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ORIGINAL ARTICLE

Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies

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Abstract

Aim and Methods: Although the antimicrobial activity of extracts from several mushroom species has been reported, studies with the individual compounds present in that extracts are scarce. Herein, the antimicrobial activity of different phenolic compounds identified and quantified in mushroom species from all over the world was evaluated. Furthermore, a structure–activity relationship (SAR) analysis and molecular docking studies were performed, in order to provide insights into the mechanism of action of potential antimicrobial drugs for resistant micro-organisms.

Results: 2,4-Dihydroxybenzoic and protocatechuic acids were the phenolic compounds with higher activity against the majority of Gram-negative and Gram-positive bacteria. Furthermore, phenolic compounds inhibited more MRSA than methicillin-susceptible *Staphylococcus aureus*. MRSA was inhibited by 2,4-dihydroxybenzoic, vanillic, syringic (MICs = 0·5 mg ml^{-1}) and *p*-coumaric (MIC = 1 mg ml^{-1}) acids, while these compounds at the same concentrations had no inhibitory effects against methicillin-susceptible *Staph. aureus*.

Conclusions: The presence of carboxylic acid (COOH), two hydroxyl (OH) groups in *para* and *ortho* positions of the benzene ring and also a methoxyl (OCH_3) group in the *meta* position seems to be important for anti-MRSA activity.

Significance and Impact of the Study: Phenolic compounds could be used as antimicrobial agents, namely against some micro-organisms resistant to commercial antibiotics.

Introduction

In recent years, there are an increasing number of reports on phenolic compounds in different mushroom species. Phenolic acids including benzoic and cinnamic acid derivatives have been pointed out as the most common. Among benzoic acid derivatives, *p*-hydroxybenzoic, protocatechuic, gallic, vanillic and syringic acids were identified in different mushroom species (Puttaraju *et al.* 2006; Kim *et al.* 2008; Barros *et al.* 2009; Heleno *et al.* 2011, 2012; Reis *et al.* 2011; Vaz *et al.* 2011a,b) (Table 1). The identification of cinnamic acid and its derivatives such as *p*-coumaric, *o*-coumaric, caffeic, ferulic and chlorogenic acids was also described (Mattila *et al.* 2001; Valentão *et al.* 2005; Puttaraju *et al.* 2006; Barros *et al.* 2009; Kim *et al.* 2008; Heleno *et al.* 2011; Reis *et al.* 2011; Vaz *et al.* 2011a,b; Heleno *et al.* 2012). The presence of some flavonoids such as quercetin, rutin and chrysin (Valentão *et al.* 2005; Ribeiro *et al.* 2006; Kim *et al.* 2008; Jayakumar *et al.* 2009; Yaltirak *et al.* 2009) and tannins like ellagic acid (Ribeiro *et al.* 2007) was reported (Table 1).

In vitro and epidemiologic studies suggest that consumption of foods rich in phenolic compounds might significantly decrease the risk of some health problems due to their antioxidant, antimutagenic, anti-inflammatory and antibacterial properties (Surh 2002; Albayrak

Table 1 Phenolic compounds identified in wild mushrooms and submitted to antimicrobial activity evaluation

Phenolic compounds	Mushroom species	Country	References
Phenolic acids: benzoic acid derivatives			
p- Hydroxybenzoic acid	*Agaricus arvensis, Agaricus bisporus, Agaricus romagnesii, Agaricus silvicola, Amanita caesarea, Amanita muscaria, Amanita pantherina, Amanita rubescens, Armillaria mellea, Auricularia auricula-judae, Boletus aereus, Boletus edulis, Boletus reticulatus, Boletus rhodoxanthus, Boletus satanas, Calocybe gambosa, Cantharellus cibarius, Chroogomphus fulmineus, Citocybe odora, Coprinus comatus, Cortinarius anomalus, Cortinarius collinitus, Cortinarius violaceus, Craterellus cornocopioides, Fistulina hepática, Ganoderma lucidum, Hygrophorus marzuolus, Hygrophorus olivaceo-albus, Ionotus obliquus, Lactarius deliciosus, Lactarius salmonicolor, Lactarius volemus, Lepista nuda, Lentinus edodes, Lycoperdon molle, Phellinus linteus, Pleurotus eryngii, Pleurotus ostreatus, Ramaria botrytis, Russula cyanoxantha, Sarcodon imbricatus, Sparassis crispa, Suillus granulatus, Suillus collinitus, Suillus mediterraneensis, Tricholoma acerbum, Tricholoma equestre, Tricholoma sulphureum*	Finland, Korea, Portugal, Spain, Turkey	Mattila *et al.* (2001), Ribeiro *et al.* (2006, 2007), Kim *et al.* (2008), Barros *et al.* (2009), Heleno *et al.* (2011, 2012), Oke and Aslim (2011), Palacios *et al.* (2011), Reis *et al.* (2011) and Vaz *et al.* (2011a,b)
Protocatechuic acid	*A. bisporus, A. blazei, A. caesarea, A. pantherina, Auricularia polytricha, B. edulis, B. rhodoxanthus, B. satanas, C. cibarius, Cantherallus clavatus, C. gambosa, C. fulmineus, C. anomalus, C. cornocopioides, F. hepática, Flammulina velutipes, G. lucidum, Helvella crispa, Hygrophorus agathosmus, H. marzuolus, Hydnum repandum, I. obliquus, L. deliciosus, Lactarius sangifluus, L. edodes, Lentinus squarrulosus, Lentinus sajor caju, L. nuda, Macrolepiota procera, Morchella anguiticeps, Morchella conica, Mycena haematopus, Pleurotus djamor, P. eryngii, P. linteus, P. ostreatus, Pleurotus sajor-caju, R. botrytis, R. brevepis, S. crispa, S. collinitus, S. mediterraneensis, Termitomyces heimii, Termitomyces microcarpus, Termitomyces mummiformis, Termitomyces shimperi, Termitomyces tylerance*	Finland, India, Korea, Portugal, Spain	Puttaraju *et al.* (2006), Kim *et al.* (2008), Barros *et al.* (2009), Heleno *et al.* (2011), Oke and Aslim (2011), Palacios *et al.* (2011), Reis *et al.* (2011) and Vaz *et al.* (2011a)

(Continued)

Table 1 *(Continued)*

Phenolic compounds	Mushroom species	Country	References
Gallic acid	*A. auricula-judae, A. bisporus, A. blazei, A. polytricha, B. edulis, C. gambosa, C. cibarius, C. clavatus, C. cornocopioides, F. velutipes, G. lucidum, Geastrum arinarius, H. crispa, H. marzuolus, H. repandum, I. obliquus, L. deliciosus, L. sangifluus, L. edodes, L. sajor caju, L. squarrulosus, M. procera, M. anguiticeps, M. conica, P. djamor, P. eryngii, P. ostreatus, P. linteus, P. sajor-caju, R. brevepis, Russula delica, S. crispa, T. heimii, T. microcarpus, T. mummiformis, T. shimperi, T. tylerance*	India, Korea, Spain, Turkey	Puttaraju *et al.* (2006), Kim *et al.* (2008), Yaltirak *et al.* (2009), Oke and Aslim (2011) and Palacios *et al.* (2011)
Vanillic acid	*A. auricula-judae, A. polytricha, C. clavatus, H. crispa, H. repandum, L. sangifluus, L. squarrulosus, L. sajor caju, L. molle, M. procera, M. conica, Pleurotus sajorcaju, P. djamor, P. eryngii, R. brevepis, T. heimii, T. microcarpus, T. shimperi, T. acerbum*	India, Portugal, Turkey	Puttaraju *et al.* (2006), Barros *et al.* (2009) and Oke and Aslim (2011)
Syringic acid	*A. blazei, C. clavatus, A. auricula-judae, H. repandum, L. sangifluus, L. sajor caju, M. procera, M. conica, M. anguiticeps, P. eryngii, P. djamor, R. brevepis, S. crispa, T. mummiformis, T. tylerance, T. microcarpus*	India, Korea, Turkey	Puttaraju *et al.* (2006), Kim *et al.* (2008) and Oke and Aslim (2011)
Cinnamic acid and derivatives			
Cinnamic acid	*A. arvensis, A. bisporus, A. blazei A. silvicola, A. romagnesii, A. caesarea, A. muscaria, A. pantherina, A. mellea, B. aereus, B. edulis, Boletus purpureus, B. reticulatus, B. rhodoxanthus, B. satanas, C. gambosa, C. cibarius, C. clavatus, C. fulmineus, C. odora, C. comatus, C. anomalus, C. collinitus, C. violaceus, F. hepática, G. lucidum, H. agathosmus, H. repandum, Hygrophoropsis aurantiaca, H. olivaceo-albus, Lactarius aurantiacus, Lactarius quietus, L. salmonicolor, L. sangifluus, L. squarrulosus, L. edodes, L. volemus, Lycoperdon perlatum, P. eryngii, Macrolepiota prócera, M. haematopus, P. sajor-caju, P. djamor, S. crispa, Russula caerulea, Russula sardónia,S. collinitus, Suillus luteus, S. mediterraneensis, T. heimii, T. mummiformis, T. shimperi, Tricholoma atrosquamosum, T. sulphureum, Tricholoma ustale*	Finland, India, Korea, Portugal, Turkey	Mattila *et al.* (2001), Valentão *et al.* (2005), Puttaraju *et al.* (2006), Kim *et al.* (2008), Barros *et al.* (2009), Heleno *et al.* (2011, 2012), Oke and Aslim (2011), Reis *et al.* (2011) and Vaz *et al.* (2011a,b)
p-Coumaric acid	*A. arvensis, A. bisporus, A. silvicola, A. muscaria, A. pantherina, B. aereus, B. edulis, C. gambosa, C. cibarius, C. fulmineus, C. odora, C. comatus, C. collinitus, F. hepática, G. lucidum, G. arinarius, H. agathosmus, H. marzuolus, L. sangifluus, L. sajor caju, L. nuda, M. procera, P. djamor, P. ostreatus S. crispa, T. heimii, T. atrosquamosum*	India, Korea, Portugal, Spain	Puttaraju *et al.* (2006), Ribeiro *et al.* (2007), Kim *et al.* (2008), Barros *et al.* (2009), Heleno *et al.* (2011, 2012), Palacios *et al.* (2011), Reis *et al.* (2011) and Vaz *et al.* (2011a,b)
o-Coumaric acid	*I. obliquus*	Korea	Kim *et al.* (2008)

(Continued)

Table 1 *(Continued)*

Phenolic compounds	Mushroom species	Country	References
Caffeic acid	*A. auricula-judae, A. bisporus, B. edulis, C. gambosa, C. cibarius, C. clavatus, F. hepática, F. velutipes, H. marzuolus,, L. sangifluus, L. deliciosus L. sajor caju, L. squarrulosus, M. anguiticeps, M. conica, M. procera, P. linteus, P. djamor, P. eryngii, R. brevepis, R. delica, S. crispa, T. heimii, T. microcarpus, T. shimperi T. tylerance*	India, Korea, Portugal, Spain, Turkey	Valentão *et al.* (2005), Puttaraju *et al.* (2006) Ribeiro *et al.* (2007), Kim *et al.* (2008), Yaltirak *et al.* (2009), Oke and Aslim (2011) and Palacios *et al.* (2011)
Ferulic acid	*A. bisporus, C. clavatus, C. gambosa, C. cibarius, C. cornocopioides, F. velutipes, I. obliquus, L. deliciosus, L. sangifluus, L. squarrulosus, M. conica, M. procera, P. djamor, P. ostreatus P. sajor-caju, S. crispa, T. heimii, T. microcarpus, T. shimperi*	India, Korea, Spain	Puttaraju *et al.* (2006), Kim *et al.* (2008) and Palacios *et al.* (2011)
5-*O*-Caffeoylquinic acid	*A. bisporus, B. edulis, C. gambosa, C. cibarius, F. velutipes, L. deliciosus, P. ostreatus, P. linteus*	Korea, Portugal, Spain	Valentão *et al.* (2005), Kim *et al.* (2008) and Palacios *et al.* (2011)
Flavonoids			
Quercetin	*A. blazei, F. velutipes, G. lucidum, I. obliquus, S. crispa, S. luteus, S. granulatus*	Korea, Portugal	Ribeiro *et al.* (2006) and Kim *et al.* (2008)
Rutin	*C. cibarius, P. ostreatus, R. delica*	India, Turkey	Valentão *et al.* (2005), Jayakumar *et al.* (2009) and Yaltirak *et al.* (2009)
Chrysin	*P. ostreatus*	India	Jayakumar *et al.* (2009)
Tannins			
Ellagic acid	*Fistulina hepatica*	Portugal	Ribeiro *et al.* (2007)

et al. 2010). Nowadays, the evidence that the increasing number of micro-organisms resistant to the available antibiotics is an emergent problem and subject for researchers and clinicians from all over the world. In general, it can be observed that the treatment of virus, bacteria, fungi and protozoa with the existent drugs is increasingly difficult. To overlap the disadvantages of the available antimicrobial drugs, other drugs with new mechanisms of action should be developed (Khalafi-Nezhad *et al.* 2005).

Although the antimicrobial activity of extracts from several mushroom species has been reported (Barros *et al.* 2007; Quereshi *et al.* 2010; Ozen *et al.* 2011; Alves *et al.* 2012), studies with the individual compounds present in that extracts are scarce, being mainly related to phenolic compounds identified in plant sources (Kuete *et al.* 2009; Orhan *et al.* 2010; Lou *et al.* 2012).

Therefore, the aim of the present study was to evaluate the antimicrobial activity of most relevant compounds identified and quantified in mushroom species from all over the world. Furthermore, a structure–activity relationship (SAR) analysis and molecular docking studies against penicillin-binding protein 2a (PBP2a) were performed, in order to provide insights into the mechanism of action of potential antimicrobial drugs for resistant micro-organisms. Molecular docking is an *in silico* tool that predicts how a ligand (substrate or drug candidate) interacts with a receptor (e.g. proteins involved in several biological processes) and has been successfully applied in several therapeutic programmes at the lead discovery stage (Ghosh *et al.* 2006).

Materials and methods

Standards and reagents

The culture media Muller Hinton broth (MHB) and Wilkins-Chalgren broth (WCB) were obtained from Biomerieux (Marcy l′ Etoile, France), respectively. The dye *p*-iodonitrotetrazolium chloride (INT) was purchased from Sigma–Aldrich (St. Louis, MO, USA) to be used as microbial growth indicator. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA) before use.

Phenolic compounds

Sixteen phenolic compounds (phenolic acids, flavonoids and tannins) already identified in tens of different wild mushroom species by our research group and by others (Table 1) were submitted to antimicrobial activity evaluation against Gram-positive and Gram-negative bacteria clinical isolates. Compounds were dissolved in water or in water with 1% DMSO (for flavonoids and tannins), at a concentration of 10 mg ml^{-1}, and stored at −20°C for further use (up to 1 week).

Micro-organisms and culture media

The micro-organisms used were clinical isolates from patients hospitalized in various departments of the Hospital Center of Trás-os-Montes e Alto Douro – Chaves, Portugal.

Six Gram-positive bacteria [methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from wound exudates, *Staphylococcus epidermidis, Enterococcus faecalis* and *Listeria monocytogenes* isolated from blood culture and *Streptococcus agalactiae* isolated from vaginal swab] and five Gram-negative bacteria (*Escherichia coli*, *Proteus mirabilis* and *Morganella morganii*, isolated from urine, *Pasteurella multocida* isolated from synovial fluid and *Neisseria gonorrhoeae* isolated from urethral exudate) were used to screen the antimicrobial activity of the selected phenolic compounds. *Escherichia coli* showed resistance to fluoroquinolones (levofloxacin and ciprofloxacin) and ampicillin, being intermedia for amoxicillin/clavulanic acid; *Pr. mirabilis* was resistant to nalidixic acid, levofloxacin, nitrofurantoin, fosfomycin and trimethoprim/sulfasoxazole and intermediate to gentamicin; *M. morganii* showed resistance to ampicillin, amoxicillin/clavulanic acid, cephalothin, cefazolin, cefuroxime, nitrofurantoin, fosfomycin and trimethoprim/sulfasoxazole; MSSA was only resistant to penicillin and ampicillin, while MRSA was resistant to oxacillin, levofloxacin and ciprofloxacin; *Staph. epidermidis* showed resistance to oxacillin and erythromycin.

All strains were identified using the MicroScan automated methodology – Siemens.

MHB and WCB were used for the determination of minimum inhibitory concentration (MIC, lowest concentration of the phenolic compound able to completely inhibit bacterial growth).

Test assays for antimicrobial activity

MICs were determined by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay following the methodology suggested by Kuete *et al.* (2011) with some modifications.

Initially, 50 μl of each filter-sterilized phenolic compound solution (1 mg ml^{-1}) was diluted in 450 μl of MHB for all micro-organisms except for *N. gonorrhoeae* where WCB was used (also with final concentration of 1 mg ml^{-1}) and then, 200 μl of this solution was added in each well (96-well microplate). Titrations (eight different final concentrations) were carried out over the wells

containing 100 μl of MHB or WCB and, afterwards, 10 μl of inoculum (1×10^8 cfu ml^{-1}) was added to all the wells.

Two negative (one with MHB or WCB and the other with the phenolic compound) controls and one positive (with MHB or WCB and the inoculum) control were performed. The plates were incubated at 37°C, for 24 h, in an oven (Jouan, Berlin, Germany) or with humidified atmosphere containing 10% CO_2 (NuAire, Plymouth, MA, USA), in the case of *N. gonorrhoeae*.

The MIC of the samples was detected following the addition of INT (0·2 mg ml^{-1}, 40 μl) and incubation at 37°C for 30 min. Viable micro-organisms reduced the yellow dye to a pink colour. MIC was defined as the lowest phenolic compound concentration that prevented this change and exhibited complete inhibition of bacterial growth. All the assays were carried out in duplicate.

Compounds and protein structure preparation

ACD/ChemSketch Freeware 12.0 software was used to design 2D structure of the compounds. The software VegaZZ 2.3.1 (Pedretti *et al.* 2004) was then used to convert all compounds from 2D to 3D structures. AutoDockTools1.5.2 (ADT) (Sanner 2005) was used to merge nonpolar hydrogens, add Gasteiger charges and set up rotatable bonds through AutoTors.

The crystal structure of PBP2a (penicillin-binding protein 2a) was obtained from the Protein Data Bank (PDB): 1VQQ (PDB entry) (Lim and Strynadka 2002). The software AutoDockTools was also used to assign polar hydrogens, add Gasteiger charges and save the protein structure in PDBQT file format. AutoGrid4 (Morris *et al.* 2009) was used to create affinity grid maps for all the atoms on the protein and phenolic compounds used.

Molecular docking

AutoDock4 (version 4.2) with the Lamarckian genetic algorithm was used to perform the docking studies. Docking parameters selected for AutoDock4 runs were as follows: 100 docking runs, population size of 200, random starting position and conformation, translation step ranges of 2·0 Å, mutation rate of 0·02, cross-over rate of 0·8, local search rate of 0·06 and 2·5 million energy evaluations. Docked conformations were clustered using a tolerance of 2·0 Å root mean square deviation (RMSD). The molecular docking experiments were performed on a dedicated cluster of 64 Core AMD 2·0 GHz, running on CentOS and using MOLA, a custom-designed software for virtual screening using AutoDock (Abreu *et al.* 2010). All figures with structure representations were produced using PyMOL [The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC. Available at: (http://www.pymol.org/)].

Results

Table 1 presents phenolic compounds that have been identified in different mushroom species from several countries, where it can be observed that different compounds were detected in the same species. Several external factors have been pointed to explain this fact, such as the heterogeneous enzymatic and oxidative decomposition after collection, different stress conditions associated with each sample, and even dissimilar methodologies applied to phenolic compounds extraction (Oke and Aslim 2011; Vaz *et al.* 2011a,b).

These compounds are well known for their antioxidant properties (Puttaraju *et al.* 2006; Ribeiro *et al.* 2007; Kim *et al.* 2008), but they also revealed antimicrobial activity (Barros *et al.* 2007; Quereshi *et al.* 2010; Ozen *et al.* 2011) emerging with potential against multiresistances. Their increasing prevalence is one of the major challenges for the healthcare systems worldwide. Antibiotic-resistant infections are associated with a 1·3- to 2-fold increase in mortality compared to antibiotic-susceptible infections (Cosgrove and Carmeli 2003). Moreover, antibiotic resistance imposes enormous health expenditure due to the higher treatment costs and longer hospital stays. In addition, the development of new generations of antibiotic drugs is stalling.

In the present study, in the range of tested concentrations (0·78–1000 μg ml^{-1}), 2,4-dihydroxybenzoic, protocatechuic, vanillic and *p*-coumaric acids showed antibacterial activity (MIC = 1 mg ml^{-1}) against *E. coli*, *Past. multocida* and *N. gonorrhoeae* (Table 2). It should be highlighted that the *E. coli* isolate used herein shows resistance to fluoroquinolones (levofloxacin and ciprofloxacin) and ampicillin, being intermedia for amoxicillin/clavulanic acid. Kuete *et al.* (2009) reported a MIC = 78 μg ml^{-1} for protocatechuic acid isolated from *Ficus ovata* against *E. coli* (β-lactamases positive). The observed difference in MIC values could be related to the use of strains with different susceptibility profiles. *Escherichia coli* resistance to fluoroquinolones and cephalosporins has drastically increased in the last decade (Rogers *et al.* 2011); the mentioned phenolic acids could be an option against this bacteria. Recently, Lou *et al.* (2012) also reported the antimicrobial activity of *p*-coumaric acid (MIC = 80 μg l^{-1}) against *E. coli*, but also against other Gram-negative bacteria such as *Salmonella typhimurium* and *Shigella dysenteriae*; this compound changes the permeability of the cell membrane and has the capacity to bind DNA, inhibiting cell function. Other authors

Table 2 MIC values (mg ml^{-1}) of wild mushroom phenolic compounds against clinical isolates of Gram-negative bacteria

Phenolic compounds	*Escherichia coli*	*Proteus mirabilis*	*Morganella morganni*	*Pasteurella multocida*	*Neisseria gonorrhoeae*
Benzoic acid derivatives					
p-Hydroxybenzoic acid	>1	>1	>1	>1	>1
2,4-Dihydroxybenzoic acid	1	>1	>1	1	1
Protocatechuic acid	1	>1	>1	1	1
Gallic acid	>1	>1	>1	1	1
Vanillic acid	1	1	>1	1	1
Syringic acid	>1	>1	>1	1	>1
Cinnamic acid derivatives					
Cinnamic acid	>1	>1	>1	>1	1
p-Coumaric acid	1	>1	>1	1	1
o-Coumaric acid	>1	>1	>1	>1	>1
Caffeic acid	>1	>1	>1	1	>1
Ferulic acid	>1	>1	>1	1	1
Chlorogenic acid	>1	>1	>1	>1	>1
Flavonoids					
Quercetin	>1	>1	>1	1	0,5
Rutin	>1	>1	>1	>1	>1
Chrysin	>1	>1	>1	>1	>1
Tannins					
Ellagic acid	>1	>1	>1	1	>1
Reference compounds					
Imipenem	≤ 1	2	4	nt	nt
Ceftriaxon	nt	nt	nt	≤ 1	≤ 1

nt, not tested.

(Teke *et al.* 2011) described the antimicrobial activity of vanillic acid against *E. coli* and *Pr. mirabilis*, which is in agreement with the results reported herein. Moreover, the *Pr. mirabilis* strain used in the present study shows resistance to nalidixic acid, ciprofloxacin, nitrofurantoin, fosfomycin and trimethoprim/sulfasoxazole, being intermedia for gentamicin. Nevertheless, it should be highlighted that the strains used herein have different antibiotic resistance profiles, while the ones used in the mentioned study did not reveal relevant resistances; this important feature could be related to the differences observed in MIC values.

Despite the absence of reports regarding the presence of 2,4-dihydroxybenzoic acid in mushrooms and its antimicrobial activity, due to the chemical similarity with other phenolic acids mentioned as antimicrobial compounds, we decided to test it and, as far as we know, this is the first report on its activity against Gram-negative bacteria.

Gallic acid, ferulic acid and quercetin exhibited activity only against *Past. multocida* and *N. gonorrhoeae*, and the latter was mainly sensible to quercetin (MIC = 0·5 mg ml^{-1}; Table 2). According to WHO report published in 2001, more than six million cases of *gonorrhoea* (infection caused by *N. gonorrhoeae*) occur in each year, and with increasing levels, mostly in developing countries; furthermore, there is an emergent resistance of this bacteria to the antimicrobial agents used in *gonorrhoea* treatment. Therefore, the mentioned phenolic compounds could be an alternative to be explored for the control of this infection. Studies evaluating the antibacterial activity of mushroom extracts or isolated compounds against *N. gonorrhoeae* are scarce, so it is important to clarify their mechanism of action upon this micro-organism as also in other Gram-negative cocci.

Although no activity was observed for rutin against the tested Gram-negative bacteria (Table 2), other authors (Orhan *et al.* 2010) reported antimicrobial activity of this compound against different strains of Gram-negative bacilli, such as *E. coli, Pr. mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter baumannii.*

Once more, 2,4-dihydroxybenzoic and protocatechuic acid were the phenolic compounds with higher activity against the majority of Gram-positive bacteria (Table 3). Protocatechuic acid showed a MIC of 1 mg ml^{-1} for MSSA and MRSA, as also for *L. monocytogenes* and *Strep. agalactiae.* Other studies reported the antimicrobial activity of this compound against *Staph. aureus* and with lower concentrations (MIC = 156 μg ml^{-1}; Kuete *et al.* 2009). Once more, the strain used herein was resistant to oxacillin and to both fluoroquinolones (ciprofloxacin and levofloxacin), which could be responsible for the higher MIC value observed in comparison with the mentioned study.

Table 3 MIC values (mg ml^{-1}) of the wild mushroom phenolic compounds against clinical isolates of Gram-positive bacteria

Phenolic compounds	MSSA	MRSA	*Staphylococcus epidermidis*	*Enterococcus faecalis*	*Listeria monocytogenes*	*Streptococcus agalactiae*
Benzoic acid derivatives						
p-Hydroxybenzoic acid	>1	>1	>1	>1	>1	>1
2,4-Dihydroxybenzoic acid	>1	0·5	>1	1	>1	1
Protocatechuic acid	1	1	>1	>1	1	1
Gallic acid	>1	>1	>1	>1	>1	>1
Vanillic acid	>1	0·5	>1	>1	1	1
Syringic acid	>1	0·5	>1	>1	0·5	>1
Cinnamic acid derivatives						
Cinnamic acid	>1	>1	>1	>1	>1	0·5
p-Coumaric acid	>1	1	>1	>1	>1	>1
o-Coumaric acid	>1	>1	>1	>1	>1	1
Caffeic acid	1	1	1	>1	>1	>1
Ferulic acid	1	0·5	1	>1	>1	1
Chlorogenic acid	>1	>1	>1	>1	1	>1
Flavonoids						
Quercetin	>1	>1	>1	>1	1	>1
Rutin	>1	>1	>1	>1	1	>1
Chrysin	>1	>1	>1	>1	>1	>1
Tannins						
Ellagic acid	>1	>1	>1	>1	0·5	>1
Reference compounds						
Gentamicin	≤1	4	≤1	nt	nt	nt
Penicillin	nt	nt	nt	2	0·25	≤0·03

MSSA, Methicillin-susceptible *Staphylococcus aureus*; MRSA, Methicillin-resistant *Staphylococcus aureus*.

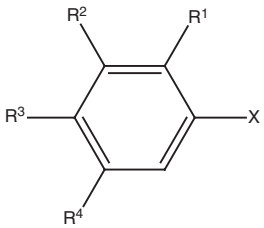
Regarding *Staphylococcus*, ferulic and caffeic acids were the only phenolic compounds inhibiting *Staph. aureus,* MRSA and *Staph. epidermidis.* Nevertheless, other authors reported antimicrobial activity of *p*-coumaric acid, quercetin and rutin against *Staph. aureus* (Kuete *et al.* 2009; Orhan *et al.* 2010; Lou *et al.* 2012). The absence of antimicrobial activity observed in the present study could be related to the different dissolution solvent used, water and not ethanol/hexane and Tween 80 as used by the mentioned authors. Nevertheless, some of those solvents might have some inherent toxicity and should be carefully used.

Syringic and ellagic acids showed a MIC of 0·5 mg ml^{-1} against *L. monocytogenes* (Table 3). Cinnamic acid seemed to be the most active upon *Strep. agalactiae* (CMI 0·5 mg ml^{-1}). Among all the tested phenolic compounds, only 2,4-dihydroxybenzoic acid inhibited *Ent. faecalis* (MIC = 1 mg ml^{-1}); nonetheless, other authors described antimicrobial activity of rutin (MIC = 128 mg ml^{-1}), protocatechuic acid (MIC = 39 μg ml^{-1}) and vanillic acid (zone of inhibition 16 mm) (Kuete *et al.* 2009; Orhan *et al.* 2010; Teke *et al.* 2011). Isolates of *Ent. faecalis* and *Enterococcus faecium* are the third- to fourth-most prevalent nosocomial pathogen worldwide; an increasing number of isolates acquired resistance most prominently to penicillin/ampicillin, aminoglycosides (high-level resistance) and glycopeptides, and the therapeutic spectrum in these cases is limited. Therefore, therapeutic alternatives to treat infections with multi- and vancomycin-resistant enterococci (VRE) are restricted to antibiotics introduced recently into clinical practice such as quinupristin/dalfopristin, linezolid, tigecyclin and daptomycin. However, these drugs are only approved for certain indications and resistance has already been reported (Montero *et al.* 2008; Werner *et al.* 2008), which emphasizes the importance of the discovery of new alternative drugs.

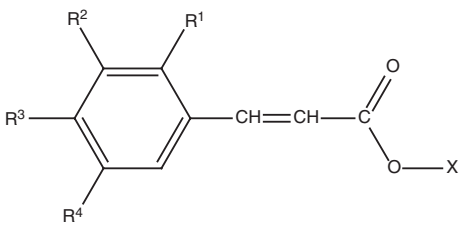
It should be noticed that the differences among the results reported by several authors could be related to the use of strains with different resistance profiles, but also to different methodologies used including different solvents for compound solution preparation or different techniques to determine MICs. In the present study, water was chosen for being the most innocuous solvent; however, in the case of flavonoids and tannins, water with 1% DMSO was used to assure the total solubility of the compounds.

MRSA has been indicated as one of the major causes of nosocomial infections and its increasing prevalence has been observed in the last decade. Furthermore, the treatment of MRSA infections is difficult due to the restrict spectra of efficient antibiotics (Chambers 2001). The

Table 4 Phenolic acids identified in mushrooms submitted to structure–activity relationship analysis



Benzoic acid derivatives	Substitutions				
	X	R 1	R 2	R 3	R 4
2,4-Dihydroxybenzoic acid	COOH	OH	H	OH	H
p-Hydroxybenzoic acid	COOH	H	H	OH	H
Protocatechuic acid	COOH	H	H	OH	OH
Gallic acid	COOH	H	OH	OH	OH
Vanillic acid	COOH	H	OCH_3	OH	H
Syringic acid	COOH	H	OCH_3	OH	OCH_3



Cinnamic acid derivatives	Substitutions				
	X	R^1	R^2	R^3	R^4
Cinnamic acid	CHCHCOOH	H	H	H	H
p-Coumaric acid	CHCHCOOH	H	H	OH	H
o-Coumaric acid	CHCHCOOH	OH	H	H	H
Caffeic acid	CHCHCOOH	H	OH	OH	H
Ferulic acid	CHCHCOOH	H	CH_3O	OH	H

obtained data in the present study (Table 3) show that phenolic compounds inhibited more MRSA than methicillin-susceptible *Staph. aureus*. MRSA was inhibited by 2,4-dihydroxybenzoic, vanillic, syringic (MICs = 0·5 mg ml^{-1}) and *p*-coumaric (MIC = 1 mg ml^{-1}) acids, while these compounds at the same concentrations had no inhibitory effects against methicillin-susceptible *Staph. aureus*. Ferulic acid inhibited both MRSA and methicillin-susceptible *Staph. aureus*, but in a lower concentration for MRSA (Table 3).

Discussion

Regarding these results, it is interesting to notice that the two *Staph. aureus* tested showed different susceptibility towards the compounds tested, possibly explained by the different resistance mechanisms exhibited by each strain. To understand these differential effects, a SAR study was carried out by analysing the different chemical structure patterns of the evaluated compounds.

Only phenolic acids (benzoic and cinnamic acid derivatives) showed activity, highlighting the importance of the carboxylic group in the molecule structure (proton acceptor). Furthermore, all the compounds with anti-MRSA activity have OH (proton donor) and OCH_3 (proton acceptor) groups in the *para* and *meta* positions of the benzene ring, respectively (Table 4). In the absence of OCH_3 group in the *meta* position (*p*-coumaric acid), the activity decreased. Nevertheless, the absence of the mentioned group in the structure of 2,4-dihydroxybenzoic acid seemed to be overlapped by the OH substitution in *ortho* position of the benzene ring. Only OCH_3 (proton acceptor) or H in position 5 of the benzene ring allowed anti-MRSA activity, because when OH is presented in that position, the activity disappears (see the examples of protocatechuic and gallic acids in Table 4).

MRSA is resistant to all β-lactam antibiotics and this ability is due to the acquisition of mecA gene (Lowy 2003). This gene encodes the PBP2a protein, and when it is challenged by β-lactams, MRSA will use the transpepti-

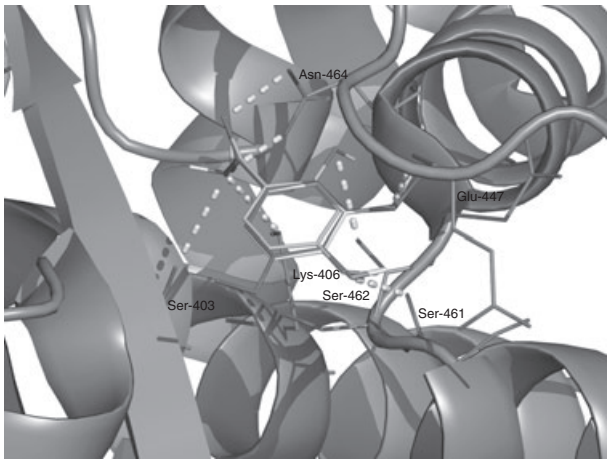


Figure 1 2,4-Dihydroxybenzoic acid (grey), syringic acid (dark grey) and vanillic acid (light grey) docking poses (lines) in PBP2a (carton). Hydrogen bonds are present in dash.

dase functionality of PBP2a to synthesize the cell wall (Wilke *et al.* 2005).

Because the major difference between MSSA and MRSA is mecA, studies of molecular docking were performed using 3D crystal structure of PBP2a (PDB:1QVV) as target to understand the inhibition mechanism of the phenolic compounds with activity against MRSA. The docking results revealed a superimposition of the docking poses for the three benzoic acid derivatives (vanillic, 2,4-dihydroxybenzoic and syringic acids) (Fig. 1).

The binding pose shows several hydrogen bonds (H-bonds) that validate SAR analysis described above. The carboxylic group is stabilized by H-bonds with the amino (NH_2) group of Lys-406 side chain, the hydroxyl (OH) group of Ser-403 side chain and the carboxamide (NH_2CO) group of Asn-464 side chain. Furthermore, OH in the *para* position of the benzene ring, which contributes to the anti-MRSA activity of the compounds, establishes a hydrogen bond with serine (Ser-461) carbonyl group of the peptide bond. The OCH_3 group in the *meta* position of the benzene ring (as in vanillic and syringic acids) is stabilized by a hydrogen bond with glutamate (Glu-447) amine group of the peptide bond. The OH in *meta* position of the benzene ring (as in 2,4-dihydroxybenzoic acid) is stabilized by a hydrogen bond with serine (Ser-462) carbonyl group of the peptide bond.

Overall, 2,4-dihydroxybenzoic, protocatechuic, vanillic and *p*-coumaric acids were the compounds that showed higher antimicrobial activity against Gram-positive and Gram-negative bacteria. Cinnamic acid derivatives revealed higher antimicrobial activity against Gram-positive and Gram-negative cocci.

The presence of carboxylic acid (COOH), two hydroxyl (OH) groups in *para* and *ortho* positions of the benzene ring and also a methoxyl (OCH_3) group in the *meta* position seems to play an important role in the studied phenolic compounds anti-MRSA activity. The docking studies provided strong evidence that the molecular basis for this activity is probably due to PBP2a inhibitors. The mentioned compounds could be a solution for multiresistance problem, but their mechanism of action in different micro-organisms should be better understood.

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