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Evaluation of grape-derived compounds with antimicrobial properties as alternatives to sulphites in wine

MSc. Thesis

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Certification

I, Tereza Žáková, declare that this diploma thesis, submitted in partial fulfilment of the requirements for the degree of Master of Science in the Institute of Tropics and Subtropics of the Czech University of Life Sciences Prague, is wholly my own work unless otherwise referenced or acknowledged.

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Abstract

Sulphites are widely used in the food industry as preservatives including wine production. On the other hand, health risks associated with their consumption and demand for foods without artificial preservatives lead to the tendency to reduce their use and substitute them with suitable alternatives. With this aim, the antimicrobial activity of 15 natural compounds present in wine was tested against yeasts and bacteria using the broth microdilution method at two different levels of pH (3.5 and 5.5) in the frame of this thesis. The efficiency of selected compounds was evaluated as the minimal inhibitory concentration (MIC) and further compared with the effectiveness of potassium metabisulphite. The results demonstrated that pterostilbene (MICs 8–128 μg/mL) possessed the strongest antimicrobial activity, followed by resveratrol (MICs 64–256 μg/mL) and luteolin (MICs 128–512 μg/mL). Myricetin, p-coumaric acid and ferulic acid proved only a selective inhibitory effect against some tested bacteria and yeasts (MICs 256–512 μg/mL). Moreover, the tested microorganisms also showed to be more sensitive to the natural compounds than to the sulphite. In summary, the results ascertained that natural wine compounds possess antimicrobial effect against undesirable microorganisms in wine and can thus contribute to the reduction of sulphite usage.

Key words: *Vitis vinifera*, wine spoilage, yeasts, bacteria, antimicrobial activity, sulphur dioxide, natural compounds.

Abstrakt

Siřičitany jsou stále často používaným potravním konzervantem, který je běžný také při výrobě vína. Vlivem zdravotních rizik spjatých s jejich konzumací a poptávky po potravinách bez chemické úpravy ovšem narůstají tendence nahrazovat tyto konzervanty vhodnými alternativami. Proto byla v této práci pomocí mikrodiluční bujónové metody testována antimikrobiální aktivita 15 látek přirozeně se vyskytujících ve víně při pH 3,5 a 5,5 proti kvasinkám a bakteriím, které vedou ke zhoršení kvality vína. Jejich účinnost byla následně hodnocena pomocí minimálních inhibičních koncentrací a porovnána s účinností disiřičitanu draselného. Podle výsledků vykazoval nejsilnější účinnost pterostilbene (MICs 8–128 μg/mL) a dále následovaly resveratrol (MICs 64–256 μg/mL) a luteolin (MICs 128–512 μg/mL). Myricetin, p-coumaric acid a ferulic acid prokázaly pouze selektivní inhibiční účinek proti některým z testovaných bakterií a kvasinek (256 – 512 μg/mL). Výsledky dále ukázaly, že všechny testované mikroorganismy byly k účinným testovaným látkám citlivější než k siřičitanu. Výsledky testů potvrdily, že látky přirozeně se vyskytující ve víně mají antimikrobiální účinky proti nežádoucím mikroorganismům ve víně a mohou tak přispět k redukci používání siřičitanů.

Klíčová slova: *Vitis vinifera*, kažení vína, kvasinky, bakterie, antimikrobiální aktivita, oxid siřičitý, přírodní látky

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AAB	Acetic acid bacteria
DMSO	Dimethyl sulfoxide
GRAS	Generally Recognised as Safe
LAB	Lactic acid bacteria
MIC	Minimum inhibitory concentration

1 Foreword

The history of sulphite usage goes back to the time of ancient Greece where wine barrels were sterilised using sulphur dioxide. Nowadays, sulphites are still applied to inhibit the wine spoilage microorganisms during the process of wine production. In general, they are employed in foods for their antimicrobial effect to suppress growth of bacteria, yeasts and moulds but also for their antioxidant properties to prevent colour changes especially in sour foods such as. Despite their favourable characteristics, sulphites have also adverse effects on the food quality and human health and can cause allergies, diarrhoea, rash, nausea or asthma. As a result, searching for alternatives to sulphite became a research objective focusing also on natural substances such as wine phenolic compounds. Moreover, there has been an increase in popularity of functional and organic foods recently and therefore it can be assumed that unsulphurised wines would be welcomed by many consumers. It may find popularity among allergy sufferers but also those who are interested in healthy ways of living and prefer healthy foods.

As wine itself possesses antimicrobial properties, the subject of this thesis is the study of the antimicrobial compounds naturally present in wine, which could substitute for sulphites. Additionally, beneficial effects of wine consumption on human health have been observed and therefore, the enrichment of wine by these natural substances would support its health promoting effects. Subsequently, the substitution of artificial compounds with natural ones might further increase the health promoting effects of wine. Since I personally know several allergy sufferers and I myself am sensitive to some food additives, I decided to address the topic of the possibility of their substitution in my thesis.

2 Introduction

Wine is a product of microbial fermentation of grape juice, which starts spontaneously by yeasts and bacteria naturally present in winery environment but also on grape skin, stems, leaves or in the air. The spectrum of microorganisms depends on the quality of the harvested grapes and on sanitation applied during the production to prevent undesirable yeasts or bacteria negatively affecting final wine quality (Ingr, 2006; Kling, 1989). Therefore, winemakers endeavour to ensure desirable spectrum of microorganisms, which are essential for proper fermentation (Kling, 1989). The main role of microorganisms in winemaking is to convert grape sugars to alcohol, reduce wine acidity and introduce an interesting and desirable aroma and flavours to the wine. Although grape must have a relatively complete nutrient composition, it can support only a limited number of microorganisms, and wine, with its limited nutrients, is even less inviting. The strongest selection pressures against yeast and bacteria in grape must are high sugar content and low pH, whereas, in wine, it is high ethanol, acidity, SO_2 content and limited nutrients. One of the aims of winemaking is to minimise potential for microbial spoilage (Batrowsky, 2008). Microbial wine spoilage is commonly the result of the combined activities of yeasts, moulds and bacteria.

2.1 Microorganisms in wine

There have been determined three stages of winemaking in which the processed juice can be exposed to contamination. The first stage of the process involves the contact of grapes with winery equipments as a possible source of contamination. The natural microflora is affected indirectly by external conditions such as grape variety, the state of grapes at harvest, the health of grapes, temperature, rainfall, soil, the use of insecticides and fungicides, and other vinicultural practices (Toit and Pretorius, 2000; Moreno-Arribas and Polo, 2005). As for yeasts, the amount and type of yeasts (Table 1) in grapes and grape must depend on the growing season (Farkaš, 1987). The yeasts on the surface of the fruit are carried through grape harvest and therefore, crushing, and pressing into the juice becomes the major source of yeast and bacteria (Kling, 1989).

The second stage of possible contamination may occur during fermentation. At this point, the grape juice contains the natural flora of the grapes along with the microflora of the wine cellar and storage tanks. The composition of the grape juice (high sugar and acid content, and low pH) and the addition of sulphur dioxide exert a selective pressure on

the development of yeasts and bacteria during alcoholic fermentation. *Saccharomyces cerevisiae* is the dominant yeast during fermentation, and the increase in ethanol concentrations further suppresses the development of certain fungi and bacteria. In natural fermentation, the initiators of this process are yeast species belonging to the genera *Candida, Hanseniaspora, Koloeckera* and *Metschnikowia* (Toit and Pretorius, 2000).

Bottling and filling of storage barrels is the third stage of possible exposure to spoilage microorganisms. During this stage, the critical factors are cellar sanitation, limitation of oxygen and the adequate amount of antimicrobial agents to ensure a stable product and prevent growth of undesired yeasts, bacteria but also fungi such as *Actinomyces* and *Streptomyces* presented at the corks and oak barrels (Toit and Pretorius, 2000).

Table 1: Spoilage of wines by yeasts

Yeasts	**Spoilage**
Brettanomyces intermedius *Anamorph: Dekkera intermedia*	Produces volatile phenols causing medicinal, phenolic, horsy, barnyard taints; mousy off-flavour, results from isomers of tetrahydropyridines and produces high levels of acetic acid
Candida spp. *C. vini* *C. stellata* *C. pulcherrima* *C. krusei* *Anamorph: Issatchenkia orientalis*	Wine exposed to air will develop film layers; oxidize ethanol with resulting high concentration of acetaldehyde, volatile acids and esters
Hanseniaspora uvarum *Anamorph: Kloeckera apiculata*	High levels of acetic acid and its esters, and produces killer toxins
Hansenula anomala (now Pichia anomala)	High levels of acetic acid; ester taint, large amounts of ethyl acetate, isoamyl acetate and methylbutyl acetate and development of film layer
Metschnikowia pulcherrima	Grows as a film layer and produces high levels of ethylacetate and acetaldehyde
Pichia spp. *P. farinosa* *P. membranaefaciens* *P. vini*	Produces chalky film layer and high levels of acetaldehyde
Saccharomyces cerevisiae	Re-fermentation of wine with residual sugars
Saccharomycodes ludwigii	High concentrations of acetaldehyde, flocculent masses settle as chunks and form a slimeness
Schizosaccharomyces pombe	Re-fermentation of bottled wine, deacidification
Zygosaccharomyces bailii	Secondary fermentation of wine with large amounts of $CO_{2;}$ turbidity and sediment, high levels of acetic acid and esters
Z.rouxii [1]	Influence enological characteristics of wine, production of malic and succinic acids

Table adapted from (Toit and Pretorius, 2000; Combina, M. *et al*., 2008)

Various types of yeasts are found in sugar solutions excreted by trees, in blossoms, and on fruits. The various yeasts found in grape juice are grouped into non-useful (wild yeasts) and useful (fermentative yeasts) (Kling, 1989) including *Saccharomyces spp.*, which can properly complete the fermentation of grape juice. These yeasts are tolerant to high concentration of ethanol and sugar (Toit and Pretorius, 2000). Factors influencing wine spoilage include acidity, water activity and temperature, low pH, low water activity and low temperature, all having the effect of largely suppressing bacteria. Yeasts show remarkable tolerance to low pH, and thus, are particularly associated to spoilage of wine As for wine, yeasts that are ethanol resistant prevail (Farkaš, 1989). Wild yeasts refer to non-*Saccharomyces* yeasts (e.g. *Candida, Debaryomyces, Hanseniaspora, Hansenula, Kloeckera, Metschnikowia, Pichia, Saccharomycodes* and *Torulaspora*) which can perform a partial alcoholic fermentation, often with the formation of esters. These three species are associated with grape juice and result in spoilage at the early stages of alcoholic fermentation. *Z. rouxii* is the main yeast which causes spoilage in wine due to its resistance to preservatives (Loureiro and Malfeito-Ferreira, 2003). One of the major wine spoilage yeasts re-fermenting juice or wine during storage is *Zygosaccharomyces bailii*, which is highly resistant to preservatives (such as SO_2, sorbic and benzoic acid) and tolerant to a high level of ethanol (>15%) and low pH (<2.0) (Toit and Pretorius, 2000) and only a few viable its cells in a bottle of wine may be sufficient to cause spoilage (Deák, 2008). Its high tolerance to weak acid preservatives and ethanol makes it a notorious spoilage agent of chemically preserved wine (Kalathenos *et al*., 1995). Growth of these *yeasts* may also lead to an increase in acetic and succinic acid, a decrease of L-malic acid and a contaminant reduction in total acidity and an altered ester concentration. *Z. bailli* contamination originates partly from habitats in the winery, but mainly from concentrated grape juice used in wine production (Deák, 2008). *Saccharomycodes ludwigii*, found in bottled wines, is often regarded as "the winemaker's nightmare". This yeast species is highly tolerant to ethanol and resistant to SO_2 and sorbate. It produces high levels of acetaldehyde and has been isolated as a slimy flocculent mass. *Hansenula anomala* (now known as *Pichia anomala*), *Kloeckera apiculata* and *Hanseniaspora uvarum* are mainly associated with the ester taint of faulty wines, which correlates with large amounts of acetic acid. The ester taint can be linked to the presence of ethyl acetate and methylbutyl acetate, which are most prominent in wines possessing this off-flavour. Wines with concentrations of >200 mg/l ethyl acetate and 0.6 mg/L of acetate are regarded as spoiled. *Brettanomyces* is the non-sexual, non-sporulating form of *Dekkera* (Toit

and Pretorius, 2000). Grape berries are the primary source of *Dekkera bruxellensis*, which produce high amounts of acetic acid, but their most objectionable metabolites are volatile phenols causing mousy off-flavour (Deák, 2000). Descriptive words for wines contaminated with *Brettanomyces* include mousy, barnyard-like, horsy, wet dog, tar, tobacco, creosote, leathery and pharmaceutical. Contaminated wines often display an increase in volatile acidity, due to the oxidation of acetaldehyde to acetic acid instead of ethanol. Some yeasts, called film yeasts, can form a film layer on top of stored wine, species of genera *Candida, Metschnikowia* and *Pichia* have been associated with this trait. These yeasts not only create a cosmetic problem, they may also be detrimental to the quality of wine, imparting an oxidised flavour due to the production of acetaldehyde. The development of these yeasts is highly dependent on available oxygen and will thus proliferate in wine exposed to air and in partially filled barrels. The main products formed from ethanol by these yeasts are acetic acid, acetaldehyde and acetate esters (Toit and Pretorius, 2000; Martorell, P *et al.*. 2006; Yurdugul, S. and Bozoglu, F., 2008).

Their occurrence is also significant in cellars, on the cellar equipment and in the working facilities. Furthermore, bacteria are present in wine at all stages of processing and storage. Generally, they are more demanding than yeasts. In wine, two genera of acetic acid bacteria (AAB) and lactic acid bacteria (LAB) occur (Table 2), which can cause favourable but also undesired changes (Farkaš, 1987). Lactic acid bacteria predominate under the anaerobic conditions of vinification and wine storage, and are important in the wine making process for their role in malolactic fermentation (Kling, 1989). They can also be responsible for wine deterioration. LAB are gram-positive bacteria, which produce mainly lactic acid as the end product of carbohydrate fermentation. Therefore, the LAB are divided into three groups according to their metabolic activity: obligatory homofermetative, facultatively heterofermentative and obligatory heterofermentative. The LAB associated with grape juice and wine belong to four genera: *Lactobacillus, Leuconostoc, Oenococcus* and *Pediococcus*. The LAB can tolerate the stress of wine characterized by low pH, presence of ethanol, SO_2, low temperature and the availability of nutrients (Fleet, 1998; Moreno-Arribas and Polo, 2008). Lactic acid-fermenting bacteria have been the subject of many studies. However, in wine, acetic acid-fermenting bacteria prevail and therefore, they will receive more attention in the thesis.

AAB belong to the family *Acetobacteriaceae* and are commonly known as the vinegar bacteria. AAB are gram negative, aerobic, microorganisms producing acetate from sugars and ethanol. The habitat of these bacteria is ubiquitous; they are found on flowers and fruit, in wine as spoilage microorganisms. *Acetobacter* and *Gluconobacter* are of importance to the wine industry. They are linked by the fact that they can oxidise ethanol to acetic acid and are differentiated in that *Acetobacter spp.* can overoxidise acetic acid and lactic acid to CO_2 and H_2O via the tricarboxylic acid cycle (Toit and Pretorius, 2000; Wibowo, D. *et al*., 1985). They produce large amounts of acetate and small amounts of other fatty acids. Wines containing more than 1.1–2.5 g l^{-1} acetic acid are considered spoiled, but concentrations of less than 1 g l^{-1}can already reduce the wine quality. Usual concentrations of acetic acid in wine are 0.3–0.5 g l^{-1}. Growth of *Acetobacter* may produce acetaldehyde at concentrations exceeding the threshold value of 100–200 mg/L (Toit and Pretorius, 2000). They are present during all stages of vinification. Yet in juice prepared from undamaged grapes which are free of mould (e.g. *Botrytis*) they carry only few bacteria and the concentration of acetic acid bacteria is very low contrary to the juice prepared from damaged fruit (Kling, 1989).

Table 2: Spoilage of wines by bacteria - lactic acid bacteria

Bacteria	**Spoilage**
Lactobacillus brevis	Produces ethyl carbamate precursors, tartaric acid utilisation; acidification of wine through the production of acetic and lactic acids; mannitol is formed by the reduction of fructose, mousy taints
L. cellobiosus *L. hilgardii*	Mousy taints from tetrahydropyridine; bitterness arising from glycerol metabolism
L. kunkeei	Production of high levels of acetic acid that is implicated in stuck fermentations
L. plantarum	Tartrare degradation; produce elevated diacetyl levels
L. trichodes	Flocculent growth
Leuconostoc mesentoroides	Forms ropiness; bitterness from glycerol metabolism
Oenococcus oeni	Degrades arginine to produce ethyl carbamate precursors; produces histamine as a biogenic amine; implicated in stuck fermentation; buttery flavour due to increased diacetyl levels
Pediococcus damnosus	Produces histamine, synthesise polysaccharides
P. parvulus	Acrolein formation, from glycerol contributes to bitterness
P. pentosaceus	Produce polysaccharides that increase viscosity

Table adapted from (Toit and Pretorius, 2000)

The genus *Gluconobacter* is represented by three species *G. asaii, G. frateurii* and *G. oxydans*, of which *G. oxydans* is important to the winemaking process. *Gluconobacter* has a preference for sugar-rich environments, where alcohol is present in low concentrations. *Acetobacter spp.* are more ethanol tolerant and can survive through the alcoholic fermentation to exert influence in the final product, where the ethanol produced by the yeasts may be converted to acetic acid reaching concentration up to 3.9 g/L with the legal limit for wine being only 1.2–1.4 g/L. The glycerol produced by yeast and moulds serves as a carbon source for *A. aceti* and *G. oxydans*. These two species can convert glycerol into dihydroxyacetone under aerobic conditions. Dihydroxyacetone can affect the sensory quality of the wine with a sweet/etherish property (Toit and Pretorius, 2000).

Table 3: Spoilage of wines by acetic acid bacteria

Bacteria	Spoilage
Acetobacter aceti *A. estunensis* *Gluconobacter oxydans*	Oxidation of ethanol to acetaldehyde and acetic acid; production of ethyl acetate; production acetion from lactic acid; metabolism of glycerol to dihydroxyacetone; ropiness
Acetobacter Oeni[1]	Strong and pungent odour of vinegar as a result of high levels of acetic acid, accompanied by high concentrations of glycerol, ethyl acetate, ethanol and acetaldehyde

Table adapted from (Toit and Pretorius, 2000; Bartowsky, E.J. and Henschke, P.A[1]., 2008)

2.2 Methods of wine preservation

Wine management employs several preservation strategies covering physical (e.g. pasteurisation, hot bottling and filtration pre-clarification of the juice) and chemical treatments, which traditionally use ascorbic acid, potassium sorbate, benzoic acid but the most used compound is sulphur dioxide (Delfini and Formica, 2001; Batrowsky, 2008; Wedzicha, 1984). They possess antimicrobial activity and inhibit both enzymatic and non-enzymatic browning reactions (VVP, 2004).

2.3 Sulphites in wine production

The use of SO_2 gas as a fumigant in wineries dates back to Roman times, when sulphur was burnt inside wine casks in order to "freshen" them. There are various references to its occasional use in winemaking from the 15th century onwards and there are indications as to the more general use of SO_2 in this capacity towards the end of the 18th century, but the widespread deliberate use of the gas in viniculture is very much a 20th century phenomenon (Hornsey, 2007). For the best product, careful and judicious use of SO_2 is required (Ough, 1988). Until now, it has been the oldest and most widespread preservative in our food supply. Sulphites serve a multifunctional role in foods. They are currently applied to sterilise storage barrels, to stabilise the wine flavour and colour (VVP, 2004). Sulphiting agents include sulphur dioxide (SO_2) and several forms of inorganic sulphite that liberate SO_2 under the conditions of their use (SO_3^{2-} sulphite, HSO_3- bisulphite and $S_2O_5^{\ 2}$-metabisulphite) (Marshall, 2007).

At present, the inhibition of wild, spoilage yeasts prior to the onset of fermentation is achieved by the addition of SO_2 to freshly prepared must. The substance is then re-added at the end of fermentation when its anti-oxidant qualities are sought. Most of the SO_2 found in wine is deliberately added at some stage during processing, but small amounts are contributed by the fermenting yeast which not exceed 10 mg L^{-1}. When dissolved in water it behaves as a fairly strong acid, commonly known as "sulphurous acid" (H_2SO_3). Despite the fact that in wine it can be found in different compounds, for the sake of simplicity it is always identified as sulphur dioxide (Farkaš, 1980). Molecular SO_2 exists as a gas or as single molecules in juice and wine, and is the most important for anti-microbial activity. In addition, its volatility is responsible for the "sulphury" odour and taste. Bisulphite is the predominant form of free SO_2 in juice and wine, with bitter and slightly salty taste possessing a very low anti-fungal activity (Hornsey, 2007). However, several bacterial species are resistant to high concentrations of sulphur dioxide. Physical removal of micro-organisms through filtration typically is mainly conducted prior to bottling and hence is not used to remove micro-organisms during winemaking (Batrowsky, 2008; Loureiro, 2000).

The steps involved in the processing of grapes into wine vary depending on whether the wine produced is white or red. White winemaking requires extracting the juice from the berries (skin and seeds are separated from the juice) as quickly as possible and transforming the grape juice into wine through a temperature-controlled fermentation. Red winemaking requires a period of maceration of the juice, skin and seeds to extract not only colour but also the tannins that will contribute to the structure and body of the final wine. The different phases of the process can be summarised as follows (FAO, 2009):

a) Harvesting method is often closely linked to grape variety and the style of wine intended. Grapes should be as cool as possible at harvest in order to minimise oxidation and unwanted microbial growth. Some wineries make their first addition of SO_2 into the picking bin. Red and white grape varieties contain roughly equal amounts of non-pigmented tannins, but the skins of red varieties contain around two times more amount of the phenolic compounds than white varieties. The flesh consists of large cells with conspicuous, sap-filled vacuoles, which yield most of the free-run juice after crushing and draining. It has the lowest pH (3.0–3.8) of all grape bunch components, and less than 5% of all the phenolics of the berry. Grape skin can contain high levels of sugar. In white grape varieties, 10% of total phenolics are located in the skin, while in red varieties up to 65%. The waxy bloom of skins contains fatty acids and sterols, which are thought to stimulate yeast growth (Hornsey, 2007).

b) Depending on the variety of grape, water content of a ripe berry will range between 70 and 80%. In any crushing, macerating and pressing operation applied to a mass of berries, there is an inevitable mixing of both solid and liquid components. A reasonably complete separation of liquid (juice) components from the grapes, therefore, requires more than one crushing or squeezing operation. The amount of components picked up from skins and stems has a marked effect on the wine's characteristics, sometimes beneficial, sometimes detrimental. Sulphite dioxide is used for its antiseptic and antioxidant properties in the treatment of must (dosage usually 100–200 ppm) (FAO, 2009) as well as to suppress the growth of the components of their natural micro-flora, and to bind with anthocyanin pigments to make them more soluble (Hornsey, 2007). For red wines, small quantities are added to fully eliminate spoilage bacteria and unwanted yeast. In white wine, the functions of SO_2 are similar and in addition, SO_2 prevents the development of a brownish colouring (FAO, 2009).

c) Red wine fermentations are generally regarded as being more of an art than those required for white wine production. The reason for this is that the composition and quality of a red wine generally depend on far more processing variables than are applicable to a white wine. During red wine fermentation, a mass of skin debris called the cap floats to the surface, carried there by bubbles of CO_2. If allowed to remain suspended, this cap will overheat and dry out, thus minimising extraction of colour and flavour components. A dried cap also becomes a repository for undesirable microbes such as acetic acid bacteria. Pressing the grape mass occurs after the free-run wine has been removed from the fermentation vat, and takes place when the winemaker decrees that the required amounts of colour, flavour and tannin have been extracted. With red wines, practicalities demand that they be almost completely fermented in tank before being transferred into a barrel for the completion of fermentation (Hornsey, 2007).

d) Depending on factors such as the type of wine, the size of winery and traditional practices, wine may go to large or small storage tanks or it may remain in the fermentation tanks for several days (Pátek, 1998).

e) In various stages, “green” wine matures into an acceptable market product by settling of finely divided solid particles and colloidal materials and the subtle and slow chemical reactions involving aldehydes and esters that enter into the ultimate bouquet of a wine. Before bottling the wine, SO_2 is added to stabilise the final product (FAO, 2009).

The most commonly used food preservatives are sorbic and benzoic acids, their salts, nitrites, nitrates, compounds with sulphur, particularly sulphur dioxide and sulphites (Vrbová, 2001). Pre-occupation with food additives throughout the Western world during the last decades of the 20th century has to some extent cast SO_2 in villainous role on the world stage and its use in the food industry is now coming under increasing scrutiny (Wedzicha, 1984). Although sulphites are very effective food preservatives, they are subject to regulatory restrictions owing to their potentially adverse effects on health. Many reports have described allergic reactions and asthmatic attacks. Its amount is subject to regulation (Farkaš, 1980). Therefore, they do not have the GRAS status anymore and their content higher than 10 mg/kg must be clearly declared on the ingredient label (Kim *et al.*, 2007). Its intake usually leads to headaches or increased acidity of stomach fluids. As reported, not all individuals are equally sensitive to it. 10–20% of people show decreased tolerance of sulphur dioxide compounds, especially those who suffer from stomach acid deficiency or excess. A certain percentage of the population, mainly asthmatics, have an allergy to the gas (Hornsey, 2007). There is a WHO/FAO recommendation on its maximum daily intake from all sources of 0.7 mg kg^{-1} per body weight. In the EU, wine producers put "E220" on bottle labels of wine containing SO_2 (Hornsey, 2007).

As a result, there has been a considerable focus on identifying appropriate sulphite substitutes for use in foods. Besides some specific technological approaches, the great attention is paid to the natural products with antimicrobial properties, which are described in chapter 2.5.

2.4 Natural products as alternatives to sulphites in wine

In recent years, the growing demands for safer, fresher, more nutritious and novel food products have stimulated research of alternative preservation technologies. The exploration of naturally occurring antimicrobials for food preservation receives increasing attention due to consumer awareness of natural food products and a growing concern of microbial resistance towards conventional preservatives. Consequently, new classes of antimicrobial drugs are urgently required (Moreno-Arribas and Polo, 2005).

As far as plant compounds with antimicrobial properties are concerned, there are certain microorganisms that serve as natural preservatives. Natural products such as lysozyme and bacteriocins have been successfully utilised to inhibit bacterial growth in various

pharmaceutical and food industries for almost 50 years, and lysozyme has recently been approved for use in winemaking. Lysozyme, a small single peptide with muramidase activity, is ineffective against eukaryotic cells; that is, it cannot be used to control spoilage yeasts, such as *Dekkera/Bretanomyces* (Mckenzie, 2002). Bacteriocin production is a characteristic typical of many LAB. Bacteriocins of LAB are ribosomally synthesised antimicrobial peptides that inhibit closely related bacteria by destabilising the function of the cytoplasmatic membrane. Bacteriocin-producing strains resist its own bacteriocin by producing a highly specific immunity factor. Bacteriocins of LAB have received considerable attention due to their potential application as natural preservatives. They may provide a valuable, additional and controllable tool for the inhibition of some deleterious wine-associated organisms.

Nisin is one of the LAB bacteriocin possessing GRAS status that inhibits growth of the most undesirable LAB at low concentrations. Other bacteriocin with GRAS status is bacteriolytic enzyme lysozyme with bactericidal properties. Zymocins are toxins in yeasts, produced by many yeast genera. They kill closely related species (Toit and Pretorius, 2000). Consumers today are increasingly concerned about chemical preservatives in food and tend to choose food products that are natural, save and with multi-health benefits (Wu *et al.*, 2008). These preveservatives could have an activating or inhibiting effect on microbial growth (Frias, 2002). Structural differences between the cell wall of Gram-positive and Gram-negative bacteria also limit its use for controlling AAB species. Lysozyme can be added at various stages throughout grape vinification to inhibit LAB (Gerbaux, 1997). Different LAB vary in their susceptibility to lysozyme in wine (Batrowsky, 2003), however, uses of lysozyme include the inhibition of *Lactobacillus* species during alcoholic fermentation thus reducing the risk of increased volatile acidity, delaying or blocking the onset of malolactic fermentation, controlling LAB populations during sluggish or stuck alcoholic fermentation, and to inhibit onset of malolactic fermentation post bottling. The aroma of wine is not affected by the addition of lysozyme (Batrowsky, 2004; Gerbaux, 1997). As with all treatments of wine, the addition of lysozyme must be considered carefully as it is able to bind with tannins and polyphenols in red wines and typically results in slight decrease in wine haze. Bacteriocins such as nisin, pediocin and plantaricin, produced by some LAB, are small polypeptides that are inhibitory to other bacterial species. These polypeptides act on the cell wall of bacteria to induce cell lysis (Bruno *et al*., 1999; Fleet, 1992). Species of *Lactobacillus* and *Pediococcus* are more resistant to nisin than *O. oeni* strains, and pediocin and plantaricin have been shown to successfully kill *O. oeni* cells (Nel *et al.*, 2002; Mendes Faia and Radler,

1990). A combination of nisin and sulphur dioxide has been proposed as a means to reduce the use of sulphur dioxide in winemaking (Rojo-Bezares *et al*., 2007). More recently, a bacteriocin-like inhibitory substance has been shown to be affective against wine *Lactobacillus* species (Yurdugul and Bozoglu, 2008). Although the use of bacteriocins to control LAB in wine has a great potential, its use in winemaking has not yet been approved. The preservatives are efficient against yeasts or bacteria and cannot substitute antimicrobial agents such as sulphites. Oenological products such as phenolic compounds have been demonstrated to have antimicrobial activity against pathogenic bacteria and several types of these compounds (hydroxycinnamic and hydroxybenzoic acids) can hinder wine bacterial growth (Vivas *et al*., 1997; Reguant *et al*., 2000, Papadopoulou *et al*.,. 2005). Limited investigations have been undertaken in using individual phenolic compounds to control spoilage bacteria (Garcia-Ruiz *et al.*, 2008). Phenolic compounds are found in fruit, vegetables, nuts, seeds, stems, and flowers as well as tea, wine, propolis, and honey and represent a common constituent of the human diet (Vivas, *et al*., 1997). They have been proposed to have a variety of biological effects on human health, including anti-inflammatory activity, enzyme inhibition, anti-allergic activity, antioxidant activity, vascular activity, and cytotoxic anti-tumor activity. Interest in phenolic compounds in wine has increased in recent years because of their potential beneficial effects on human health. Phenolic compounds are responsible for some of the major organoleptic properties of wines, in particular colour and astringency (Jayaprakasha *et al*., 2003; Matthews *et al*., 2004). The value of plants lies in some chemical substances that produce a definite action on the microbiological, chemical and sensory quality of foods, and these phytochemicals have been grouped in several categories including polyphenols, flavonoids, tannins, alkaloids, terpenoids, isothiocyanates, lectins, polypeptides or their oxygen-substituted derivatives. Besides antimicrobial effects, several plants are being used in different areas of human health such as traditional medicine, functional foods, dietary supplements and recombinant protein manufacturing. Phytochemicals, especially flavonoids, polyphenols, anthocyanins and carotenoids, share the major market (Negi, 2012; Gutiérrez-Larraínzar, *et al*., 2010; Kim and Lee, 2004.).

3 Hypothesis

Since it has previously been proved that some of wine compounds possess antimicrobial effects, we assume that substances with antimicrobial effects naturally present in wine could be able to suppress undesirable organisms causing wine spoilage.

4 Thesis objectives

The objective of this thesis is an evaluation of the antimicrobial activity of grape phenolics using *in vitro* methods in order to verify their potential application as alternatives to sulphites in wine production. Specific objectives are: (i) to identify the most prospective antimicrobial phenolic compounds present in grapes using literature analysis and (ii) to test susceptibility of the most important wine spoilage agents to selected compounds.

5 Materials and methods

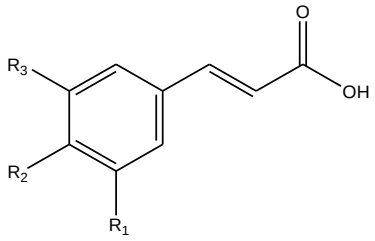
5.1 Strains of microorganisms and growth media

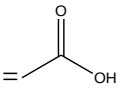
The microbial cultures covering bacteria (*Acetobacter aceti* DSM 3508, *Acetobacter estunensis* DSM 4493, *Acetobacter oeni* DSM 23926) and yeasts (*Dekkera bruxellensis* DSM 3429, *Hanseniaspora uvarum* DSM 70788, *Zygosaccharomyces bailii* DSM 70492, *Zygosaccharomyces rouxii* DSM 70540) were purchased from Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Stock cultures were stored on Sabouraud dextrose agar slant (Oxoid, Basingstoke, UK) at the temperature of 4°C. Microorganisms were cultivated in Sabouraud liquid medium (Oxoid) at the temperature of 26°C for 48 hours (yeasts) and for 24 hours (bacteria).

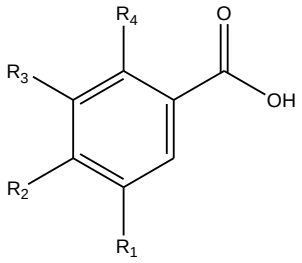
5.2 Tested compounds and other chemicals

Standards of plant compounds (Fig.1) caffeic acid, (-) - catechin, 3,4-dihydroxybenzoic acid, ferulic acid, gallic acid, 4-hydroxybenzoic acid, chlorogenic acid, myricetin, p-coumaric acid, potassium metabisulphite, pterostilbene, resveratrol, sinapic acid and vanillic acid were purchased from Sigma-Aldrich (Prague, Czech Republic); luteolin and rutin from Roth Carl GmbH. All compounds were diluted in dimethyl sulfoxide (DMSO; Lach-Ner, Neratovice, Czech Republic).

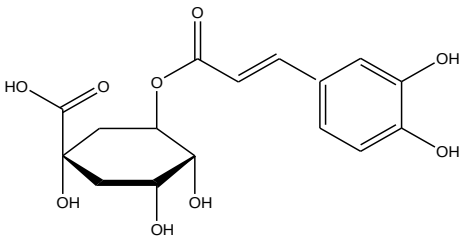
Caffeic acid	$R_1 = R_2 = OH$
p-coumaric acid	$R_2 = OH$
Ferulic acid	$R_1 = OCH_3$; $R_2 = OH$
Sinapic acid	$R_1 = R_3 = OCH_3$; $R_2 = OH$



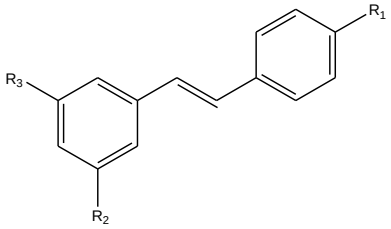
3,4-dihydroxybenzoic acid	$R_1 = R_2 = OH$
Gallic acid	$R_1 = R_2 = R_3 = OH$
Vanillic acid	$R_1 = OCH_3$; $R_2 = OH$
4-hydroxybenzoic acid	$R_1 = OH$; $R_4 =$ 



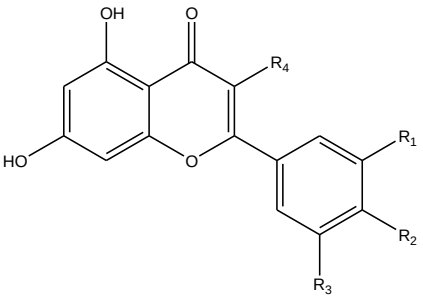
Chlorgenic acid



Pterostilbene	$R_1 = OH$; $R_2 = OCH_3$; $R_3 = OCH_3$
Resveratrol	$R_1 = OH$; $R_2 = OH$; $R_3 = OH$



Myricetin	R1 = R2 = R3 = R4 = OH
Luteolin	R2 = R3 = OH
Rutin	R1 = R2 = OH; R4 = rutinosa



Catechin

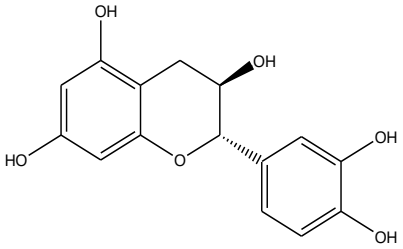


Figure 1: Chemical structure of wine phenolics

5.3 Antimicrobial assay

The microbial assay was performed by the broth dilution method (Wayne, 2008) using 96-well microtiter plates, modified according to the recommendations proposed for effective assessment of anti-infective potential of natural products (Cos *et al*., 2006). The tested concentration ranged from 0.25 to 512 μl/ml. The microtiter plates were inoculated with a bacterial suspension (10 μL) at a density of 10^7 colony-forming units (CFU)/mL and then incubated for 48 hours (yeasts) or 24 hours (bacteria). The growth of microorganisms was determined spectrophotometrically as turbidity using Multiscan Ascent Microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 405 nm. The MICs (minimum inhibitory concentrations) were determined as the lowest dilution that resulted in an 80% reduction in growth compared with the compound-free growth control. The solution of DMSO (1%) was assayed as the negative control did not inhibit any tested strain. All tests were performed in three independent experiments, each carried out in triplicate. We performed the tests at pH 5.5 a 3.5 for which medium was adjusted with 0.1 M HCl.

6 Results and discussion

Among 15 tested compounds, six of them possessed significant growth-inhibitory effect against at least on of the wine spoilage microorganisms assayed in this study. At pH 3.5 (Table 4), pterostilbene, resveratrol and luteolin showed a significant antimicrobial effect against all yeasts and bacteria at the concentration ranging from 32 to 512 μg/mL. Pterostilbene as the most efficient inhibitor of all microorganisms showed a significant activity against yeasts (MICs = 32–128 μg/mL) as well as bacteria (MICs = 64–128 μg/mL). Resveratrol and luteolin inhibited growth of all tested yeasts and bacteria ranging from 64 to 512 μg/mL but their average MICs showed higher efficiency in resveratrol (yeasts: MIC = 282 μg/mL, bacteria: MIC = 256 μg/mL) than in luteolin (yeasts: MIC = 410 μg/mL, bacteria: MIC = 256 μg/mL).

Considering other effective compounds, myricetin showed inhibitory effect only against yeast *Z. rouxii* and bacteria *A. aceti*, *A. pasterianus* and *A. oeni* at concentrations ranging from 256–512 μg/mL. As for the less effective compounds, p-coumaric acid, it was efficient against yeast *D. bruxellensis* and bacteria *A. pasteriaunus* and *A. oeni* at the concentration of 512 μg/mL. Ferulic acid was active against yeasts *D. bruxellensis*, *Z. bailli*, *Z. rouxii* and bacteria *A. aceti*, *A. pasterianus*, *A. oeni* at concentrations of 256–512 μg/mL. And lastly, Caffeic acid, chlorogenic acid, (-) – catechin, 4-hydroxybenzoic acid, 3,4 dihydroxybenzoic acid, gallic acid, sinapic acid, rutin, vanillic acid did not inhibit any of the tested yeasts or bacteria.

Table 4: *In vitro* inhibitory effect of selected compounds against wine spoilage bacteria and yeasts at pH 3.5

Tested compounds	**Bacterium+Yeasts / pH / MIC [**μg/mL**]**							
	AA	***AE***	***AO***	***DB***	***HU***	***ZB***	***ZR***	***Average***
caffeic acid	-	-	-	-	-	-	-	
catechin	-	-	-	-	-	-	-	
chlorogenic acid	-	-	-	-	-	-	-	
p-coumaric acid	-	512	512	512	-	-	-	512
3,4-dihydroxybenzoic acid	-	-	-	-	-	-	-	
ferulic acid	512	512	512	512	-	512	256	469
gallic acid	-	-	-	-	-	-	-	
4-hydroxybenzoic acid	-	-	-	-	-	-	-	
luteolin	256	256	256	256	512	512	256	329
myricetin	512	256	512	-	-	-	512	448
pterostilbene	64	128	64	64	128	64	32	78
resveratrol	256	256	256	256	256	256	512	293
rutin	-	-	-	-	-	-	-	
sinapic acid	-	-	-	-	-	-	-	
vanillic acid	-	-	-	-	-	-	-	
potassium metabisulphite	1024	1024	1024	512	512	1024	1024	878

MIC - minimum inhibitory concentration AA - *Acetobacter aceti*, AE - *Acetobacter estunensis*, AO - *Acetobacter oeni*, DB - *Dekkera bruxellensis*, HU - *Hanseniaspora uvarum*, ZB - *Zygosaccharomyces bailii*, ZR - *Zygosaccharomyces rouxii*

With aim to determine influence of different acidity level on antimicrobial effects of tested compounds, the second part of the experiments was performed at pH 5.5. As it is shown in Table 5 the higher pH significantly altered efficiency of the tested phenolics. However, pterostilbene, resveratrol and luteolin inhibited the same spectrum of tested yeasts and bacteria, whereas. pterostilbene possessed the strongest growt-inhibitory effect against all yeasts (MICs = 8–128 μg/mL) and bacteria (MICs = 16–128 μg/mL). Both, resveratrol and luteolin were more active against bacteria (64–128 μg/ml; 128 μg/mL, respectively) than yeasts (128 -256 μg/mL).

Some compounds demonstrated only selective efficiency. Coumaric acid was active against yeast *D. bruxellnesis*, and bacteria *A. aceti* and *A. oeni* (256 μg/mL). Ferulic acid inhibited all bacteria (512 μg/mL) and yeast *Z. rouxii* (512 μg/mL). Myricetin inhibited the growth of yeasts *H. uvarum* and *Z. rouxii* at the concetration of 256 μg/mL and all bacteria at concentraion from 128 μg/mL to 256 μg/mL. Caffeic acid, chlorogenic acid, (-) – catechin, 4-hydroxybenzoic acid, 3,4 dihydroxybenzoic acid, gallic acid, sinapic acid, rutin and vanillic acid did not demonstrate any antimicrobial activity against the tested microorganisms. For bacteria, it was registered the noticeable differences for resveratrol, myricetin, coumaric acid and luteolin, slight difference for pterostilben and no difference for ferulic acid. On the other hand, the antimicrobial activity against yeasts was affected more significantly. However, a slight difference was noticed for ferulic acid, and more distinct differences for resveratrol and luteolin. The most significant difference was for pterostilbene, myricetin and coumaric acid.

Table 5: *In vitro* inhibitory effect of selected compounds against wine spoilage bacteria and yeasts at pH 5.5

	MIC [μg/mL**]**							
	Bacterium			**Yeasts**				
Tested compounds	***AA***	***AE***	***AO***	***DB***	***HU***	***ZB***	***ZR***	***Average***
caffeic acid	-	-	-	-	-	-	-	
catechin	-	-	-	-		-	-	
chlorogenic acid	-	-	-	-		-	-	
p-coumaric acid	256	-	256	256	-	-	-	256
3,4-dihydroxybenzoic acid	-	-	-	-	-	-	-	
ferulic acid	512	512	512	-	-	-	512	512
gallic acid	-	-	-	-	-	-	-	
4-hydroxybenzoic acid	-	-	-	-	-	-	-	
luteolin	128	128	128	128	256	256	256	183
myricetin	256	128	256	-	256	-	256	230
pterostilbene	32	128	16	16	32	16	8	35
resveratrol	128	64	128	128	128	256	256	155
rutin	-	-	-	-	-	-	-	
sinapic acid	-		-	-	-	-	-	
vanillic acid	-	-	-	-	-	-	-	
potassium metabisulphite	4096	2048	4096	4096	4096	4096	4096	3803

MIC - minimum inhibitory concentration AA - *Acetobacter aceti*, AE - *Acetobacter estunensis*, AO - *Acetobacter oeni*, DB - *Dekkera bruxellensis*, HU - *Hanseniaspora uvarum*, ZB - *Zygosaccharomyces bailii*, ZR - *Zygosaccharomyces rouxii*

According to our results, pterostilbene shown very effective antifungal and antibacterial activity demonstrated especially by inhibition of yeast *Z. rouxii* (16 μg/mL) and bacteria *A. oeni* (8 μg/mL), which has not been assayed previously. Resveratrol inhibited very effectively yeast *H. uvarum* (128 μg/mL) and bacterium *A. estunensis* (64 μg/mL). According to Garcia-Ruiz (2011), resveratrol is the phenolic compounds with prominent antimicrobial activity also against lactic acid bacteria such as *O. oeni* (698 μg/mL), *P. pentosaceus* (715 μg/mL) and *L. hilgardii* (855 μg/mL). However, according our results, this compound shown higher efficiency for inhibition of AAB. The most effective inhibitory

activity of luteolin was shown on the inhibition of yeast *D. bruxellensis* (128 μg/mL) and all bacteria (128 μg/mL) corresponding with antimicrobial activity reported by Keute et al. (2007).

The antimicrobial activity of p-coumaric acid, which was very effective against yeast *D. bruxellensis* (256 μg/mL) and bacteria *A. aceti* and *A. oeni* (256 μg/mL), corresponding with Pokorny *et al.* (2003) who confirmed the inhibitory effect against bacteria (*E. coli* and *B. cereus)* and moulds *(Aspergillus flavus* and *A. parasiticus*) but at higher concentrations (400 μg/mL, 300 μg/mL, respectively) and also Gacia-Ruiz (2002) who reported the inhibition of *L.hilgardii* (1260 μg/mL), *P. pentosaceus* (994 μg/mL) and *O. oeni* (818 μg/mL).

For ferulic acid, our results of inhibitory concentrations, covering *Z. rouxii* (256 μg/mL) and acetic acid bacteria (512 μg/mL), shown the significantly higher efficiency in comparison with Garcia-Ruiz (2002), who reported the inhibition activity at the concentration of 2110 μg/mL for *L. hilgardii*, at 1580 μg/mL for *P. pentosaceus* and at 843 μg/mL for *O. oeni.* Results for potassium metabisuphite shown that yeasts and bacteria were inhibited by concentations (512 to 1024 μg/mL) which proved its lower efficiency compered to tested phenolics and was more effective against yeasts.

Myricetin was very active against yeasts *H. uvarum* and *Z. rouxii* (256 μg/mL) and bacteria *A. estunensis* (128 μg/mL) and also according Gutierrez (2012) against *O. oeni* (854 μg/mL). On the other hand, *L. hilgardii* and *P. pentosaceus* were not inhibited at all.. These LAB were not susceptible to the, sinapic acid, tryptophol, myricetin and gallic acid which support our results. However gallic acid was quiete successful in other studies. Gutierrez (2012) reported inhibitory activity against *Pseudomonas fluorescen*s (3200 μg/mL), *E. coli* (3200–6400 μg/mL) and *B. cereus* (2400-4800 μg/mL.); Vaquero (2007) against *L. monocytogenesthan*; and Kubo (2004) against ten strains of *S. aureus* (560 μg/mL) Our results did not demonstrated any antimicrobial activity of caffeic acid and hydroxybenzoic acids against wine pathogens. However, these compunds were more effective in inhibiion of *E. coli* and *L. monocytogenes*, resprectively. All tested compounds, which shown inhibitory activity agastin *Z. bailli* and *Z. rouxii,* were significantly more effective than those reported by Martorel (1999) (3005 μg/mL; 1100 μg/mL, respectively).

The differences between inhibitory activties of the most potent compounds (resveratrol and pterostilbene) were not significant which correspond with Rimando *et al.*. (2002), that pterostilbene is a naturally occurring analogue of resveratrol with similar biological activities

(Rimando et al., 2002). Both stilbenes were reported to be harmless for human consumption even possessing health beneficial effects (such as anticancer, anti-inflammatory activity, cardiovascular protecting and lipid-lowering effect (Remsberg et al., 2008; Satheesh and Pari, 2006. Toxicological studies did not shown any cotoxic effects of pterostilbene (Kim *et al.*., 2009) and Mikstacka et al., (2007) reported also its reducing risk of mutagenesis.

Resverastrol is considered to be the most studied wine compound mainly for its cardioprotective effect, which explained the effective inhibition of oxygesic enzymes by stilbenoids in general, preventing blood cell aggregation, thrombosis, cholesterol storage and high blood pressure increases. Since, this compounds possess status GRAS (Generally Recognised as a Safe) its is considered to be safe for human consumption and is also available in form of food supplement (Harmatha, 2002; Jang et al., 1997; de la Lastra and Villegas, 2007).

Both stilbenes have shown excellent result for inhibitory activity which suggested them as promising items in the field of wine preservative research.

7 Conclusion

This study has clearly demonstrated that the polyphenols naturally occurring in grape wine possess antimicrobial activity against pathogens frequently occurring in wine. Stilbenes (pterostilbene and resveratrol) and flavonoid luteolin inhibited the growth of all selected microorganisms at low MICs and displayed a significant potency to cope with the microorganisms causing wine spoilage, whereas myricetin, p-coumaric acid and ferulic acid proved only a selective effect against some microorganisms at higher MICs. Moreover, according to our best knowledge, this is the first report on growth-inhibitory activity of pterostilbene against the wine-spoiling microorganisms, which suggests that this compound is a promising agent for further research in the field of plant-derived alternatives to sulphites. In addition, the positive effects of stilbenes on human health (e.g. antioxidative, antiaging, cardioprotective or anticancer activities) and safe statute supports the possibility of their application in food industry.

In summary, these findings uncovered possible applications of pterostilbene, resveratrol and luteolin as preservative agents for the purpose of winemaking and indicated that the proved biological activity of the selected grape constituents creates a great potential for grapes in the field of food additives. However, further investigations regarding the safety and technological properties of these natural compounds are still necessary before their possible practical application in the food industry, which might prove useful in saturating the increasing demand for foods without artificial additives.

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