 CFU/mL) cells suspended in PBS were treated with different concentrations (64, 128 and 256 μg/mL) of carvacrol and thymol for 6 h. After incubation, cells were separated by centrifugation and absorbance of the supernatant at 260 nm was measured.

2.6. Quantitation of sterols

Extraction and quantitation of total sterols after the treatment of *D. hansenii* cells with carvacrol and thymol was done as described by Ahmad et al. (2011). The concentrations of carvacrol and thymol tested were 0, 32 and 64 μg/mL and fluconazole was used as negative control.

2.7. Hemolysis assay

The toxicity of carvacrol and thymol was checked by the red blood cell (RBC) lysis assay as described by Khan and Ahmad (Khan & Ahmad, 2011). The concentrations of the compounds tested were in the range of 2–1024 μg/mL.

2.8. Statistical analysis

All the experiments were performed in triplicate. For antimicrobial effect of carvacrol and thymol in wine, mean values were used to plot line graphs. The OD values were compared by one-way analysis of variance and by using the Bartlett's test for equal variances and the Bonferroni's multiple comparison post-test. $P < 0.05$ was considered as significant. The analysis was performed with GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA).

3. Results and discussion

During wine fermentation, *S. cerevisiae* does not rapidly suppress the growth of natural yeast flora from grape berries and significant growth of the yeasts like *Kloeckera apiculata*, *Candida stellata*, *Candida colliculosa*, *Candida pulcherrima*, and *Hansenula anomala*, *M. pulcherrima* and *H. uvarum* takes place during early fermentation (Heard & Fleet, 1985; Schütz & Gafner, 1993). These yeast species may affect the wine quality favourably or adversely.