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Phytochemicals and antibacterial activity screening of three edible mushrooms *Pleurotus ostreatus*, *Ganoderma lucidum* and *Lentinula edodes* accessible in Bangladesh

Maafi Rizwana Islam[1], M. Omar[1], M. Moyen Uddin PK[1, *], Md. Rubel Mia[1], Kamrunnahar[2]

[1]Department of Biochemistry, Primeasia University, Dhaka, Bangladesh
[2]Department of Microbiology, Primeasia University, Dhaka, Bangladesh

Email address
moyen.uddinpk@primeasia.edu.bd (M. M. U. PK)

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Abstract

In this study methanolic extract of dried fruiting bodies of *Pleurotus ostreatus*, *Ganoderma lucidum* and *Lentinula edodes* were evaluated for their phytochemical analysis. The crucial objectives of this investigation were to check out the relative abundances of the selected eleven phytochemical constituents and preliminary antibacterial activity screening of selected three edible mushrooms. The screening was accomplished for alkaloids, polyphenols, flavonoids, tannins, saponins, phyto-sterols, vitamin-C, coumarins, terpenoids, cardiac glycosides and anthocyanin. The color strength or the precipitate formation was used as analytical answers to these tests. Eleven selected phytochemicals were found in fruiting body extracts of *Ganoderma lucidum* with polyphenols, flavonoids, tannins, coumarins, vit-C, and anthocyanin were in highest concentration category. Excepting polyphenols, *Lentinula edodes* showed the rest of 10 selected phytochemicals thru vit-C and tannin were in maximum concentration category. Saponins, coumarins, and anthocyanin were absent in fruiting bodies of *Pleurotus ostreatus* while steroids, terpenoids, cardiac glycosides were found in uppermost concentration category. The distribution difference of eleven selected phytochemicals in three edible mushrooms, *Pleurotus ostreatus* {$p > 0.05$ (0.965)}, *Ganoderma lucidum* {$p > 0.05$ (0.307)} and *Lentinula edodes* {$p > 0.05$ (0.484)} were statistically non-significant. Mushroom extracts were also exposed to preliminary antibacterial activity screening by disc diffusion method and found to be active against selected bacterial strains. Fruiting bodies of *Ganoderma lucidum* was exhibited highest antibacterial activity against both selected gram negative (*Escherichia coli* and *Pseudomonas fluorescens*) and gram positive bacteria (*Bacillus cereus*, *Stapylococcus aureus*) with zones of inhibition ranging from 11 mm to 16 mm. The *Pleurotus ostreatus*, and *Lentinula edodes* showed less antibacterial activities with less than 10 mm of inhibition zones. The relative phytochemicals distribution is responsible for its' biological activities. It is predicted that the phytochemical belongings is acknowledged in our study in the innate medicinal plants of *Pleurotus ostreatus*, *Ganoderma lucidum* and *Lentinula edodes* will be advantageous for clarification and footing of Pharmacognosy outlining of edible mushrooms.

Keywords

Mushrooms, Medicinal Plants, Statistical Inference, Phytochemicals, Fruiting Bodies

1. Introduction

There are over 200 edible mushroom species. The mushrooms *Pleurotus ostreatus* (oyster), *Ganoderma lucidum* (reishi), and *Lentinula edodes* (shiitake) possess immense medicinal properties. The fruiting bodies of *Pleurotus spp.* possesses a number of therapeutic properties like anti-inflammatory, immunostimulatory, immunomodulatory

[1], anticancer activity [2], and ribonuclease activity [3]. Oyster mushroom has been explored to combat simple and multiple drug resistant isolates of *Escherichia coli, Staphylococcus epidermidis*, *S. aureus* [4] and species of *Candida, Streptococcus*, *and Enterococcus* [5]. It has been reported that *Ganoderma lucidum* has exhibited a greater antibacterial potential with their inhibition zones greater than that of some commercial antibiotics [6]. Shiitake mushrooms are the second most commonly cultivated edible mushrooms in the world. Extracts from this mushroom, and sometimes the whole dried mushroom, are used in herbal remedies. This mushroom is used as an anti-carcinogenic, anti-inflammatory, antioxidant, antifungal, antibacterial, antiviral as well as antithrombotic in cardiovascular disorders [7]. Studies in animals have found antitumor, cholesterol-lowering, and virus-inhibiting effects in compounds in shiitake mushrooms [8]. The medicinal properties that the mushroom species possess are due to the presence of certain phytochemicals. The phytochemical screening of edible mushrooms revealed the presence of alkaloids, flavonoids, cardiac glycosides, tannins, phenols, steroids, and terpenoids [9, 10, and 11]. Hence, the purposes of the existing work were to evaluate the phytochemicals and antibacterial properties of the edible mushrooms (*Pleurotus ostreatus*, *Ganoderma lucidum*, and *Lentinula edodes*) in order to establish the logical basis for some of their therapeutic properties in folkloric uses.

2. Materials and Methods

2.1. Sample Collection and Preparation of Extracts

Fruiting bodies of *Pleurotus ostreatus*, *Ganoderma lucidum* and *Lentinula edodes* were collected from National Mushroom Development and Extension Centre and identified by strengthening mushroom development officer, Savar, Dhaka-1304, Bangladesh.

The fruiting bodies of the selected mushrooms were collected and air dried for few days. The fruiting bodies were grinded off into powder and kept in polythene bags for future uses. The extracts of selected sample powder were organized by saturated 50 gm of dried powder in 100 ml 80 % methanol and traumatized well. The solution then filtered with the help of filter paper and filtered extracts of the selected plant sample were reserved and used for additional phytochemicals and antibacterial screenings.

2.2. Phytochemicals Screening

Phytochemicals screening of the three edible mushrooms extracts were done by using standard established methods [12, 13, 14, and 15].

2.3. Anti-Bacterial Activity Screening

Bacterial cultures of *Bacillus cereus*, *Stapylococcus aureus, Escherichia coli, Pseudomonas fluorescens* were obtained from the culture collection centre, Department of Microbiology, Primeasia University, Bangladesh, were used for antibacterial test organisms. The bacteria were maintained on nutrient broth (NB) at 37°C. The gram positive (*Bacillus cereus* and *Staphylococcus aureus*) and gram negative bacteria (*Escherichia coli, Pseudomonas fluorescens)* were pre-cultured in nutrient broth overnight in a rotary shaker at 37°C, centrifuged at 10,000 rpm for 5 min, pellet was suspended in double distilled water and the cell density was standardized spectrophotometrically (A_{610} nm). The methanolic extracts of fruiting bodies of *Pleurotus ostreatus*, *Ganoderma lucidum*, and *Lentinula edodes* were tested by the disc diffusion method [16]. A defined concentration of the extracts (100 µg ml^{-1}) was prepared by reconstituting with sterile distilled water. The test microorganisms were seeded into respective medium by spread plate method 10 µl (10^6 cells/ml) with the 24h cultures of bacteria growth in nutrient broth. After solidification the filter paper discs (5 mm in diameter) impregnated with the extracts were placed on test organism-seeded plates. The diameters of the inhibition zones were measured in millimeter.

3. Statistical Analysis

The screening of eleven selected phytochemicals among three nominated mushrooms Pleurotus ostreatus, Ganoderma lucidum and Lentinula edodes was statistically evaluated. Chi-square test was used to find out correlation of phytochemicals distribution in selected three mushrooms. Mean of zone inhibition with error bars generated with standard error of mean. This analysis was carried out using SPSS version 21.

4. Results

This study has uncovered the presence of phytochemicals painstaking as active medicinal chemical ingredients. The all selected mushrooms show the 11 selected phytochemicals with different concentrations. Graphical presentation of 11 selected phytochemicals such as alkaloids, flavonoids, polyphenols, saponins, steroids, coumarins, terpenoids, vit-C, tannins, anthocyanin and cardiac glycosides are exhibited in figure 1.

In *Ganoderma lucidum*, flavonoids, polyphenols, coumarins, vit-C, tannins, and anthocyanin while steroids, terpenoids and cardiac glycosides in *Pleurotus ostreatus* as well as vit-C and tannins in *Lentinula edodes* remained in utmost concentration category. The phytochemicals distributions in *Pleurotus ostreatus*, *Ganoderma lucidum* and *Lentinula edodes* were statistically non-significant ($p > 0.05$).

In the fruiting bodies of *Pleurotus ostreatus,* polyphenols and vit-C are present as medium concentration sort whereas alkaloids, flavonoids, and tannins are existed with lowest concentration category. On the other-hand, alkaloids, and terpenoids are present in *Ganoderma lucidum* and *Lentinula edodes* as medium concentration type of phytochemicals while saponins and cardiac glycosides in *Ganoderma lucidum*, and anthocyanins and cardiac glycosides in *Lentinula edodes* are recorded in lowest concentration sort of phytochemicals.

Figure 2 shows the comparative frequencies of

phytochemicals among three selected mushrooms. 6 (54.5 %) out of 11 (100 %) phytochemicals were uppermost (+++) concentration in *Ganoderma lucidum* whereas 3 (27.3 %) and 2(18.2 %) from 11 (100 %) selected phytochemicals were in *Pleurotus ostreatus*, and *Lentinula edodes*.

A trend line of 18.2 %, 27.3 %, and 45.5 % in *Pleurotus ostreatus*, *Ganoderma lucidum*, and *Lentinula edodes* was observed as sort of medium (++) concentration category despite the fact that in lowest (+) concentration group, the highest phytochemical frequency , 27.3 %, was observed in *Pleurotus ostreatus*.

Fruiting bodies extracts of *Pleurotus ostreatus* ,*Ganoderma lucidum*, and *Lentinula edodes* exhibited speckled in the zone of inhibition from 7-10 mm, 11-16 mm and 8-11mm respectively (Figure 3). *Ganoderma lucidum* fruiting bodies extract showed highest activity against *Bacillus cereus* (16 mm) whereas *Pleurotus ostreatus* and *Lentinula edodes* did not show activity against *Bacillus cereus* (0 mm). The maximum inhibition zone of *Pleurotus ostreatus* and *Lentinula edodes* was 10 mm against *Escherichia coli* and 11 mm against *Pseudomonas fluorescens* respectively while *Ganoderma lucidum* showed 13 mm, 12 mm, and 11 mm zone of injibition against *Stapylococcus aureus, Pseudomonas fluorescens,* and *Escherichia coli*.

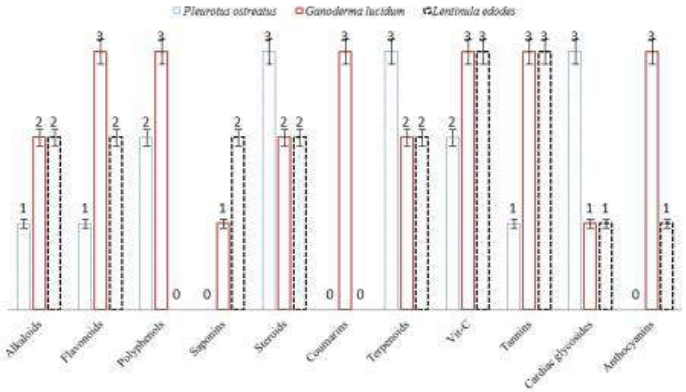

Figure 1. *Distribution of phytochemicals in three selected mushrooms. ["3" = high amount after added of reagent immediately; "2" = moderate amount after 5 minutes of reagent added; "1" = low amount after 10 minutes of reagent added and "0" = absence and parenthesis; 0 = "-", 1 = "+", 2 = "++", and 3 = "+++"].*

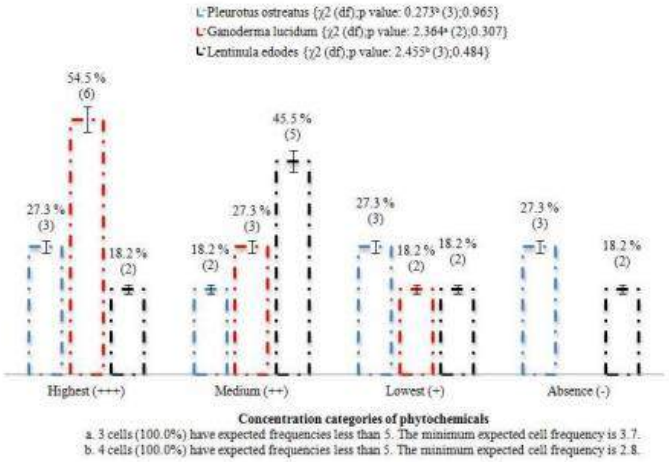

Figure 2. *Graphical presentation of eleven selected phytochemicals with their reasonable abundances categories specifications. The column (X-axis) represents the concentration categories of selected plants with three color stratified and the row (Y-axis) with frequency of phytochemicals. Error bars are generated with 5 % value from selected data chart.*

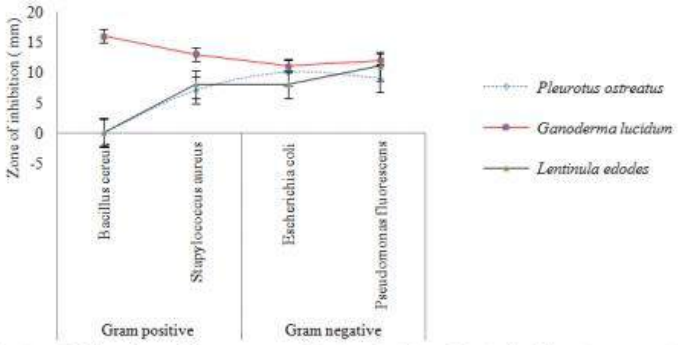

Figure 3. *Antibacterial activity of edible mushrooms, Pleurotus ostreatus, Ganoderma lucidum, and Lentinula edodes against gram positive and gram negative bacteria. Mean of zone inhibition with error bars generated with standard error of mean.*

5. Discussion

The relative distribution of eleven selected phytochemicals among the three selected mushrooms was correlated statistically in this study. In the highest concentration category, 6 of 11 phytochemicals were documented in the *Ganoderma lucidum*. Phytochemicals found in edible mushroom are known to play a vital role in upholding health. The presence of essential nutrients and minerals in the wild edible mushroom could be utilized to improve health [16, 17]. Alkaloids showed stimulant to CNS, anti-microbial activities, sympathomimetic, vasodilator, antihypertensive, antipyretics, and anti-malarial activity [18]. *Ganoderma lucidum* and *Lentinula edodes* with medium level of alkaloids bestow high medicinal values.

Several epidemiological studies have shown beneficial effects of polyphenol in cancer, cardiovascular, and neurological diseases. The health benefits associated with polyphenol containing preparation consumption have also been corroborated in animal studies of cancer chemoprevention, hypercholesterolemia, artherosclerosis, Parkinson's disease, Alzheimer's disease, and other aging-related disorders [19]. The existence of phenolic compounds in *Ganoderma lucidum* and *Pleurotus ostreatus* notice that these selected mushrooms may be used as a medicine. Tannin employs anti-inflammatory effects probably by inhibiting the release, synthesis and /or production of inflammatory cytokines and mediators, including prostaglandins, histamine, polypeptide kinins and so on [20]. Excepting *Pleurotus ostreatus* and the *Ganoderma lucidum*, and *Lentinula edodes* extracts shows highest level of tannin.

The methanolic extracts of *Pleurotus ostreatus, Ganoderma lucidum*, and *Lentinula edodes* were also reported that the flavonoids, cardiac glycosides, anthocyanins, steroids, coumarins, terpenoids, and vit-C were present at different concentration categories. It was reported that coumarins used in the treatment of asthma and lymphedema as well as this medication was a blood thinner used to keep blood flowing smoothly and prevent the formation of blood clots [21, 22].

Cardiac glycosides include a large family of naturally resulting compounds, the central structures of which contain a steroid nucleus with a five-membered lactone ring (cardenolides) or a six-membered lactone ring (bufadienolides) and sugar moieties [23]. Interestingly, the concentrations of cardiac glycosides used for cancer treatment are extremely close to those found in the plasma of cardiac patients treated with the same drugs, suggesting that the anticancer effects of these drugs are exerted at non-toxic concentrations [24]. The vitamin C content in *Pleurotus ostreatus, Ganoderma lucidum* and *Lentinula edodes* claimed that they have antioxidant activity. So, Vitamin C in humans must be ingested for survival. Vitamin C is an electron donor, and this property accounts for all its known functions. As an electron donor, vitamin C is a potent water-soluble antioxidant in humans. Antioxidant effects of vitamin C have been demonstrated in many experiments in vitro [25]. In this study, it is also reveals that the antibacterial activity of edible mushrooms show significant zone of inhibition against gram positive bacteria and gram negative bacteria. This study rationalizes the appealed uses of *Pleurotus ostreatus, Ganoderma lucidum* and *Lentinula edodes* in the traditional system of medicine to treat various infectious disease caused by microbes.

Based on the results obtained from the present study, it can be concluded that the methanolic extracts of mushroom can be fruitfully applied in the development of more effective and competent antimicrobial agents. The results of preliminary phytochemical analysis are in agreement with the reports of other workers. Thus we hope that the important phytochemical properties acknowledged by our study in the *Pleurotus ostreatus, Ganoderma lucidum* and *Lentinula edodes* will be helpful in the managing different diseases.

Supplementary effort is progress to identify the bioactive compounds and explicate their mechanism of action to scavenge the free radicals. This study is powerfully remindful that edible mushrooms can be used as antibacterial agent in the expansion of new drug for the therapy of urinary tract infections.

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Comparison of Antibacterial Activity of the Spent Substrate of *Pleurotus ostreatus* and *Lentinula edodes*

A. Zepeda-Bastida[1], D. Ojeda-Ramírez[1], S. Soto-Simental[1], N. Rivero-Perez[1] & M. Ayala-Martínez[1]

[1]Área Académica de Medicina Veterinaria y Zootecnia, Instituto de Ciencias Agropecuarias, Universidad Autónoma del Estado de Hidalgo, México

Correspondence: M. Ayala-Martínez, Área Académica de Medicina Veterinaria y Zootecnia, Instituto de Ciencias Agropecuarias, Universidad Autónoma del Estado de Hidalgo, Avenida Universidad s/n km 1, Tulancingo, Hidalgo, C.P. 43600, México. Tel: 52-771-717-2000 ext. 2449. E-mail: ayalam@uaeh.edu.mx

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Abstract

Nowadays, the uncontrolled use of antibiotics has created the problem of bacterial resistance to them, what has motivated the search for new alternatives of drug for the treatment of bacterial diseases. Here, we compare antimicrobial activity of spent substrate of mushroom *Pleurotus ostreatus* and *Lentinula edodes*, against *Escherichia coli*, *Salmonella tiphymorium*, *Staphylococcus aureus* and *Micrococcus luteus*. We designed two mixtures, barley straw to be used as a substrate of cultivation of mushroom *Pleurotus ostreatus* and oats or cedar for the cultivation of mushroom *Lentinula edodes*; and were obtained aqueous extracts from spent substrates; extracts were tested for antibacterial activity. The protocol was a completely randomized assay with a factorial arrangement design. The data were analyzed with PROC GLM, SAS. The results showed that in the case of *Escherichia coli* the greatest inhibition zone was of 12.66 mm at a concentration of 6 mg mL^{-1}, with treatment of *Lentinula edodes*/Cedar; *Salmonella tiphymorium* showed a greatest inhibition zone of 31.10 mm to a concentration of 5.12 mg mL^{-1}, with treatment of *Pleurotus ostreatus*/Barley straw; *Staphylococcus aureus* showed a greatest inhibition zone of 9.33 mm to a concentration of 100 mg mL^{-1}, with the treatment of *Lentinula edodes*/Cedar and finaly, *Micrococcus luteus* showed a greatest inhibition zone of 15.00 mm to a concentration of 50 mg mL^{-1}, with the treatment *Lentinula edodes*/Oats. In conclusion, the results suggest that it is possible to use indistinctly the spent substrate of *Pleurotus ostreatus* and *Lentinula edodes* as source of extracts with antibacterial activity.

Keywords: mushroom, *Escherichia coli*, *Salmonella tiphymorium*, *Staphylococcus aureus* and *Micrococcus luteus*

1. Introduction

The uncontrolled use of antibiotics has caused serious problems in human and animal health, causing that bacterias develop resistance to them, so World Health Organization considered to infections caused by bacteria resistant to drugs as a public health problem; therefore, it is necessary to find new pharmacological strategies, among which we can find natural products such as plants and fungi (Roca et al., 2015).

Due to the excess production of agricultural wastes it has increased world production of edible fungi to over 7 million tons, of which 70% are Basidiomycetes as *Agaricus bisporus, Pleurotus ostreatus* and *Lentinula edodes* (Sánchez, 2010; Lin, Ge, & Li, 2014). Basidiomycetes produce several bioactive substances such as polysaccharides, peptides, nucleosides and phenols which act as hypoglycemic, immunomodulatory, anti-inflammatory, antitumor, antiviral, antibacterial or antiparasitic (Wasser & Wis, 1999; Wasser, 2002; Sánchez, 2010). By increasing the production of edible mushrooms, also increases accumulation of spent substrate residues after cultivation (Grodzínskaya, Infante, & Piven, 2002).

Spent mushroom substrate containing carbohydrates as cellulose and hemicellulose, lignin, remnant of edible fungi, is a byproduct of mushroom production industry. Substrate used as growth media to produce mushroom is composed of maize cobs, wheat straw, grass straw, sugarcane bagasse, field hay, corn cobs, cotton seed hulls and some other. After several mushroom harvesting cycles, the productivity of the substrate could decrease so that the substrate is declared as spent (Guoa & Choroverb, 2006; Onyango, Palapala, Arama, Wagai, & Gichimu, 2011). One of the main problems in the production of mushrooms still the treatment and disposal of spent

mushroom substrate; many studies have already been carried out for the use of such substrates, among which we mention feeding and/or antimicrobial activity (Zhu, Sheng, Yan, Qiao, & Lv, 2012).

The fungi have an important role in the degradation of organic matter (Chang & Miles, 1984), in addition to being a source of bioactive substances to produce antibiotics or pharmaceutical drugs, such as functional food and additives in feeding stuffs (Santoyo, Ramírez-Anguiano, Reglero, & Soler-Rivas, 2009). Crop of mushrooms (*Pleurotus ostreatus*) it is a source of products agricultural, their organic waste generated can be used as a source of food with high protein content and as an alternative pharmaceutical treatment; *Pleurotus ostreatus* is a fungus that has the ability to grow on agricultural wastes, accelerates the biodegradation and recycling them, avoiding its burning and the subsequent environmental pollution (Varnero, Quiroz, & Álvarez, 2010). *Lentinula edodes*, fungi edible, is the most studied, has been shown that fruiting body and mycelium have antimicrobial properties; likewise, the lentine inhibits mycelium growth of other fungi as *Physalospora piricola, Mycosphaerella arachidicola* and *Botrytis cinerea* (Rojas, 2013; Romero-Arenas, Martínez, Damian, Ramírez, & López-Olguín, 2015).

The production of mushroom has been used with a large number of substrates; one of the main is straw, used as a source of carbon to increase the nutritional characteristics and palatability of the fruiting body, getting a better nutritional quality (Sánchez, 2010). The crop of fungi and the quick growth in mushroom production worldwide has resulted in large quantities of spent substrate mushroom (about 13.6 million t year^{-1}). The massive amounts of waste can cause environmental problems; this causes, led more research to develop technologies for its treatment or use (Lin et al., 2014).

In recent years, there has been a need study antimicrobial phytochemicals with potential to generate new pharmacological options. Our group previously demonstrated that the use of spent substrate of *Pleurotus ostreatus* mixed or not with medicinal plants, has antibacterial activity (Ayala et al., 2015). Therefore, the objective of this study was to determine the antibacterial activity of spent substrate of *Pleurotus ostreatus* and/or *Lentinula edodes* against *Escherichia coli, Salmonella tiphymorium, Staphylococcus aureus* and *Micrococcus luteus* at different concentrations.

2. Method

2.1 Strain, Substrates and Cultivation Mushrooms

The blocks (1 Kg) were obtained from Centro de Investigaciones Biológicas of the Universidad Autónoma del Estado de Hidalgo, México. The blocks were formed by a mixture of barley straw; which were purchased in Central Abastos in Pachuca Hidalgo, Mexico, the taxonomic identification did it the botanist Dr. Miguel Angel Villavicencio Nieto; the specimens were deposited at the Herbarium of the Centro de Investigación de Ciencias Biológicas, of the Universidad Autónoma del Estado de Hidalgo, México. To form the substrate, barley straw was colonized with mycelium of *Pleurotus ostreatus* UAEH-004 in solid substrate fermentation; mushrooms were harvested at 23 d and was obtained spent substrate; on the other hand, oats or cedar were colonized with mycelium of *Lentinula edodes* UAEH-015 in solid substrate fermentation; mushrooms were harvested at 90 d and were obtained spent substrate of each one.

2.2 Preparation of Organic Extracts

The extracts were obtained by mixing 100 g of spent substrate mushroom of each treatments and 300 mL distilled water in case of *Pleurotus ostreatus* and 600 mL distilled water in case of *Lentinula edodes*. Then, the mix was macerated in blender during 1 min. Subsequently, mix macerated was filtered using gauze, to separate solid and liquid parts, was filtered through filter paper Whatman® #41. The extract was placed in a water bath to 70 °C for 48 h, to obtain the sample dry; for each 25 ml of liquid extract were obtained 0.472 g of *Pleurotus ostreatus* and 0.451 g of *Lentinula edodes* of dry extract.

2.3 Colony Forming Units (CFU)

For obtention of CFU, 100 µl mother sample was placed in 50 ml of nutrient broth and incubated at 37 °C for 24 h. Subsequently, through serial dilutions of this solution, to concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 200 µl were inoculated on plates; *Escherichia coli* in medium culture Mac Conkey (DIBICO, México), and *Salmonella tiphymorium, Staphylococus aureus* and *Micrococcus luteus* in medium culture Estafilococos No. 110 (DIBICO, México), and incubated at 37 °C for 24 h, CFU they were counted.

2.4 Test Organisms

The test organisms were *Escherichia coli* (ATCC25922), *Salmonella tiphymorium* (ATCC14028), *Staphylococcus aureus* (ATCC25923) and *Micrococcus luteus* (ATCC9341).

2.5 Antimicrobial Assay

The antibacterial activity of the spent substrate of *Pleurotus ostreatus* and/or *Lentinula edodes* extracts were studied by the method of paper disc diffusion assay with slight modification (Kil et al., 2009). The bacterial pathogens strain were grown in liquid medium for 24 h to yield a final concentration of *Escherichia coli* 7.7×10^6 CFU/200 µL, *Salmonella tiphymorium* 1.11×10^7 CFU/200 µL, *Staphylococus aureus* 1.0×10^7 CFU/200 µL and *Micrococcus luteus* 1.04×10^7 CFU/ 200µL. Next, aliquots of 0.1 ml of the test microorganisms were spread over the surface of agar plates. Sterilised filter paper discs of 6 mm diameter (paper Whatman® #41) were saturated with 50 µl of different concentrations (0, 6, 12.5, 25, 50 and 100 mg mL^{-1}) spent substrate of *Pleurotus ostreatus* and/or *Lentinula edodes* extracts. The soaked discs were then placed in the middle of the plates and incubated for 24 h at 37 °C (Forma Series II Water Jacket CO_2, Incubator, Model 3100, Thermo Scientific, USA), after which the diameter (in mm) of each inhibitory zone was measured (scalimeter). Negative control was prepared with distilled water; as positive control was used commercial antibiotic (Penicillin G sodium salt, Sigma-Aldrich, St. Louis, MO, USA) to a concentration 100 mg mL^{-1} on medium culture Mac Conkey and Estafilococos No. 110.

2.6 Statistical Analysis

Data were analyzed using factorial design 3 × 3 and blocked by extract type and extract concentration and bacterial strain as factors. A PROC GLM procedure and LSMEANS option were used (SAS, 2002).

3. Results

Treatment with spent substrate of *Pleurotus ostreatus* and *Lentinula edodes* extracts were effective against four bacterial strains tested; antibacterial activity at different concentrations is showed in Table 1; the tested bacteria were quantitatively assessed by measuring the diameter of inhibition generated for each sample; each result is the mean of three replicates. The results showed that the spent substrate *Pleurotus ostreatus*/Barley straw extracts presented highest inhibitory effect against *Escherichia coli* (7.7×10^6 CFU/200 µL) at a concentration of 12.5 mg mL^{-1} with 9.86 mm inhibition halo; *Staphylococus aureus* (1.0×10^7 CFU/200 µL) at a concentration of 25 mg mL^{-1} with 9 mm inhibition halo and *Micrococcus luteus* (1.04×10^7 CFU/ 200µL) at a concentration of 50 mg mL^{-1} with 9.66 mm inhibition halo. *Salmonella tiphymorium* (1.11×10^7 CFU/200 µL) at a concentration of 12.5 mg mL^{-1} with 31.10 mm inhibition halo, showing significant differences with the concentrations 6, 25, 50 and 100 mg mL^{-1} ($P < .05$).

In case of spent substrate *Lentinula edodes*/Oats extracts, results showed that the highest inhibitory effect against *Escheriquia coli* (7.7×10^6 CFU/200 µL) to 12.5 mg mL^{-1} with 9.20 mm inhibition halo; *Salmonella tiphymorium* (1.11×10^7 CFU/200 µL) to 6 mg mL^{-1} with 30.30 mm inhibition halo; *Staphylococus aureus* (1.0×10^7 CFU/200 µL) to 50 mg mL^{-1} with 8.66 mm inhibition halo and *Micrococcus luteus* (1.04×10^7 CFU/200µL) to 50 mg mL^{-1} with 15 mm inhibition halo; in this treatment, there was no significatives differences between each of the concentrations tested for each bacteria.

Spent substrate *Lentinula edodes*/Cedar extracts, results showed the highest inhibitory effect against *Escheriquia coli* (7.7×10^6 CFU/200 µL) to 6 mg mL^{-1} with 12.66 mm inhibition halo, showing significant differences with the concentrations 12.5, 25, 50 and 100 mg mL^{-1} ($P < .05$) and *Salmonella tiphymorium* (1.11×10^7 CFU/200 µL) to 50 mg mL^{-1} with 29.80 mm inhibition halo, showing significant differences with the concentrations 6, 12.5 and 25 mg mL^{-1} ($P < .05$). The highest inhibitory effect against *Staphylococus aureus* (1.0×10^7 CFU/200 µL) was to 100 mg mL^{-1} with 9.33 mm inhibition halo and *Micrococcus luteus* (1.04×10^7 CFU/200µL) to 100 mg mL^{-1} with 10 mm inhibition halo. Positive control (penicilin) showed larger diameter of inhibition (45 and 30.6 mm), than all spent substrate extracts tested.

Table 1. Antibacterial activity of spent substrate of *Pleurotus ostreatus* and *Lentinula edodes* extracts at different concentrations i*n vitro*

Treatment	Concentration (mg mL^{-1})	Diameter of inhibition (mm)			
		Escherichia coli	*Salmonella tiphymorium*	*Staphylococcus aureus*	*Micrococcus luteus*
		Means±SD			
Pleurotus ostreatus/Barley straw	6	9.53±0.40	22.33±2.51^{c}	0.00	0.00
	12.5	9.86±0.51	31.10±8.61^{a}	0.00	0.00
	25	9.46±0.68	26.96±10.01^{b}	9.00±1.00	0.00
	50	9.10±1.05	24.63±5.22^{b}	8.86±1.69	9.66±1.52
	100	9.00±1.17	20.50±3.43^{c}	8.36±0.71	8.83±1.25
Lentinula edodes/Oats	6	8.83±1.04	30.30±2.30	0.00	0.00
	12.5	9.20±0.72	29.60±4.35	0.00	0.00
	25	8.50±0.50	29.40±2.02	0.00	0.00
	50	8.06±1.32	28.76±3.99	8.66±1.15	15.00±2.29
	100	8.83±0.28	27.66±3.78	8.33±1.52	12.00±1.80
Lentinula edodes/Cedar	6	12.66±2.88^{a}	27.83±1.44^{b}	0.00	0.00
	12.5	8.66±0.35^{b}	26.00±1.00^{b}	0.00	9.33±0.57
	25	10.10±1.92^{b}	28.46±5.68^{b}	8.66±0.57	9.66±1.15
	50	7.83±1.61^{b}	29.80±1.55^{a}	8.43±1.40	9.66±1.52
	100	8.80±1.85^{b}	29.46±3.99^{a}	9.33±1.15	10.00±1.00
Penicilin	100	30.6±3.05	45.00±0.00	45.00±0.00	45.00±0.00

Note. ^{abc} Literal different ranks indicate significant difference between the concentrations of each treatment ($P < 0.05$) with the Tukey test.

When comparing the largest zone of inhibition of the three treatments tested (*Pleurotus ostreatus*/Barley straw, *Lentinula edodes*/Oats y *Lentinula edodes*/Cedar) against each bacteria (Table 2), the results showed in the case of *Escherichia coli* the largest zone of inhibition was at a concentration of 6 mg mL^{-1} (12.66 mm) with treatment of *Lentinula edodes*/Cedar; *Salmonella typhimurium* showed the largest zone of inhibition at a concentration of 5.12 mg mL^{-1} (31.10 mm) with treatment of *Pleurotus ostreatus*/Barley straw; *Staphylococcus aureus* showed the largest zone of inhibition at a concentration of 100 mg mL^{-1} (9.33 mm) with the treatment of *Lentinula edodes*/Cedar and *Micrococcus luteus* showed the largest zone of inhibition at a concentration of 50 mg mL^{-1} (15.00 mm) with treatment of *Lentinula edodes*/Oats. *Salmonella typhimurium* and *Staphylococcus aureus* no showed significant differences between each treatments; *Escherichia coli* treated with *Lentinula edodes*/Oats is significantly different to the treatment *Lentinula edodes*/Cedar ($P < 0.05$), but without showing difference significant with treatment *Pleurotus ostreatus*/Barley straw, while *Micrococcus luteus* treated with *Lentinula edodes*/Oats is significantly different with treatment of *Lentinula edodes*/Cedar and *Pleurotus ostreatus*/Barley straw ($P < 0.05$).

Table 2. Comparison of antibacterial activity of spent substrate of *Pleurotus ostreatus* and *Lentinula edodes* extracts against *Escheriquia coli*, *Salmonella tiphymorium*, *Staphylococus aureus* and *Micrococcus luteus* in *vitro*.

Bacteria	*Pleurotus ostreatus*/Barley straw		*Lentinula edodes*/Oats		*Lentinula edodes*/Cedar	
	Concentration (mg mL^{-1})	Diameter of inhibition (mm) Means ± SD	Concentration (mg mL^{-1})	Diameter of inhibition (mm) Means ± SD	Concentration (mg mL^{-1})	Diameter of inhibition (mm) Means ± SD
E. coli	12.5	9.86 ± 0.51^{a}	12.5	9.20 ± 0.72^{b}	6	12.66 ± 2.88^{a}
S.tiphymorium	12.5	31.10 ±8.61	6	30.30 ± 2.30	50	29.80 ±1.55
S. aureus	25	9.00 ±1.00	50	8.66 ±1.15	100	9.33 ±1.15
M.luteus	50	9.66 ±1.52^{b}	50	15.00 ±2.29^{a}	100	10.00 ±1.00^{b}

Note. ^{ab} Literal different ranks indicate significant difference between treatments of each bacteria ($P < 0.05$) with the Tukey test.

4. Discussion

In recent years the bacteria have acquired the ability of multi-resistance to antibiotics (Nehra, Meenakshi, & Yadav, 2012) which has generated that recent research are focus in the search for alternative treatments, such as fungi. Edible fungi such as *Pleurotus ostreatus*, have shown a high nutritional value as food (Patel, Naraian, & Singh, 2012) and anti-inflammatory, antidiabetic, antiviral, antioxidant, anticancer, antitumor, inmunomodulatory and antibacterial activity; however, most of the research has been based on the study of the fruiting body and not in spent substrate (Hearst et al., 2009; Deepalakshmi & Mirunalini, 2014).

A water-soluble polysaccharide named PL was isolated and purified from spent mushroom substrate, the polysaccharide contained two fractions (PL1 and PL2), composed of glucose, rhamnose and mannose; the antibacterial activity of polysaccharide against *E. coli* was the strongest, while the weakest against *Sarcina lutea*, the minimal inhibition concentrations of PL2 were 12.5, 25 and 100 μg/mL, respectively (Zhu et al., 2012). We show that aqueous extract of spent substrate of *Pleurotus ostreatus*/Barley straw has antibacterial activity against *Escherichia coli* (9.86 mm), *Staphylococcus aureus* (9.00 mm), *Micrococcus luteus* (9.66 mm) and *Salmonella tiphymorium* (31.10 mm), similar to that obtained using extracts of mushroom *Pleurotus ostreatus* obtained with different organic solvents (24.56 and 14 mm) for Gram positive and Gram negative bacteria (Nehra et al., 2012).

For the cultivation of *Lentinula edodes* for many years have used various agricultural and industrial wastes, among which we mention sorghum, sugar cane, sawdust, oak, cedar (Grodzínskaya et al., 2002) as carbon source; shiitake mushrooms (*Lentinus edodes*) is of great importance, due to its attributed not only to its nutritional value, but also potential applications in industrial food and medicine as antibacterial (Hearst et al., 2009) and/or antitumor, among other features; this activity is due to the lentina (one polysaccharide isolated from fruiting body), which acts as an enhancer of host defense; has shown action against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* (Hatvani, 2001). Chowdhury, Kubrai, and Ahmed (2015) mentioned antimicrobial activity of 3 edible mushrooms (*Pleurotus ostreatus, Lentinula edodes, Hypsizigus tessulatus*) methanolic extracts, indicated considerable activity against bacteria and fungi, reveling zone of inhibition ranged from 7 ± 0.2 to 20 ± 0.1 mm; Kazue, Megumi, and Dantas (2001) found that the mycelium of 35 different strains of *Lentinus edodes*, has antibacterial activity against *B. subtilis*, with inhibition halos 5-20 mm in diameter similar to our findings.

This work is novel because for the first time is studied the use of spent substrate *Lentinula edodes* as antibacterial, since only been shown this activity in the fruiting body; they have been used different extraction techniques: high-pressure operations and low-pressure methods. The high-pressure technique was applied to obtain *Lentinus edodes* extracts using pure CO_2 and CO_2 with co-solvent or organic solvents such as n-hexane, ethyl acetate and dichloromethane (Kitzberger, Smânia Jr., Pedrosa, & Ferreira, 2007); here it is included barley straw as a substrate for the cultivation of *Pleurotus ostreatus* and oats or cedar for the cultivation of *Lentinula edodes*, in order to obtain aqueous extracts and determine its antibacterial activity; the findings suggest that it is feasible to use these substrates in the future for obtain antibacterial pharmaceutical compounds and at the same time reduce the pollution by their accumulation.

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Research Article Open Access

Lentinula edodes and *Pleurotus ostreatus*: functional food with antioxidant - antimicrobial activity and an important source of Vitamin D and medicinal compounds

Simone Parola[1,2], Luca Chiodaroli[1,2], Viviana Orlandi[2], Candida Vannini[2], Luigi Panno[3]*

[1]PreventPCB srl, via Sempione 26, 21029 Vergiate (VA) Italy; [2]University of Insubria Varese, Italy, Department of Biotecnologie e Scienze della Vita (DBSV); [3]Società Agricola IoBoscoVivo srl, via Sempione 26, 21029 Vergiate (VA) Italy

Corresponding author: Luigi Panno, Società agricola IoBoscoVivo srl, via Sempione 26, Vergiate 21029, Varese, Italy

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ABSTRACT

Background: Mushrooms produce a large amount of medicinal compounds, and are also an optimal source of fibres, proteins, vitamins (like groups B and D), and other micronutrients including potassium, magnesium, etc. Consequently, mushrooms are commonly considered to be functional foods. Many works report the high biological potentials of medicinal mushrooms involving their antibacterial, hypoglycaemic, anticholesterolemic, radical scavenging, and anti-inflammatory effects.

Context and purpose of this study: First off, this work aimed to find strains of *Lentinula edodes* and *Pleurotus ostreatus* from a bank of edible mushrooms bought from international strain banks (Table I) that could possess health benefit related properties, such as a radical scavenging activity (antioxidant effect), antibacterial effects against common pathogenic bacteria, and being able to produce interesting nutrients and secondary metabolites. As the fungal bank comprises of 20 strains of *L. edodes* and 20 strains of *P. ostreatus,* a first screening was made by the selection of 13 strains for each mushroom able to grow in multiple wood types or that were particularly productive and had proved good growth reproducibility over the last 5 years. This work also studied the correlation between culture conditions and mushroom quality in terms of the previously reported properties. Comparison among the selected strains was operated by the assessment of

antioxidant and antimicrobial activities after different sample treatments. Furthermore, an initial optimization of the analytic techniques was produced for the direct estimation of important secondary metabolites and nutrients by means of HPLC-MS/MS technique. Further research will encompass an evaluation of transformation processes (drying, freezing, rehydration, cooking, etc.) impact on radical scavenging, antibacterial activity, and possible degradation/loss of nutraceutically important substances such as vitamin D2, ergothioneine, eritadenine, lovastatin, lentinan, and lenthionine.

Results: 13 strains of each mushroom species have been cultivated on different wood logs. Seven strains of shiitake and six strains of oyster mushroom were able to produce sporocarps. Antioxidant levels in water extracts from dried mushrooms produced significatively different results on the basis of strains and of wood. Both mushrooms demonstrated higher radical scavenging activity in log cultivation than substrates cultivation, which was subsequently used as reference. Furthermore, all strains of *P. ostreatus* demonstrated the lowest level of antioxidant activity at 4°C, a significant increase towards 50°C and a limited decrease towards 80°C. The same trend was observed for shiitake extracts. Concerning the shiitake mushroom only, crude water extracts showed an interesting antibacterial activity against the model microorganisms *Pseudomonas aeruginosa* and *Staphylococcus aureus*. A comparison was also performed between the best performing strain extract and the commercial antibiotic Ceftriaxone against *P. aeruginosa*, assessing that 20 mg of crude extract corresponds to 0.2 mg of the pure antibiotic when studied by means of disk diffusion assay.

Conclusion: The results suggested that the cultivation of both shiitake and oyster mushrooms on logs could enhance the content of antioxidant and antibacterial activities, compared to the cultivation of mushrooms on sawdust substrates. Radical scavenging and antibacterial activity depends both on *L. edodes* strain and the log type. The bacteriostatic/bactericidal activity of the best performer strain may depend on a pH and solvent treatment sensitive substance. Secondary metabolites such as ergothioneine and vitamin D2 from both shiitake and oyster were released just after water extraction: this suggests that the transformation/cooking processes may produce a loss of characteristic mushroom biological properties in water. Further evaluation of biologically relevant compounds content and loss during different food transformation and cooking processes will be assessed.

Keywords: Shiitake, Oyster, *Pleurotus ostreatus, Lentinula edodes, Pseudomonas aeruginosa, Staphylococcus aureus*, Log grown mushroom, antioxidant, radical scavenging, total phenolic content, DPPH, ABTS, Folin-Ciocalteu, antibacterial, fruitbodies, sporocarps.

INTRODUCTION

Throughout history, mushrooms are well-known organisms with relevant medicinal properties in addition to the consumption of common food [1]. Edible mushrooms are valuable dietary components thanks to the high content of nutritionally relevant compounds, in addition to their

taste and organoleptic properties. Nutritional value was found in many works due to their high content of proteins, fibres, vitamins, and mineral salts, in addition to their low-fat level [2-4], and high content of micronutrients [5-6].

Lentinula edodes (shiitake) and *Pleurotus ostreatus* (oyster) are among the most cultivated mushrooms in the world [7]. These can be grown using a wide range of methods, conditions, and substrates. Moreover, the choice of the cultivation technique was previously found both to affect the fruiting yield and the production of secondary metabolites.

Oyster and shiitake are characterized by their short growing time compared to other edible species, and feasible production during all the year. Accordingly, their cultivation and experimentation, and the knowledge of their nutritional and medical value has increased over the years [8], [9], [10], [11].

Shiitake and oyster fruit bodies have actually been demonstrated to hold effective antioxidant activity due to a high phenolic compounds content [12], [13], [14], [15]. Additionally, shiitake extracts demonstrated antibacterial activity against *Staphylococus aureus, Bacillus subtilis* and *Pseudomonas aeruginosa* in previous research [16], [17], [18], [19], [20], [21].

Many studies regarding the medicinal properties of these mushrooms have been conducted, which have also allowed the recovery of interesting secondary metabolites. In the shiitake mushroom, one of the most relevant is lentinan, a β-glucan compound demonstarting several activities that comprise the antitumor effect due to induced stimulation of the host immune system [6]. Other interesting metabolites are ergothioneine, showing antioxidant activity and eritadenine, which holds an hypocholesterolemic effect [22], [23], [24].

Moreover, *Pleurotus ostreatus* also contains a large number of medicinal compounds. In particular, lovastatin is a secondary metabolite which is capable of lowering blood cholesterol levels and has been therefore suggested to prevent cardiovascular diseases [25], [26].

In this work, 13 strains of *L. edodes* and *P. ostreatus* were selected on the basis of their good and reproducible growth yield over the last five years. All the mushrooms were evaluated for antimicrobial activity against two bacterial strains, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Radical scavenging activity was studied by means of ABTS and DPPH assay, and total phenolic content was also measured by means of a Folin-Ciocalteu assay.

Currently, there are not enough studies regarding temperature and transformation/cooking processes effects on the nutrients content and the biological activity of characteristic compounds contained in mushrooms [27]. Accordingly, we started the optimization of mass spectrometry procedures to allow the analysis of crude water extracts for the direct evaluation of nutrient and metabolites loss during transformation and mushroom cooking processes involving water contact (i.e. extraction) and thermal treatments.

MATERIALS AND METHODS

Fungal strains

All strains of *Lentinula edodes* (Berk) Pegler, 1976 and *Pleurotus ostreatus* (Jacq.) P. Kumm., 1871, were collected from different strain banks in Europe (CBS, Mycelia, CNC, BCCM), USA (ACB) and Italy (Società agricola IoBoscoVivo srl)

Table I. Selected strains used in this project and reference international banks of strains origin

***Lentinula edodes* strains**

Bank	Strain	IoBoscoVivo Code	Origin
CBS (CBS-KNAW Fungal biodiversity centre)	134.85	KCS0157	China
CBS (CBS-KNAW Fungal biodiversity centre)	225.51	KCS0158	Japan
CBS (CBS-KNAW Fungal biodiversity centre)	833.87	KCS0159	Germany
BCCM (Belgian collection of micro-organisms)	28773	KCS0138	Belgium
BCCM (Belgian collection of micro-organisms)	29756	KCS0139	China
Mycelia	M3710	KCS0141	Far east
Mycelia	M3770	KCS0142	Far east
Mycelia	M3790	KCS0143	Far east
Aloha colture bank	Jumbo	KCS0144	Ukraine
Società agricola IoBoscoVivo	KCS0140	KCS0140	China
Società agricola IoBoscoVivo	KCS0127	KCS0127	China
Società agricola IoBoscoVivo	KCS0128	KCS0128	China

***Pleurotus ostreatus* strains**

Bank	Strain	IoBoscoVivo Code	Origin
CBS (CBS-KNAW Fungal biodiversity centre)	145.22	KCS0153	Germany
CBS (CBS-KNAW Fungal biodiversity centre)	291.47	KCS0154	France
CBS (CBS-KNAW Fungal biodiversity centre)	342.69	KCS0155	Netherland
BCCM (Belgian collection of micro-organisms)	28511	KCS0146	Belgium
Mycelia	M2181	KCS0147	Europe
Mycelia	M2191	KCS0148	Europe
Mycelia	M2153	KCS0149	Europe
Aloha colture bank	JB	KCS0150	USA
Società agricola IoBoscoVivo	KCS0160	KCS0160	Italy
Società agricola IoBoscoVivo	KCS0050	KCS0050	Italy
Società agricola IoBoscoVivo	KCS0152	KCS0152	Italy

Bacterial strains and growth conditions

Bacterial strains used in this study includes Gram-positive *Staphylococcus aureus* MSSA (ATCC29213) and Gram-negative *Pseudomonas aeruginosa* PAO1, both grown in Luria Bertani (LB) broth under aerobic conditions at 37°C for 24 h [28].

Standard compounds

Standards for HPLC-MS analysis of lentinan and eritadenine were bought from Carbomer Incorporation (San Diego) and Alpha Chemistry, Holtsville, NY 11742, USA. Vitamin D2, Lovastatin, and Ergothioneine were obtained from Sigma-Aldrich, Darmstadt, Germany. Antibiotic Ceftriaxone disodium salt was purchased from Sigma Aldrich.

Reference strains

KCS0140 for shiitake and KCS0160 for oyster strains grown on sawdust substrate, were used as reference for antioxidant and antibacterial analysis. Substrates composition were the property of Società Agricola IoBoscoVivo srl (via Sempione 26, Vergiate, Varese, Italy).

Log cultivation

The grain spawn method was used for mycelia cultivations [11], [29], [30]. Spawns of different strains of *Lentinula edodes* were inoculated in fresh woods of *Quercus robur* (oak), *Robinia*

pseudoacacia (robinia), and *Fagus sylvatica* (beech). For *Pleurotus ostreatus* logs of *Populus tremula* (poplar), *Salix alba* (willow), *Tilia platyphyllos* (tilia), and *Robinia pseudoacacia* (robinia) were used. For each mushroom strain, 20 logs of each wood species were used, resulting in 1040 logs for *Lentinula edodes* and 1040 logs for *Pleurotus ostreatus*. Spawn was driven into holes in each wood species by means of an inoculator gun. Afterwards, holes were covered with plastic foam plugs and logs were stored in sprayed greenhouses for four months to achieve the complete colonization of the mycelia. Finally, logs were put outside in order to obtain the production of fruitbodies [29]. Within 12 months, every harvest of fruitbodies was performed.

Sample thermal treatments

Each mushroom strain was dried at 37°C for 2 days, subsequently 5g aliquots of each substrate grown strain (KCS0140 and KCS0160) were subjected to thermal treatments either at 4°C or 37°C or 50°C or 80°C for 2h in dark conditions. After treatment, materials were powdered and immediately subjected to extraction.

Sample water extraction

The extraction method reported by N.M. Tonucci *et al.* (2015) was modified. Briefly, 5 g of all fruitbodies that underwent thermal treatment were powdered and extracted for 72 h at 4°C in 100 ml of water [31]. The final water extracts were centrifuged and the supernatants were filtered on 0.2 µm nitrocellulose Millipore membranes and freeze dried. After lyophilization, all the samples (coded **W1**-*strain-log*) were conserved at -20°C.

This procedure was repeated more than 5 times during the assessments. Extraction yields were reported as the average yield values.

Fractionation of KCS0140 strain crude water extract

Solvent counter-extraction: The crude water extract W1-kcs0140-beech was subjected to counter-extraction with an increasing polarity series of solvents, starting from ciclohexane (kcs0140-CHE), then dichloromethane (kcs0140-DCM), ethanol (kcs0140-EtOH), methanol (kcs0140-MeOH), and finally water (kcs0140-WR). An aliquot of 1 g of the freeze dried extract was resuspended in 10 ml of milliQ water and extracted with 20 ml of the solvents. Every solution was centrifuged at RT for 30 min at 4000 rpm in Falcon tubes on an Eppendorf 5810R centrifuge, then filtered on paper disks, and finally evaporated under vacuum conditions at 30°C. The last water fraction was freeze dried. Each solid was resuspended in the same extraction solvent at 250 mg/ml. The ethanol/water 2:1 solution resulting from ethanol extraction was evaporated, freeze dried, and resuspended in water at the same concentration of 250 mg/ml as the other fractions.

Acid-basic separation: An aqueous solution of 1g of the extract in 10 ml was prepared and adjusted to pH 3 with HCl 2.5N, allowed to precipitate at RT for 3h, and then centrifuged at 4000 rpm in Falcon tubes. The supernatant (kcs0140-pH3) was recovered and filtered on paper disks, and the precipitate was extracted with 10 ml of water adjusted to pH 9 by the addition of NaOH 2.5N.

After precipitation of 3 h at RT, supernatant (kcs0140-pH9) was recovered by centrifugation and filtration on paper disks. The last precipitate was extracted again with 10 ml of water and pH 5, producing a suspension (kcs0140-pH5). After the centrifugation of this fraction, the last insoluble material was used as a suspension (kcs0140-pellet) at 500 mg/ml. All the solutions were adjusted to pH 5 for the subsequent assays.

Oversaturation-based separation: An aliquot of 1 g of the freeze dried extract was resuspended in 1 ml of water and vigorously shook for 30 min at RT, before being allowed to precipitate at 4°C for 24 h. The suspension was centrifuged at 13000 rpm at 4°C in Eppendorf centrifuge 581 0R.

The supernatant (kcs0140-S) was transferred and the precipitate (kcs0140-P) freeze dried, weighed, and resuspended in 100 µl of milliQ water.

ABTS radical scavenging activity

Antioxidant activity of the water extracts was performed by ABTS radical cation decolorization assay [32]. Freeze dried extracts were resuspended in water at a concentration of 0.1 mg/ml, allowing a complete dissolution. In a cuvette, 500 µl of the sample solution and 500 µl of $ABTS^{\bullet+}$ was added for spectrophotometric analysis. After 15 minutes, the OD_{734nm} has been measured and was compared with the Trolox titration curve and reported on a Trolox equivalents scale.

DPPH scavenging activity

Radical scavenging activity was performed on all W1 extracts following the method of Villano et al. (2007), which was modified by Baba and Malik (2014) and adapted to our samples [33], [34]. Briefly, 100 µl of each extract ranging from 100 to 800 µg/ml were mixed with 1.9 ml of DPPH reagent to a final concentration of 80 µM (Sigma) and incubated in a dark chamber for 1 h. Solutions absorbance was measured at 517 nm. Ascorbic acid was used as positive control. IC_{50} values were calculated from each sample scavenging – concentration curve.

The percentage of DPPH scavenging activity was determined following the formula:

DPPH scavenging % = [(Control OD – Sample OD) / Control OD] x 100

Total phenolic content estimation

Total phenolic content of each W1 extract was assessed by means of Folin-Ciocalteu assay (Kaur et al. 2002, Baba and Malik. 2014) adapted to our samples. Extracts were prepared in methanol at a concentration of 100 µg/ml. Sodium carbonate was prepared as a 20% (w/v) stock solution. 20 µl of sample solution was mixed with 1 ml of milliQ water and 100 µl of Folin-Ciocalteu reagent (Sigma). After 8 min of incubation in a dark chamber at RT, the solution was added with 400 µl of sodium carbonate and 490 µl of milliQ water, and then incubated for 1 h. The absorbance at 750 nm of the solution was then measured. Gallic acid was used as a reference compound and the results were expressed as its equivalents [34], [35].

Antibacterial assays

Disk diffusion assay

Antibacterial activity of crude water extracts was assessed by a modified Kirby-Bauer agar disk-diffusion assay. An inoculum of the selected bacterial strain grown overnight in LB was diluted to approximately 10^6 cfu/ml and seeded in LB-agar plates, using a cotton swab. Freeze dried extracts were suspended in water at a concentration of 500 mg/ml, allowing complete dissolution. Each paper disk (BD, Blank Paper Disks) was loaded with 100 µl of sample solution and allowed to dry for 20 min. Dried disks were placed onto the LB-agar layer and allowed to incubate for 24 h before reading the results [36]. Ceftriaxone was used as antibiotic control: ceftriaxone powder was resuspended in water solutions at 4, 2, 1, or 0.5 mg/ml. Inhibition zone (halo) diameter was reported as the average of measurements in three directions of the inhibition zone diameter subtracted by disk diameter.

Minimal inhibitory concentration (MIC)

The MIC of each water extract stored at 4°C (i.e. control condition) was established according to the official CLSI (Clinical and Laboratory Standards Institute) protocol for each model microorganism (*S. aureus*, *P. aeruginosa*). A 96-wells microplate was loaded with 20µl of a twofold dilution of 10 mg/ml mother solution of each water extract up to 5 µg/ml [37]. Next, each well was inoculated with 80 µl of 10^6 cfu/ml MH broth suspension of the appropriate bacterial culture. Control samples were set up loading the wells with sterile growth medium or the crude extracts or the bacterial inoculum. The lower extract concentration which demonstrated no visible growth was considered the MIC [37].

Viable counts

Viable counts (expressed as colony forming units per mL, CFU ml^{-1}) were estimated by a plate count technique: a volume (0.01 ml) of undiluted or serially diluted samples was plated on LB Agar plates and incubated for 24 h at 37°C. Detection limit < 100 cfu/ml.

Time-kill assay

A time-kill assay was performed for the extract W1-kcs0140-beech against *P. aeruginosa* bacterial strain. A diluted inoculum of the appropriate bacterial culture was prepared to 10^6 cfu/ml in MH broth. An aliquot of 5 ml was treated with 25 mg/ml of powdered extract. The bacterial biomass, expressed as OD_{600}, and cellular concentration (cfu/ml), were checked after 2h, 4h, and 6h treatment with gentle shaking at 37°C. Samples, collected after 2 and 24 h treatment, were observed by means of phase-contrast microscopy (100x magnification). At least four images were acquired for each sample. A detail of each capture field is reported for comparison purpose.

HPLC-MS/MS analysis

Freeze dried extracts obtained from dried samples treated at 4°C (W1) were resuspended in water at 100 mg/ml and added with acetonitrile 1:1 v/v, the solutions were allowed to precipitate and centrifuged at 13000 rpm in 1.5 ml vials for 20 min. Each supernatant recovered was diluted 1:50 for MS analysis. HPLC-MS/MS analyses of each sample were performed on a Perkin Elmer UHPLC system with a OD-300 Aquapore column. The elution gradient used was based on (A)

Water and (B) Acetonitrile. Starting from A 80% for 5 min, then linear gradient to A 10% in 15 min. Isocratic to 25 min. MRM scan was used for the quantification of lovastatina (MW 405; F1 199, F2 225), vitamin D2 (MW 397; F1 379, F2 309), ergothioneine (MW 230, F1 143, F2 127), eritadenina (MW 254; F1 178, F2 136), and lenthionine (MW 191, F1 168, F2 150) [22].

Statistical analysis

Data was compared on the basis of significance levels obtained by one-way ANOVA test followed by Tukey HSD post-hoc test. Probability levels of 0.05 were marked with a single sign (*) or different letters [38]. All the extractions and assessments were repeated and reported as the average (n = 3) with standard deviation.

RESULTS

Water extraction of dried mushrooms yield

Water extracts of selected strains of *L. edodes* produced from 0.7 g to 1.8 g of raw material after freeze drying process. Regarding *P. ostreatus*, extracts from 0.9 to 3.1 grams were obtained (Table1).

Fruitbodies production from log cultivation

Shiitake strains KCS0128, KCS0138, KCS0139, KCS0141, KCS0142, and KCS0144, showed the production of fruitbodies after one year from the inoculum and only on oak, while KCS140 was able to grow on substrate, oak, and beech logs (Table 1).

Oyster strains KCS0050, KCS0146, KCS0147, KCS0148, KCS0150 and KCS0152, showed the production of fruitbodies after six-month from the inoculum. Concerning *Pleurotus ostreatus*, KCS0160 strain was able to grow only on substrate. All the oyster strains produced fruitbodies on poplar logs. Strains KCS0050, KCS0146, and KCS0152 showed fruitbodies on robinia logs. Strains KCS0147, KCS0148, KCS0150, and KCS0152 showed fruitbodies production on willow logs. Only the strain KCS0150 showed fruitbodies production on tilia logs (Table 1).

Antioxidant activity of W1 extracts of shiitake and oyster

Water extracts from thermal treated substrate grown *L. edodes* KCS0140 and *P. ostreatus* KCS0160 were assessed for heat induced alterations in the antioxidant activity. This aspect was investigated by means of the ABTS radical scavenging assay using the extracts of shiitake and oyster at a concentration of 100 µg/ml, as this produced the most repeatable results.

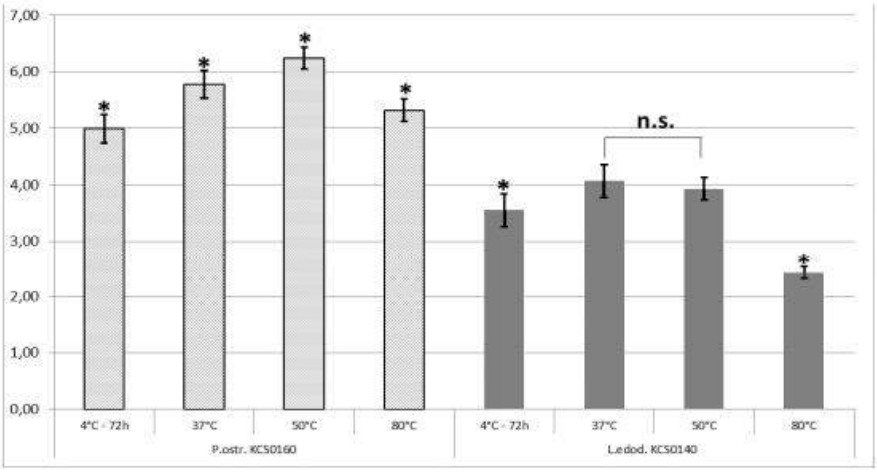
As it concerns shiitake, this strain showed the highest activity both at 37°C and 50°C, a significatively ($p < 0.05$) lower activity was observed either at 4°C and 80°C (Figure 1).

Regarding the oyster strain, the highest activity was recorded at 50°C and a significatively ($p < 0.05$) lower activity was observed either at 4°C, 37°C, or 80°C (Figure 1).

When *P. ostreatus* ABTS radical scavenging activity was assessed, substrate grown strain KCS0160 was found to possess the lowest efficacy, while KCS0147 showed the most evident activity when grown on poplar wood, with a slight less efficiency when cultivated on willow logs. Log species produced significant alteration in the activity of strain KCS0146, that was most effective when grown on robinia than on poplar. KCS0050 resulted more active when cultivated on poplar than on robinia. KCS0148 was more performing when grown on willow than on robinia logs (Table 1, Figure 2).

Reference strain	Thermal treatment	Extract Weight (g ± sd)	Trolox eq. (mM)
P. ostreatus KCS0160 on substrate	4°C	3.13 ± 0.43	4.99
	37°C	1.54 ± 0.43	5.78
	50°C	1.74 ± 0.33	6.24
	80°C	1.24 ± 0.35	5.32
L. edodes KCS0140 on substrate	4°C	1.50 ± 0.50	3.55
	37°C	1.50 ± 0.51	4.07
	50°C	1.52 ± 0.35	3.93
	80°C	1.47 ± 0.19	2.43

A



B

Figure 1. Antioxidant levels (Trolox equivalents) in dried, thermal treated samples of shiitake and oyster (0.1 mg/ml water extracts W1) grown on substrates and used as reference (A). Levels comparison of antioxidant activity (ABTS radical scavenging) (B). Significant samples are indicated by single sign ($p < 0.05$).

As DPPH radical scavenging activity was concerned, the same strains demonstrated a similar trend. The lowest IC_{50} (i.e. highest efficacy) was recorded for KCS0147 grown on poplar and significatively higher IC_{50} was observed when cultivated on willow wood. KCS0146 resulted in an about 30% lower IC_{50} when grown on poplar in respect to willow (Table 1, Figure 2). KCS0152 was more effective on poplar, demonstrating a lower IC_{50} when compared to the cultivation on

robinia (Table 1, Figure 2). KCS0148 instead showed the same IC_{50} as the previous strain whether grown on poplar or willow logs. A higher value in respect to the previous mentioned strains was observed for KCS0050 and also this strain did not yield significant differences either on poplar or robinia. KCS0150 yielded a slightly lower IC_{50} on robinia comparing to the cultivation on poplar and willow.

In regards to *L. edodes* ABTS radical scavenging activity, substrate grown KCS0140 had the lowest efficacy. The same strain grown on oak wood revealed the second highest activity, a lower efficacy was detected when it was cultivated on beech. Strain KCS0144 allowed the observation of the highest activity among shiitake strains. KCS0139 demonstrated an activity less than 5 % lower than oak grown KCS0140. All the other strains (KCS0128, KCS0138, KCS0141, and KCS0142) resulted in a similar and lower activity as compared with the previously mentioned strains (Table 1, Figure 3).

As DPPH assay was concerned, the most efficient were KCS0140 and KCS0144 both grown on oak logs that demonstrated the lowest IC_{50}. Substrate grown KCS0140 showed the lowest activity, resulting in the highest IC_{50}. When KCS0140 grown on beech and oak were considered, significatively different results were observed, with the former showing an IC_{50} about 60% higher than the latter (Table 1, Figure 3).

Table 1. Water extracts (W1-) dry weights yielded from 5 g of dried mushrooms. IC_{50} and Trolox equivalents in 0.1 mg/ml water extract solutions (reported with standard deviations) from log/substrate grown strains; reference strain for each mushroom species is underlined.

Strain	Growth substrate	W1 extract weight (g)	IC_{50} (mg/ml)	Trolox eq. (mM)
P. ostreatus KCS0050	Poplar	1.75	2.061 ± 0.151	10.48 ± 0.277
	Robinia	2.5	1.458 ± 0.156	5.73 ± 0.173
P. ostreatus KCS0146	Poplar	2.36	1.236 ± 0.103	7.00 ± 0.329
	Robinia	2.17	3.695 ± 0.390	8.88 ± 0.485
P. ostreatus KCS0147	Poplar	1.17	0.980 ± 0.177	10.98 ± 0.173
	Willow	2.56	1.663 ± 0.035	10.44 ± 0.624
P. ostreatus KCS0148	Poplar	1.23	0.926 ± 0.032	6.73 ± 0.606
	Willow	2.26	1.094 ± 0.019	8.03 ± 0.225
P. ostreatus KCS0150	Poplar	1.65	1.225 ± 0.050	8.72 ± 0.277
	Tilia	2.13	0.978 ± 0.137	8.65 ± 0.502
	Willow	0.92	1.657 ± 0.422	8.88 ± 0.433
P. ostreatus KCS0152	Poplar	1.16	0.923 ± 0.026	9.15 ± 0.294
	Robinia	2.5	1.907 ± 0.210	9.11 ± 0.208
	Willow	1.72	1.188 ± 0.210	8.62 ± 0.346
P. ostreatus KCS0160	Sawdust	3.13	6.718 ± 0.633	4.99 ± 0.433
L. edodes KCS0128	Oak	1.98	1.127 ± 0.093	6.40 ± 0.381
L. edodes KCS0138	Oak	1.02	1.070 ± 0.093	5.76 ± 0.242
L. edodes KCS0139	Oak	1.08	0.981 ± 0.128	8.80 ± 0.277
L. edodes KCS0140	Oak	1.04	0.231 ± 0.009	9.83 ± 0.485
	Beech	1.65	3.375 ± 0.669	6.50 ± 0.104
	Sawdust	1.5	4.994 ± 0.246	3.55 ± 0.502
L. edodes KCS0141	Oak	1.87	0.648 ± 0.163	5.71 ± 0.468
L. edodes KCS0142	Oak	1.17	1.275 ± 0.257	5.51 ± 0.485
L. edodes KCS0144	Oak	0.73	0.254 ± 0.016	13.01 ± 0.312

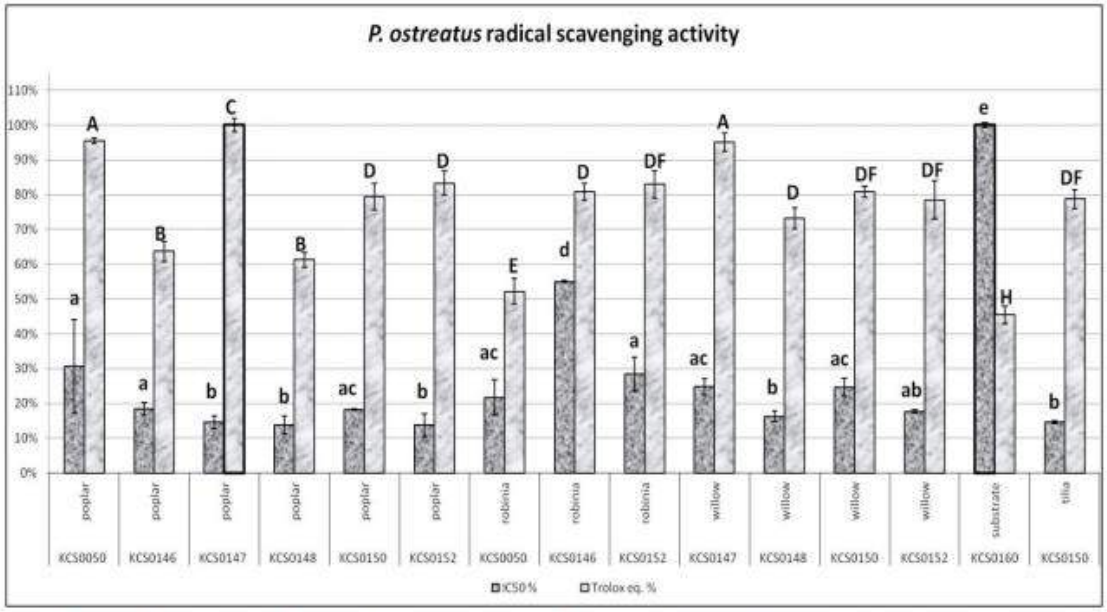


Figure 2. Comparison between *P. ostreatus* strains antioxidant levels either substrate or log grown. Activities are reported as normalized percentages to the highest one in each series (highlighted). Significant differences ($p < 0.05$) between strains and log species are indicated with different letters on top of each bar.

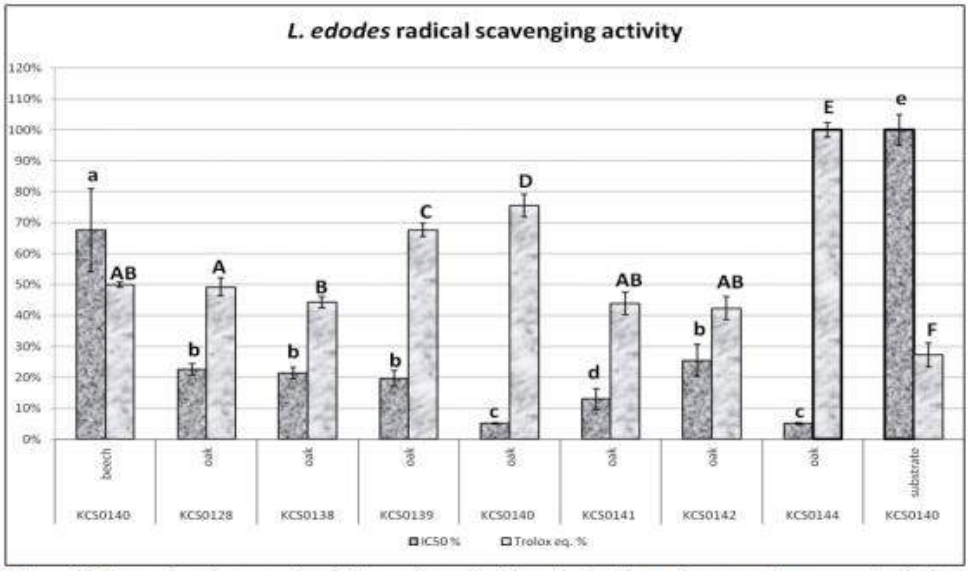


Figure 3. Comparison between *L. edodes* strains antioxidant levels either substrate or log grown. Activities are reported as normalized percentages to the highest one in each series (highlighted). Significant differences ($p < 0.05$) between strains and log species are indicated with different letters on top of each bar.

Total phenolic content

The Folin-Ciocalteu reagent was used to assess the total phenolic content in all the mushroom strains. Different *P. ostreatus* strains were found to change in phenolic content when cultivated on different wood species. Poplar wood resulted in the highest phenolic content for strains KCS0146 and KCS0147, robinia allowed the production of the highest content in KCS0152 only, while willow allowed a higher content for the strain KCS0148. Tilia allowed the highest content of phenols among all the samples and only for KCS0150 strain (Figure 4).

Table 2. Folin-Ciocalteu assay gallic equivalents measures in 0.1 mg/ml water solution of the W1 extracts for *P. ostreatus* (left) and *L. edodes* (right).

Oyster	growth substrate	Gallic eq. (mM)
KCS0146	poplar	0.872 ± 0.010
KCS0147	poplar	0.995 ± 0.016
KCS0148	poplar	0.583 ± 0.010
KCS0150	poplar	0.855 ± 0.010
KCS0152	poplar	0.694 ± 0.017
KCS0050	robinia	0.945 ± 0.008
KCS0146	robinia	0.291 ± 0.010
KCS0152	robinia	1.000 ± 0.013
KCS0147	willow	0.933 ± 0.036
KCS0148	willow	1.021 ± 0.005
KCS0150	willow	0.779 ± 0.026
KCS0152	willow	0.795 ± 0.008
KCS0160	sawdust	1.025 ± 0.010
KCS0150	tilia	1.561 ± 0.025

Shiitake	growth substrate	Gallic eq. (mM)
KCS0140	beech	0.746 ± 0.023
KCS0128	oak	1.159 ± 0.010
KCS0138	oak	0.958 ± 0.013
KCS0139	oak	1.401 ± 0.029
KCS0140	oak	1.013 ± 0.008
KCS0141	oak	0.682 ± 0.032
KCS0142	oak	0.780 ± 0.019
KCS0144	oak	1.541 ± 0.022
KCS0140	sawdust	0.583 ± 0.047

Concerning *L. edodes*, different strains on the same oak logs produced significantly different phenolic content. KCS0144 grown on tilia logs allowed the observation of highest content. KCS0140 has showed a higher content when grown on oak with significantly lower levels when cultivated on beech. The lowest content was observed for the same strain grown on sawdust substrate (Figure 5).

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Figure 4. Phenolic content in *P. ostreatus* strains grown on different log types and substrate. Values reported are normalized to the highest content observed (KCS0150 on tilia) and indicated as the respective percentages.

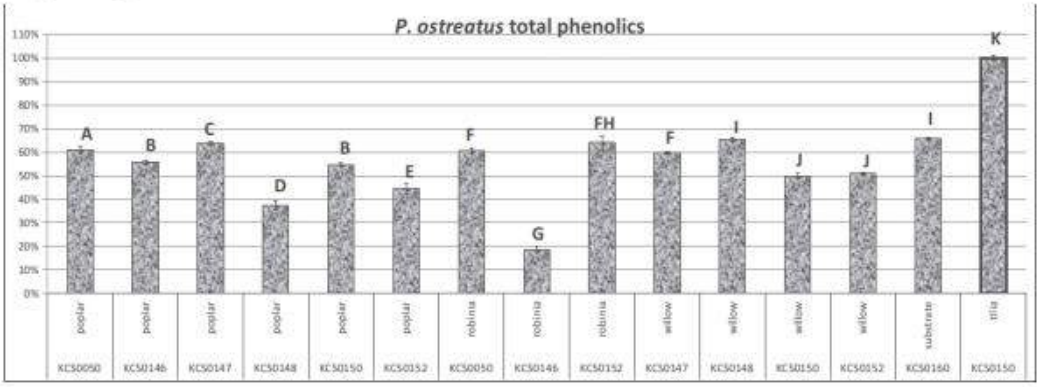
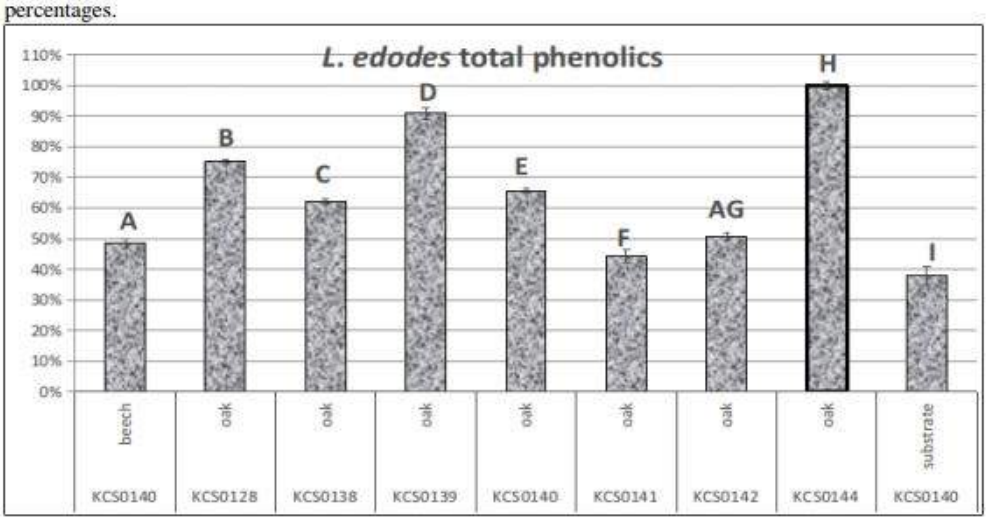


Figure 5. Phenolic content in *L. edodes* strains grown on different log types and substrate. Values reported are normalized to the highest content observed (KCS0144 on oak) and indicated as the respective percentages.



Counter-extractions of W1-kcs0140-beech

In concerns to the increasing polarity solvents counter-extraction of the water extract of KCS0140 grown on beech (W1-kcs0140-beech), it yielded five fractions with different weight and consistence, as reported in Table 2. All the extracts were dried and dissolved back into the respective extraction solvent not producing any precipitate. The last pellet that was produced in the extraction (after methanol) did not completely dissolve back into water, thereby used as a suspension (kcs0140-WR) for the subsequent assays.

The extract W1-kcs0140-beech resuspended showed pH 5 and was subjected to pH guided counter-extraction using acid (pH 3) and basic (pH 9) conditions. This yielded four fractions of different weight as reported in Table 3.

Table 3. W1-kcs0140-beech water extract 1 g yields after solvent and pH based counter-extractions. The last two extracts (kcs0140-S and kcs0140-P) were reported as the weights obtained from 100 mg of the crude extract.

Fraction	Weight (mg/g_{crude})	Consistence
kcs0140-CHE	60	Oily
kcs0140-DCM	51	Oily
kcs0140-EtOH	287	Powder
kcs0140-MeOH	115	Powder
kcs0140-WR	421	Powder
kcs0140-pH3	615	Powder – sticky
kcs0140-pH9	173	Powder
kcs0140-pH5	92	Powder – sticky
kcs0140-pellet	106	Powder
kcs0140-S	675	Brown solution
kcs0140-P	325	Brown-white powder

Antibacterial activity

Antibacterial activity of shiitake water extracts obtained both from log and substrate was assessed by means of agar disk diffusion assay. All strains were able to induce an inhibition zone (halo) in both the tested model microorganisms.

When the water extracts (W1) from *L. edodes* strains were investigated for their antimicrobial activity against the chosen model microorganisms, *S. aureus* and *P. aeruginosa*, the extracts showed significantly different activities. Shiitake extracts can be divided in three groups with increasing activities: the less active (KCS0128 and KCS0140 grown on substrate), the intermediate active (KCS0138, KCS0142, KCS0139 and KCS0144), and the most active (KCS0140 grown on oak and beech, and KCS0141).

KCS0141 and KCS0140 cultivated on beech logs showed the highest antibacterial activities both against *S. aureus* and *P. aeruginosa*. In particular, KCS0140 showed an higher antimicrobial activity when grown on oak and beech logs respect with substrate, showing an inhibition zone against *S. aureus* six and seven fold larger than substrate, respectively. A similar antibacterial activity was observed in *P. aeruginosa* (Figure 6): the inhibition halos of oak and beech were six and eight fold larger than substrate, respectively (Figure 6). The low antimicrobial activity of KCS0140 substrate grown was comparable to that of KCS0128.

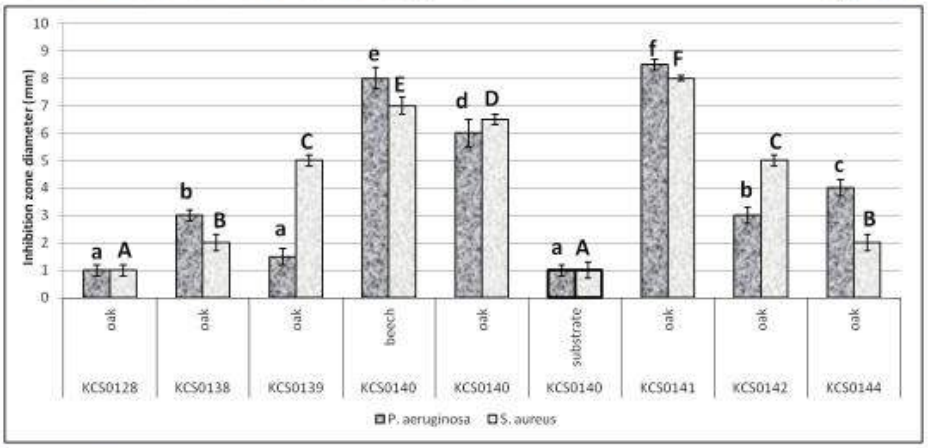


Figure 6. Inhibition zones diameter of water extracts from the shiitake strains grown on logs or substrate. The same strain KCS0140 cultivated on substrate is highlighted. Significance ($p < 0.05$) reported with different letters.

When the minimal inhibitory concentration (MIC) was assessed, *P. aeruginosa* was revealed to be more tolerant than *S. aureus* to the shiitake extracts tested (Table 4). KCS0141 and KCS0140 cultivated on beech produced the only extracts that were found active against both microorganisms. All the other strains extracts showed activity only against *S. aureus* in a concentration range between 3.3 and 6.7 mg/ml. The strains KCS0141 and beech grown KCS0140 showed the best antibacterial activity against *S. aureus*. A significantly lower activity was observed for KCS0142, KCS0139, and KCS0128. The lowest activity was found for KCS0140 oak grown and KCS0128 strains. However, no activity was observed in KCS0140 and KCS0138 strains up to 10 mg/ml of crude W1- water extracts.

Concerning *P. aeruginosa*, the only active strains with antimicrobial activity was KCS0141 and KCS0140 cultivated on beech logs (Table 4).

Oyster mushroom did not show any antibacterial activity.

Table 4. MIC values (reported with standard deviations) of log/substrate grown shiitake strains against *P. aeruginosa* and *S. aureus*. Different uppercase letters indicate significantly differences in the activities against *S. aureus*. Different lowercase letters indicates significantly differences in the activities against *P.aeruginosa*.

Strain	Growth medium	MIC *S. aureus* (mg/ml)	Significance (*S. aureus*)	MIC *P.aeruginosa* (mg/ml)	Significance (*P. aeruginosa*)
***L. edodes* KCS0141**	oak	3.3 ± 1.4	A	2.5 ± 0	a
***L. edodes* KCS0142**	oak	5.0 ± 0	B	> 10	d
***L. edodes* KCS0139**	oak	4.2 ± 1.4	B	> 10	d
***L. edodes* KCS0140**	beech	3.3 ± 1.4	A	2.5 ± 0	a
	oak	6.7 ± 2.8	C	> 10	d
	sawdust	> 10	D	> 10	d
***L. edodes* KCS0128**	oak	5.0 ± 0	B	> 10	d
***L. edodes* KCS0138**	oak	> 10	D	> 10	d
***L. edodes* KCS0144**	oak	6.7 ± 2.8	C	> 10	d

The extract W1 from KCS0140 grown on beech was subjected to solvent counter-extraction with increasing polarity. CHE, DCM, and MeOH yielded fractions depleted of antibacterial activity in a 50 mg/disk diffusion assay against *P.aeruginosa* (Table 4). EtOH and WR fraction demonstrated a very low antibacterial activity when tested at 50 mg/disk against *P. aeruginosa* (Table 5). When the pH counter-extraction was concerned, it was observed a complete depletion of the antibacterial activity against *P. aeruginosa* (Table 5).

Table 5. inhibition zone diameter (with standard deviation) in disk diffusion assay for 50 mg/disk of the fractions obtained by different counter-extraction of the crude extract W1-kcs0140-beech.

Fraction	diameter (mm)
kcs0140-CHE	0
kcs0140-DCM	0
kcs0140-EtOH	2.0 ± 0.8
kcs0140-MeOH	0
kcs0140-WR	2.5 ± 0.5
kcs0140-pH3	0
kcs0140-pH9	0
kcs0140-pH5	0
kcs0140-pellet	0
kcs0140-S	5.2 ± 1.2
kcs0140-P	6.5 ± 1.1

A time-kill assay showed that KCS0140 grown on beech decreased *P. aeruginosa* inoculum of 6 x 10^5 cfu/ml of one log unit upon 10 minutes incubation, and four log unit upon two hours incubation reaching the detection limit (< 10^2 cfu/ml). Phase-contrast microscopy images showed the detrimental effect of KCS0140 after 24-hour treatment on *P. aeruginosa* cells (Figure 7).

Furthermore, 20 mg of W1-kcs0140-beech produced an inhibition zone comparable with Ceftriaxone 0.2 mg, antibiotic that was chosen as the control (Table 6).

Table 6. Comparison of inhibition zone diameters in disk diffusion assay between the water extract of beech grown KCS0140 and the antibiotic Ceftriaxone

Sample	**Concentration (mg)**	**Inhibition zone diameter (mm)**
W1-kcs0140-beech	5	2.1 ± 0.4
W1-kcs0140-beech	10	4.7 ± 0.6
W1-kcs0140-beech	20	7.7 ± 0.8
W1-kcs0140-beech	40	8.1 ± 0.8
Ceftriaxone	0.05	3.5 ± 0.4
Ceftriaxone	0.1	6.7 ± 0.7
Ceftriaxone	0.2	8.2 ± 1.1
Ceftriaxone	0.4	14.3 ± 1.7

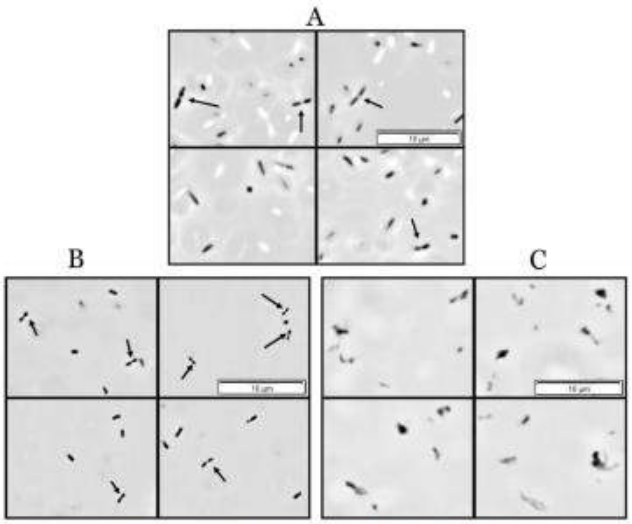


Figure 7. (A) fresh inoculum of *P. aeruginosa* 30 min after dilution of an overnight LB inoculum to 10^6 cfu/ml; arrow indicates an example of cell under division. (B) bacteria after 2 h of incubation with 25 mg/ml of treatment; arrows indicate cell presumably under division. (C) treated inoculum after 24 h of incubation; arrows indicate aggregates and presumably degraded bacterial cells.

Analyses of nutritional compounds loss

An early analysis of the medicinal compounds in water extracts was performed in all *Pleurotus* strains. Optimization of the HPLC-MS/MS analysis method was achieved to evaluate the concentration of vitamin D2 and ergothioneine in water extracts. Almost all mushrooms strains released vitamin D2 and ergothioneine in detectable concentrations. Release of vitamin D2 ranged from 12 to 46 µg/100g of dried mushrooms. Ergothioneine was found in a range from 20 to 80 µg/100g of dried material. In regards to eritadenine, lovastatin, and lenthionine, no extract showed a detectable concentration of any of these compounds.

DISCUSSION

The same strain and culture condition produced reproducible extraction yields over the multiple water extraction performed. Extraction yield were found dependant on both the strain and the culture conditions. Thermal treatment of dry mushroom material before water extraction was used as a stress test, which allowed the assessment of alteration to important substances in the mushroom matrix, thereby simulating transformation and cooking processes [39].

As far as the thermal treatment effects on the antioxidant levels were concerned, temperatures of up to 80°C showed higher antioxidant activities than 4°C treatment in oyster. In contrast, shiitake revealed a decreased activity when the same temperatures were applied. Thus, it is conceivable that differences depended on thermal-induced modifications concerning some compounds in the dried sample [15].

When log grown strain of *P. ostreatus* and *L. edodes* were assessed after an exhaustive 72 h extraction, it was noticeable that most of the strains on each wood had significantly different levels

of antioxidant activity. Furthermore, when both DPPH and ABTS radical scavenging assays were concerned, it was clear that all the strains of *P. ostreatus* and *L. edodes* performed more significantly when grown on logs than when cultivated on sawdust substrate, respectively. And with more in depth results, each strain resulted in performance levels from none to slightly correlated when tested either with the former or the latter assay. As the IC_{50} was not discovered to correlate to the activity as measured by ABTS, it is conceivable that multiple antioxidant compounds classes are present in the extracts [40]. This also suggests there is a need for development of further purifications to reveal the most interesting substances owing the activity [30]. As the scavenging activity of various strain was found significantly dependant on log species, further studies will be necessary to better to investigate the interactions between selected strains and the different species of logs [41].

In regards to the assessment of total phenolic compounds, significant differences were observed when different logs were used for the cultivation of same strain; furthermore, there were significant differences among the strains for each log. It was noticeable that tilia logs show a concentration over the average in such compounds. On the other hand, only a strain was able to grow on tilia logs. Moreover, the Folin-Ciocalteu method that was extensively used to estimate phenols [42], [43], [44]. However, it was demonstrated this was possibly biased by the presence of some interfering compounds from the crude extract [45].

As the antibacterial activity of oyster and shiitake W1 extracts were tested by means of agar disk-diffusion assay, *P. aeruginosa* was discovered to be more sensitive than *S. aureus* to most of the extracts. All the log cultivated strains were more effective than the corresponding substrate cultivated strains. Log species was found to influence KCS0140 activity, suggesting that logs plays a critical role in the interaction with the mushroom, thereby resulting in different antibacterial activity yields. In consideration with the differentiation of antioxidant activity, this further suggests the need for a more thorough study focusing on the interaction that takes place at a proteomic and metabolic level [46], [47], [48].

As a subsequent MIC assay was concerned, it is noteworthy that sawdust grown shiitake strains did not show any efficacy, while most of the log grown strains were effective against *S. aureus*. On the other hand, only two strains demonstrated activity against *P. aeruginosa*. More in depth analyses will be performed to characterize the antibacterial activity, particularly against *P. aeruginosa*. W1-kcs0140-beech was chosen as it demonstrated the highest antibacterial activity against both *S. aureus* and *P. aeruginosa*. *P. aeruginosa* cells after 2 h treatment, seem to be compromised in cell division machinery as no complete duplication event was found to occur. Indeed, bacterial cells showed proper motion, as swimming and tumbling were detectable; however, cellular viability was compromised as confirmed by viable counts. After 24 h treatment, no cells were observable, suggesting that this extract also has a bacteriolytic activity [49].

As counter-extractions were performed, the resulting fractions were depleted of antibacterial activity. It is significant that the EtOH and the WR last fraction demonstrated a low but still present activity while the MeOH fraction did not show any. This suggests that antibacterial compound/s are water soluble and particularly sensitive to the organic solvents. The same loss of activity was observed when the crude extract was subjected to acid and basic pH extractions. Furthermore, it conveys that antibacterial substances have complex structures and seem to require water as a solvent and specific pH values to exert its antibacterial effect.

Finally, a first evaluation of specific nutraceutical compounds in water extracts in both mushrooms showed that Vitamin D2 and ergothioneine are present in similar amounts in all strains of oyster and shiitake. No eritadenine in shiitake and lovastatin in oyster was noticed. Future studies will be carried out in order to achieve the best method to detect and quantify secondary metabolites of pharmacological interest and to research further how these metabolites are modified and/or degraded during food transformation processes.

CONCLUSION

Both shiitake and oyster demonstrated interesting radical scavenging (antioxidant) properties; additionally, shiitake was also able to produce antimicrobial metabolites. These data envisage their use as main ingredients for functional food formulation. Future studies will be needed in order to increase secondary metabolites of pharmacological interest production. In particular, a special effort will be made in selecting the best strains, formulating the best substrates and assessing the best parameters and conditions for mushroom incubation and fructification. Future researches will be focused both on the production of mushrooms with a high nutritional value and assess their possible use as food supplement and the purification of antimicrobial and antioxidant compounds for a more thorough and in depth assessment of the interesting results obtained.

List of Abbreviations: *L. edodes, Lentinula edodes*; *P. ostreatus, Pleurotus ostreatus*; *CHE, ciclohexane*; *DCM, dichloromethane*; *EtOH, ethanol*; *MeOH, methanol*; *W, water;* LB, Luria Bertani; MH, Mueller-Hinton; MIC, Minimal inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute.

Authors' Contribution: All authors contributed to this study.

Competing Interests: There are no conflicts of interest to declare.

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Contributed Paper

Antibacterial, Antioxidant Properties and Bioactive Compounds of Thai Cultivated Mushroom Extracts against Food-borne Bacterial Strains

Mathurot Chaiharn* [a], Waya S. Phutdhawong [b], Doungporn Amornlerdpison [c] and Weerachai Phutdhawong [d]

[a] Division of Biotechnology, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand.
[b] Department of Chemistry, Faculty of Science, Silpakorn University, Nakhon Pathom 73000, Thailand.
[c] Faculty of Fisheries Technology and Aquatic Resources, Maejo University, Chiang Mai 50290, Thailand.
[d] Department of Chemistry, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand.
*Author for correspondence; e-mail: mathurot@mju.ac.th

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ABSTRACT

A fine-dried edible mushroom was extracted and investigated for its antibacterial activity against food-borne pathogenic bacteria, i.e. *Bacillus cereus*, *Enterobacter aerogenes*, *Escherichia coli*, *Micrococcus luteus*, *Proteus vulgaris*, *Salmonella typhimurium* and *Staphylococcus aureus*. The extracts of *Flammulina velutipes*, *Ganoderma lucidum*, *Pleurotus ostreatus* and *Pleurotus pulmonarius* inhibited both Gram-positive and Gram-negative bacteria. Water extract of *Pleurotus pulmonarius* significantly inhibited the growth of Gram-positive, i.e. *Bacillus cereus* and *Micrococcus luteus* (15 mm), while Gram-negative, i.e. *Escherichia coli* and *Salmonella typhimurium* resisted to most extracts. Only water extract from *Pleurotus pulmonarius* showed antibacterial activity against all tested bacteria. The minimum inhibitory concentrations (MICs) of *Bacillus cereus* had the lowest MIC range (1.25-5.00 mg/ml), whereas those of *Salmonella typhimurium* had the highest MICs (12.5-22.5 mg/ml) in *Flammulina velutipes* in ethylacetate extract, *Ganoderma lucidum* in methanol extract, *Pleurotus ostreatus* in ethylacetate extract and *Pleurotus pulmonarius* in water extract. Mushroom crude extracts were investigated for antioxidant capacity using the ABTS system. Results showed an effective antioxidant activity against ABTS radicals (91-94 %) and vitamin E derivatives (97 %), respectively. The IC_{50} values of *Ganoderma lucidum*, *Pleurotus pulmonarius* and *Flammulina velutipes* were 2.81 ± 0.02 mg/ml to 10.57 ± 0.27 mg/ml. *Ganoderma lucidum* exhibited maximum antioxidant potential as compared to *Pleurotus pulmonarius* and *Flammulina velutipes*, respectively. Positive correlations were found between total phenolic content in the mushroom extracts and their antioxidant activities. Water-extracted bioactive compounds produced by *Pleurotus pulmonarius* were characterized and identified on the basis of ^{1}H-NMR as a polysaccharide. The study reveals that cultivated mushroom extracts have higher free radical scavenging potential with high levels of antioxidant compounds. These compounds showed broad-spectrum activity, were non-toxic, and might be applicable for human use. Results suggest that cultivated mushrooms may have potential as natural antibacterial and antioxidant properties and could be used as potential natural source for the development of nutraceuticals.

Keywords: antibacterial activity, antioxidant activity, cultivated mushroom, food-borne pathogenic bacteria, polysaccharide

1. INTRODUCTION

Food-borne pathogenic bacteria are the group of bacteria that cause food spoilage and they may produce toxins, off-flavors, lytic enzyme and rotting. The most dangerous bacteria consist of *Bacillus cereus*, *Enterobacter aeroginese*, *Escherichia coli*, *Micrococcus luteus*, *Proteus vulgaris*, *Staphylococcus aureus* and *Salmonella typhimurium* that cause toxin-contaminated foods which promote diarrhea in human and animal [1]. The lytic enzyme derived from these pathogens includes lipase, protease and carbohydrase. These enzmes can deteriorate food sensorial properties [2]. Moreover, their enterotoxins are significant to human health because toxin-contaminated foods have been associated with liver and kidney tumors [3]. Multidrug resistance in pathogenic microorganisms has been developed due to indiscriminate use of commercial antimicrobial drugs in the treatment of infectious disease. This has led to the urgent need for new antibiotics to treat infections caused by this group of resistant bacteria including the infection by food-borne pathogenic bacteria. Edible mushrooms in the genera of *Lentinula*, *Hericium*, *Grifola*, *Flammulina*, *Pleurotus* and *Tremella* have been reported to possess medicinal properties [4] such as anti-carcinogenic, anti-inflammatory, immune-suppressing and antimicrobial activities [5, 6]. The cell wall glucans are well known for their immunomodulatory properties, and many of the externalized secondary metabolites (extracellular secretion by the mycelium) combat bacteria [7] and viruses [8]. The exudates from mushroom mycelia are active against protozoa such as the parasite that causes malaria, *Plasmodium falciparum* and other microorganisms [9]. Some edible mushrooms are considered to be a good source of proteins and phenolic antioxidants [10]. Secondary metabolites including phenolic compounds, polyketides, terpenes and steroids derived from many polypores and agarics have been responsible for their antimicrobial activity [11]. These compounds could inhibit lipoprotein oxidation [12] and inhibit the occurrences of atherosclerosis and cancer [11]. Glucosylceramide isolated from *Pleurotus citrinopileatus* was found to be active against *Escherichia coli* and *Staphylococcus aureus* with IC_{50} values of 275.1 µM and 323.2 µM, respectively [13]. The importance of the Chinese Shiitake mushroom (*Lentinus edodes*) has been demonstrated to increase the host resistance to bacterial and viral infection [14]. Several compounds extracted from Shiitake mushroom revealed antifungal and antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*. Thus, chloroform and ethyl acetate extracts of dried mushroom have antibacterial activity against *Streptococcus mutans* and *Prevestella intermedia* [15]. Researchers revealed antimicrobial activity of several mushroom extracts [5] such as chloroform and ethyl acetate extracts of *Lactarius deliciosus*, *Sarcodon imbricatus* and *Tricholoma portensosum* which exhibited antibacterial activity against *Streptococcus mutans* and *Prevotella intermedia* [15]. *Ganoderma*, *Cantharellus*, *Lentinus*, *Russula*, *Agaricus* and *Pleurotus* extracts have been reported to show antimicrobial potentials which possess the different bioactive compounds [16, 17, 18]. The extracts of *Agaricus bisporus*, *Auricularia auricula*, *Lentinula edodes* and *Pleurotus* were against

Mycobacterium smegmatis and *Candida albicans* [19]. A few studies have been reported on the antimicrobial activity of other edible mushrooms and their bioactive compounds. The extractable products from mushrooms were the supplement diet which enhances health and fitness and can be classified as medicinal therapy. Thus, mushrooms could offer a particularly rich source of the new potential medicines.

Although a wide variety of edible mushrooms particularly in the tropical region has been consumed regularly, the information regarding the antimicrobial potentials of mushrooms in the tropical region is limit. In this study, we screened for antibacterial activity from Thai edible mushrooms against food-borne pathogenic bacteria. Elucidation of bioactive compound from mushroom extract was carried out by nuclear magnetic resonance (NMR) analysis. We aim to discover some novel natural compounds with low toxicity that could be used further to treat infections caused by multi-drug resistant strains of food-borne pathogenic bacteria.

2. MATERIALS AND METHOD

2.1 Preparation of Mushroom Extracts

Edible mushrooms (Table 1) were air-dried in an oven at 40°C before extraction. A fine-dried mushroom powder sample (20 g) was extracted by stirring with 100 ml of a series of organic solvent as hexane, ethyl acetate, 95% ethanol, 95% methanol and sterile distilled water at 30°C, 150 rpm for 24 hr and filtered through Whatman No.4 filter paper. The residue was then extracted with additional 100 ml of same solvent. Each solvent extract was combined and evaporated to dryness. The organic solvent in the extracts was removed by a rotary evaporator. For the entire analysis, compounds of extract were dissolved in 10% dimethylsulfoxide (DMSO), and filter sterilization was done through a 0.22 mM membrane filter. Extracts were kept in the dark at 4°C before use.

Table 1. List of Thai edible mushrooms used in this study.

Mushroom Family	Common name	Strain/Species of Mushrooms
Family Agaricaceae	Shimeji mushroom	*Hypsizygus marmoreus*
Family Amanitaceae	-	*Amanita vaginata* (Fr.) Quel. var. *fulva*
Family Auriculariaceae	Jelly ear mushroom	*Auricularia auricular* (Hook.) Underw.
Family Ganodermataceae	Lingzhi mushroom	*Ganoderma lucidum* (Fr.) Karst.
Family Pleurotaceae	Indian oyster mushroom	*Lentinula edodes* (Berk.) Sing.
		Lentinus polychrous Lev.
		Lentinus squarrosulus Mont.
	Oyster mushroom	*Pleurotus ostreatus* (Fr.) Guel.
		Pleurotus ostreatus (Fr.) Kummer.
		Pleurotus pulmonarius (Fr.) Quelet .
		Pleurotus sajarcaju (Fr.) Sing.
Family Russulaceae	Chestnut mushroom	*Agrocybe cytindracea* (Fr.) Gill.
Family Tremellaceae	White jelly mushroom	*Tremella fusiformis* Berk.
Family Tricholomataceae	Enokitake mushroom	*Flammulina velutipes* (Fr.) Curt.
Family Volvariaceae	Straw mushroom	*Volvariella volvacea* (Bull. Ex. Fr.) Sing.

2.2 Antimicrobial Activity

2.2.1 Microorganisms

The following strains of tested bacteria were used: *Bacillus cereus* ATCC 11778, *Enterobacter aerogenes*, *Escherichia coli* O157:H7, *Micrococcus luteus* ATCC 9341, *Proteus vulgaris* ATCC13315, *Salmonella typhimurium* ATCC13311, and *Staphylococcus aureus* ATCC 25923. Each bacterial stain was sub-cultured on Nutrient agar (NA) (Merck, Germany) to ensure the purity of the culture. The bacteria were grown in Nutrient broth (NB) overnight at 37°C before use in antimicrobial assay.

2.2.2 Screening of Antibacterial Activity of Mushroom Extracts

Antibacterial activity of mushroom crude extracts was determined by paper disc diffusion assay. Each bacterial strain was grown in NB at 37°C for 48-72 hr and the bacterial suspensions were adjusted to the concentration of 10^6 cfu/ml. A sterile cotton swab was used to apply each bacterial suspension onto the entire surface of the NA plate. Twenty µl of 100 mg/ml of crude extracts in DMSO were applied to the blank discs (1 mg/disc) and placed on a bacteria-seeded plate and incubated at 37°C for 72 hr. Amoxicillin (20 mg/disc) was used as a positive control. Antibacterial activities were determined by measuring the diameter of the inhibition zone, and the mean value was calculated. All experiment was performed in triplicates.

2.3 Determination of Minimum Inhibitory Concentration (MIC)

MIC tests were used to determine the lowest concentration of each mushroom extract that could inhibit the growth of test bacteria by the modified technique described by Hirasawa et al., (1999) [15]. Bacterial suspensions were prepared to contain approximately 10^6 cfu/ml. They were inoculated on 96-well microlitre plates containing mushroom extracts (10-100 mg/ml) on NB and incubated at 37°C for 48 hr. Bacterial growth was monitored by microlitre plate reader at 600 nm and culture broth (0.1 ml) were spread on NA to confirm the absence of bacterial growth. MIC was defined as the concentration of mushroom extracts that inhibited the visible growth of tested strain.

2.4 Antioxidant Activity

The ABTS (2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic) radical cation scavenging activity was performed with slight modifications described by Re et al. (1999) [20]. The ABTS radicals were produced by the reaction between 7 mM ABTS in water and 140 mM potassium persulfate, stored in the dark at room temperature for 12 hr. Prior to use, the solution was diluted with ethanol to get an absorbance at 734 nm. Free radical scavenging activity was assessed by mixing 200 µl of test sample with 1.8 ml of ABTS working standard in a microcuvette. The decrease in absorbance was measures exactly after 6 min. The percentage inhibition was calculated according to the formula: $[(Ao-A1)/Ao] \times 100$, where, Ao was the absorbance of the control, and A1 was the absorbance of the sample.

Trolox, a derivative of vitamin E, was used as a standard. Antioxidant activity of each sample was expressed as Trolox equivalent antioxidant capacity (TEAC) which represented the concentration (µM) of Trolox, having the same activity as 1 mg of sample. All determinations were carried out in triplicates. The IC_{50} was calculated from dose-response curve.

2.5 Bioassay-guided Separation

Mushroom extract which showed broad spectrum antibacterial activity was fractionated by preparative Thin layer chromatography (TLC), using Whatman 20×20 cm^2 silica gel plates with fluorescent indicator and eluted with benzene-acetone-acetic acid (7:3:0.5 v/v/v). Following development of the TLC plates, the active fraction with activity against tested bacteria was visualized under UV light (254 nm). Structural analyses were performed using proton NMR spectroscopy (300 MHz).

2.6 ^{1}H-NMR Spectroscopy of Mushroom Extracts

^{1}H-NMR spectra were obtained using a Bruker DRX 400 spectrometer, with a 5-mm inverse probe. The samples being dissolved in DMSO-d_6 followed by a drop of D_2O, for OH group exchange. Chemical shifts (d) are expressed relative to the resonance of Me_4Si (d = 0) obtained in a separate experiment. Coupling constants and chemical shifts were obtained from a first-order analysis of the spectra.

2.7 Statistical Analysis

All assays were carried out in triplicates and results are expressed as mean ± standard deviation (SD). The data were analyzed using SPSS software. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were used to analyze the difference among scavenging activity and IC_{50} of various extracts for antioxidant assays with least significance difference (LSD) at $P \leq 0.01$ as a level of significance. Experimental results were further analyzed for Pearson correlation coefficient of total phenolic with antioxidant assays.

3. RESULTS

3.1 Total Yields of Thai Edible Mushroom Extracts using Different Solvents

Fifteen edible mushroom species (Table 1) were extracted and screened for their antibacterial activity. Total yields of the mushroom extracts were varied depending on mushroom strains and solvents used (Table 2). The yields from mushroom extractions were in the range of 0.3-27.8 %, and always higher in water extracts, followed by ethanol, methanol, ethyl acetate, and hexane extracts (Table 2), respectively. Straw mushroom (*Volvariella volvacea*) in water extract showed significantly highest yield of crude extract (55.6 mg) followed by oyster mushroom (*Pleurotus ostreatus* (Fr.) Kummer (44.5 mg) and *Pleurotus sajarcaju* (Fr.) Sing. (40.3)) in water extract, while shiitake mushroom (*Lentinula edodes* and *Lentinus polychrous*) in hexane extract gave least yield of crude extract (0.6 mg).

3.2 Antibacterial Activities of Thai Edible Mushroom Extracts

In an attempt to find new drugs to treat infections, 75 Thai edible mushroom extracts were tested for antibacterial activity by agar disc diffusion assay against food-borne pathogenic bacteria. Forty-seven extracts showed inhibitory effects against tested bacteria, and 23 extracts showed broad-spectrum activity against both Gram-positive and Gram-negative bacteria. However, only 4 extracts, i.e. Flammulina velutipes in ethyl acetate extract, *Ganoderma lucidum* in methanol extract, *Pleurotus pulmonarius* in water extract and *Pleurotus ostreatus* in ethyl acetate extract showed inhibition zones against test bacteria greater than 10 mm

(Table 3). Negative control (DMSO) displayed no inhibition zone, whereas amoxicillin (20 mg/disc) (a positive control) displayed a large inhibition zone (20-25 mm) against all tested bacteria. Ethyl acetate extracts showed broad-spectrum activity against all tested bacteria in *Flamulina velutipes*, *Ganoderma lucidum*, *Hypsizygus marmoreus*, *Pleurotus ostratus* (Fr.) Kummer, *Pleurotus pulmonarius* and *Volvariella volvacea*, while hexane extracts in *Auricularia auricular*, *Flammulina velutipes*, *Ganoderma lucidum*, *Lentinus polychrous*, *Pleurotus ostreatus* (Fr.) Guel and *Tremella fusiformis* showed weak activity against only *Bacillus cereus*. *Ganoderma lucidum* in methanol extract showed significantly highest activity against *Bacillus cereus* and *Micrococcus luteus* (20 mm), while, aqueous extract of *Pleurotus pulmonarius* showed significantly highest activity against all bacteria tested (Table 3). The cultivated mushroom strains, i.e. *Flammulina velutipes*, *Ganoderma lucidum*, *Pleurotus pulmonarius* and *Pleurotus ostreatus* (Fr.) Guel. showed significant broad-spectrum activity against all tested bacteria (Table 3 and Figure 1). They were tested for the MIC values of extracts.

Table 2. Total yield of crude extract obtained from mushroom species using different extraction solvents.

Strain/Species of Mushroom	Yield (mg) of crude extract / Solvent				
	Hexane	Ethyl acetate	Methanol	Ethanol	Water
Agrocybe cytindracea (Pers.) Fayod.	0.7±0.1 k	2.1±0.7 j	19.3±1.1 e	8.0±0.7 g	16.5±1.1 f
Amanita vaginata (Bull. Ex. Fr.) Vitt.	9.6±0.7 g	12.6±1.1 f	28.8±1.5 cd	21.8±1.3 d	29.2±1.5 cd
Auricularia auricular (Hook.) Undrew.	0.8±0.1 k	1.4±0.7 j	2.7±0.7 i	1.6±0.1 j	6.0±0.1 h
Flammulina velutipes (Curtis) Sing.	0.8±0.3 k	1.9±0.7 j	23.8±1.3 d	14.6±0.9 f	25.0±1.3 d
Ganoderma lucidum (Fr.) Karst.	0.7±0.1 k	5.4±1.1 h	7.0±0.7 h	5.1±0.1 h	13.5±1.1 f
Hypsizygus marmoreus	0.8±0.1 k	1.5±0.5 j	3.1±0.1 i	1.9±0.1 j	5.9±0.3 h
Lentinula edodes (Berk.) Sing.	0.6±0.1 k	1.6±0.3 j	2.9±0.1 i	1.7±0.1 i	6.8±0.3 h
Lentinus polychrous Lev.	0.6±0.1 k	1.4±0.1 j	3.5±0.1 i	2.1±0.1 i	14.0±0.7 f
Lentinus squarrosulus Mont.	0.7±0.1 k	1.3±0.1 j	2.5±0.1 i	1.5±0.1 j	7.5±0.1 g
Pleurotus ostreatus (Fr.) Quel.	1.3±0.3 j	1.5±0.1 j	12.0±0.1 f	7.3±0.3 h	32.0±1.7 c
Pleurotus ostreatus (Fr.) Kummer	1.0±0.1 k	2.9±1.1 i	20.1±1.3 e	9.9±0.5 g	44.5±1.5 b
Pleurotus pulmonarius (Fr.) Quel.	0.8±0.3 k	1.8±0.7 j	15.6±1.3 f	5.7±0.3 h	24.5±0.7 d
Pleurotus sajarcaju (Fr.) Sing.	0.9±0.3 k	2.5±0.7 i	19.0±1.1 e	8.8±0.5 g	40.3±1.3 b
Tremella fusiformis Berk.	0.7±0.1 k	5.7±1.1 h	4.0±0.3 h	1.7±0.1 j	15.0±0.3 f
Volvariella volvacea(Bull. Ex. Fr.) Sing.	0.9±0.3 k	1.5±0.7 j	30±1.7 c	12±1.0 f	55.6±1.1 a

* The results are means ± SD. Means with different letter are significantly different from each other. The same letters of homogeneity groups denote non-significant difference of ANOVA test ($P \leq 0.01$) among average of yield of crude extract.

Table 3. Inhibitory effect of some crude extracts derived from mushroom on some food-borne pathogenic bacteria.

Mushroom	Solvent	Inhibition zone (mm)						
		Gram-positive bacteria				Gram-negative bacteria		
		BC[1]	ML[1]	SA[1]	EA[2]	EC[2]	PV[2]	ST[2]
Agrocybe gibberata	Ethyl acetate	7.2±0.25 d	-	6.1±0.10 d	6.2±0.15 d	-	-	-
	H_2O	6.5±0.25 d	6.6±0.20 d	6.2±0.25 d	6.2±0.15 d	-	6.6±0.15 d	6.2±0.25 d
Amanita vaginata	Ethyl acetate	6.2±0.15 d	-	-	6.1±0.10 d	-	6.2±0.25 d	6.4±0.35 d
	Ethanol	7.0±0.10 d	6.2±0.15 d	-	-	-	-	6.5±0.25 d
Auricularia auricula	Ethyl acetate	7.0±0.35 d	-	-	6.2±0.25 d	-	6.3±0.15 d	6.1±0.20 d
Flammulina velutipes	Ethyl acetate	7.0±0.35 d	9.0±0.35 c	-	10.0±0.50 b	9.0±0.35 c	8.0±0.15 cd	7.0±0.10 d
Ganoderma lucidum	Ethyl acetate	8.0±0.35 c	8.5±0.35 c	8.5±0.25 c	6.2±0.25 d	8.5±0.35 c	7.0±0.20 d	8.0±0.15 c
	Methanol	20.0±0.50 a	11.0±0.35 b	14.0±0.25 b	-	11.0±0.35 b	-	13.0±0.25 b
	Ethanol	8.0±0.20 c	8.5±0.20 cd	8.5±0.35 cd	6.2±0.25 d	8.5±0.20 cd	7.0±0.15 d	8.0±0.35 c
	H_2O	8.5±0.25 c	6.2±0.25 d	-	-	6.2±0.25 d	-	-
Hybrigyga marmorens	Ethyl acetate	7.2±0.15 d	6.9±0.35 d	6.1±0.15 d	6.5±0.15 d	6.9±0.35 d	8.0±0.35 cd	-
Lentinula edodes	Ethyl acetate	6.5±0.15 d	6.3±0.15 d	6.2±0.35 d	6.8±0.10 d	6.3±0.15 d	6.9±0.25 d	6.8±0.15 d
	H_2O	6.1±0.10 d	-	6.1±0.25d	-	-	6.2±0.15 d	6.3±0.35 d
Lentinus polychrous	Ethyl acetate	-	6.1±0.10 d	-	6.1±0.35 d	6.1±0.10 d	6.2±0.15 d	-

Table 3. Continued.

Mushroom	Solvent	Inhibition zone (mm)						
		Gram-positive bacteria				Gram-negative bacteria		
		BC[1]	ML[1]	SA[1]	EA[2]	EC[2]	PV[2]	ST[2]
Lentinus polychrous	H_2O	-	-	6.1±0.15 d	6.2±0.25 d	-	6.4±0.10 d	6.2±0.25 d
Lentinus squarrosulus	Ethyl acetate	7.2±0.15 d	6.2±0.25 d	-	6.5±0.10 d	6.2±0.25 d	-	6.1±0.10 d
Pleurotus ostreatus (Fr.) Guel.	Ethyl acetate	9.0±0.45 c	9.0±0.75 c	-	7.0±0.25 d	9.0±0.75 c	8.5±0.25 c	7.0±0.25 d
	Methanol	6.5±0.10 d	6.3±0.20 d	-	-	6.3±0.20 d	-	6.5±0.25 d
	Ethanol	12.0±0.45 b	-	-	8.0±0.50 cd	-	6.1±0.25 d	7.1±0.25 d
Pleurotus ostreatus (Fr.) Kummer	Ethyl acetate	7.1±0.25 d	6.4±0.15 d	6.1±0.25 d	6.5±0.15 d	6.4±0.15 d	7.0±0.45 d	6.5±0.15 d
Pleurotus pulmonarius	Ethyl acetate	7.1±0.25 d	6.3±0.20 d	6.1±0.20 d	6.2±0.15 d	6.3±0.20 d	6.3±0.15 d	6.3±0.25 d
	H_2O	15.0±0.50 b	8.0±0.35 cd	7.5±0.25 d	8.5±0.35 c	8.0±0.35 cd	8.0±0.50 cd	9.0±0.15 c
Pleurotus sajarcaju	Ethyl acetate	-	-	-	6.1±0.15 d	-	-	-
Tremella fusiformis	Hexane	9.0±0.35 c	-	-	-	-	-	-
Volvariella volvacea	Ethyl acetate	7.0±0.25 d	6.1±0.15 d	6.1±0.15 d	6.2±0.10 d	6.1±0.15 d	6.1±0.15 d	6.1±0.20 d
Amoxicillin	H_2O	20±0.50 a	-	-	20±0.25 a	25±0.25 a	20±0.35 a	20±0.25 a
DMSO	H_2O	-	-	-	-	-	-	-

Extracts were used at the final concentration of 20 µl /ml. Each paper disc was soaked with 20 µl of the aliquot. -; Not detect, *Bacillus cereus* (BC), *Enterobacter aerogenes* (EA), *Escherichia coli* (EC), *Micrococus luteus* (ML), *Proteus vulgaris* (PV), *Salmonella typhimurium* (ST), *Staphylococcus aureus* (SA). Inhibition zone was measured (n=3) after 24 hr at inhibition at 37°C. Mean with the same letter is not significantly different by ANOVA test ($P \leq 0.01$) among average of inhibitory effect of crude extracts.

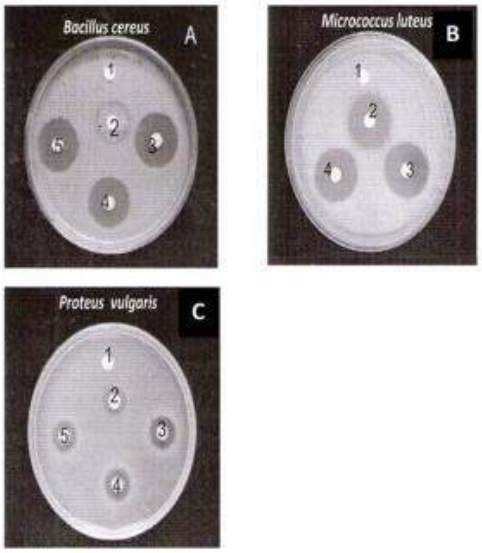

Figure 1. Inhibitory effects of *Pleurotus pulmanarius* in water extract on the bacterial growth, *Bacillus cereus* (A), *Micrococus luteus* (B), *Proteus vulgaris* (C). 1 = DMSO 20 µl, 2 = amoxicillin (20 mg/ml), 20 µl and 3, 4, 5 = crude extract 20 µl.

3.3 MICs of Most Distinctive Thai Cultivated Mushroom Extracts

The MIC values of selected cultivated mushroom strains, i.e. *Flammulina velutipes*, *Ganoderma lucidum*, *Pleurotus pulmonarius* and *Pleurotus ostreatus* (Fr.) Guel. showed significant broad-spectrum activity against all tested bacteria (Table 4). *Bacillus cereus* was most sensitive as it had lowest MICs range (1.25-5.0 mg/ml), while *Salmonella typhimurium* had highest MICs (12.5-22.5 mg/ml). The lowest MIC value of the extracts was 1.25 mg/ml, while amoxicillin had significantly higher inhibitory effect against most bacteria tested (1.25-2.75 mg/ml). *In vitro* activity of some mushroom extracts suggested that the compound has weak activity against *Salmonella typhimurium*. *Pleurotus pulmonarius* extract had significantly highest activity against all tested bacteria (1.25-15.5 mg/ml), followed by *Pleurotus ostreatus* (Fr.) Guel. extract (1.50-17.5 mg/ml), *Ganoderma lucidum* extract (1.50-37.5 mg/ml) and *Flammulina velutipes* extract (2.5-22.5 mg/ml) (Table 4).

Table 4. The minimal inhibitory concentration (MIC) of crude extracts from cultivated mushroom.

Mushroom	Solvent	MIC (mg/ml)						
		Gram-positive bacteria				Gram-negative bacteria		
		BC	ML	SA	EA	EC	PV	ST
Flammulina velutipes Karst.	Ethyl acetate	5.00±1.1d	6.25±1.3e	6.25±1.1e	6.25±1.0e	5.50±0.3d	2.50±0.5b	22.5±1.7g
Ganoderma lucidum (Fr.) Karst.	Methanol	1.50±0.1ab	2.75±0.9b	3.00±0.1c	25.0±1.7g	5.25±0.1d	3.75±0.7c	12.5±1.0f
Pleurotus pulmonarius (Fr.) Quelet	H_2O	1.25±0.1a	1.75±0.7ab	2.50±0.5b	10.0±0.3f	5.25±0.1d	5.50±0.9d	15.5±1.3f
Pleurotus ostreatus (Fr.) Guel.	Ethyl acetate	1.50±0.3ab	3.00±0.5c	3.25±0.5c	12.5±0.3f	5.50±0.3d	5.25±0.7d	17.5±1.3fg
Amoxicillin (positive control)	H_2O	1.25±0.1a	1.25±0.1a	1.75±0.3ab	2.25±0.1b	2.75±0.1b	2.25±0.3b	2.25±0.1b

Each paper disc was soaked with 20□l of the aliquot with the varied final concentration (1.25-50 mg/ml), amoxicillin was used as a positive control. *Bacillus cereus* (BC), *Micrococus luteus* (ML),*Staphylococcus aureus* (SA), *Enterobacter aerogenes* (EA), *Escherichia coli* (EC),*Proteus vulgaris* (PV), *Salmonella typhimurium* (ST).

3.4 ABTS Radical Scavenging Activity

The ABTS radical scavenging test is used to determine the antioxidant activity of hydrophilic and lipophilic compounds. ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants and chain-breaking antioxidants [21]. The scavenging ability of mushroom extracts and Trolox (vitamin E derivative) on ABTS radicals were 91-94 % and 97 %, respectively. The scavenging activity was better in *Gannoderma lucidum*, followed by *Pleurotus pulmonarius* and *Flammulina velutipes*, respectively. The ABTS radical scavenging activity of various extracts indicates their ability to scavenge free radicals by preventing lipid oxidation via a chain-breaking reaction. The IC_{50} values varied from 2.81 mg/ml to 10.57 mg/ml for all samples tested (Table 5). The IC_{50} values for mushroom extracts were simplified as *Ganoderma lucidum* (2.81 mg/ml), followed by *Pleurotus pulmonarius* (3.89 mg/ml) and *Flammulina velutipes* (10.57 mg/ml), respectively. Methanolic extracts of *Ganoderma lucidum* was assumed to be the strongest inhibitor which showed 94% inhibition of ABTS free radicals at the lowest concentration (2.81 mg/ml) among all tested extracts.

Table 5. Comparison of antioxidant activity and IC_{50} value of the aqueous extract of mushroom.

Crude extracts of mushroom	Antioxidant activity (TEAC)	IC_{50} (mg/ml)
Flammulina velutipes	3.38±0.02 c	10.57±0.27 c
Ganoderma lucidum	12.69±0.41 a	2.81±0.02 a
Pleurotus pulmonarius	9.19±0.50 b	3.89±0.13 b
Standard vitamin E	ND	0.01±0.0002 d

TEAC expressed as mM Trolox (Vitamin E derivative) per gram extract, IC_{50} = inhibitory concentration.
The values of IC_{50} are expressed as mean ± SD (n=3). ND = Not detectable.

3.5 NMR Structure Elucidation

Water extract of Indian oyster mushroom (Pleurotus pulmonarius) was a light yellow color and the major compound was separated using preparative TLC with the R_f value of 0.48. Thus, the separated compounds were analyzed using ^{1}H-NMR, focusing on the interpretation of mainly functional groups. In the ^{1}H-NMR spectrum of the main compound, which indicated the presence of polysaccharides, there were signals of different intensities as well, including the anomeric proton signals for four rhamnose residues at d 4.98, 5.04 and 5.06 (2H) (broadened singlet) and one Fuc3HAc residue at d 5.11 (doublet). Only two anomeric proton signals of the minor series were clearly observed, at d 4.94 (broadened singlet, Rha H1) and 5.13 (doublet, Fuc3HAc H1).

4. Discussion

The crude extracts of edible mushroom were tested against food-borne pathogenic bacteria. In this study, the mushroom crude extracts were low in yields due to the gel formation leading to the difficulty in filtration [5]. Water extraction resulted in significantly highest yields, while, hexane extraction gave the lowest yield (Table 2). Due to the application of mushroom extracts in traditional medical treatments, the water extraction can be an option of advantageous and safety process. Antibacterial activities from mushroom extracts were detected at varied levels (Table 2). This could be due to the difference in solubility of organic solvents and water of the mushroom constituents in each species. On the other hand, these test strains may have different level of intrinsic tolerance to mushroom crude extracts and the inhibition effect differs from strain to strain. In this study, the water extract of *Volvariella volvacea* gave significantly higher yield than other solvent extracts (Table 2), however, the extract had less antibacterial activities (Table 3). Although, the water extracts of *Flammulina velutipes*, *Ganoderma lucidum*, *Pleurotus pulmonarius* and *Pleurotus ostreatus* (Fr.) Guel significantly inhibited the growth of all tested bacteria, only aqueous extracts from *Pleurotus pulmonarius* showed significant antibacterial activity against all tested bacteria, and also with greater inhibitory activity compared to ethyl acetate extracts. Our results showed higher antibacterial activity of Indian oyster mushroom than those activities found in previous reports [22, 23]. This study indicated that there are differences in antibacterial effects of mushroom strains (Table 3), due to phytochemical differences among species and the sensitivity of microorganisms to the chemotherapeutic compounds which can change even against different strains [23]. Thus, the aqueous

extract of *Pleurotus pulmonarius* may contain antibacterial compounds that can dissolve in water. The difference in the inhibitory effect of water extract may be attributed to the production of secondary metabolites from the shikimic acid and cinnamic acid pathways during lignocellulosic degradation by *Pleurotus* sp., which may have antibacterial activity [24].

Antibacterial assay of hexane extracts from *Auricularia auricular*, *Flammulina velutipes*, *Ganoderma lucidum*, *Pleurotus ostreatus* and *Tremella fuciformis* showed weak activity only against *Bacillus cereus* (6.1-9.0 mm) (Table 3). Ethyl acetate extracts of *Flammulina velutipes*, *Ganoderma lucidum*, *Hypsizygus marmoreus*, *Lentinula edodes* and *Pleurotus ostreatus* significantly inhibited both Gram-positive and Gram-negative bacteria and extracts of *Flammulina velutipes*, *Ganoderma lucidum* and *Pleurotus ostreatus* significantly inhibited *Escherichia coli*. Methanol extract of *Ganoderma lucidum* showed significantly strong activity against all tested Gram-positive bacteria, and also moderate activity against some Gram-negative bacteria (Table 3). *Salmonella typhimurium* was resistant to all mushrooms extracts (12.5-22.5 mg/ml). The standard amoxicillin presented lower MICs (2.25 mg/ml) than the mushroom extracts (Table 4) because antibiotics and pure active compounds revealed more activity than crude extracts. Cell membrane of Gram-negative bacteria contains outer membrane, which is formed by lipoproteins, lipopolysaccharides and phospholipid that show lipophilicity. However, the major compound derived from mushroom extracts showed water-solubility, which makes the compound difficult to transport through outer membrane of the Gram-negative cell [25]. Our results agree with methanol extract of *Ganoderma lucidum* from India, which demonstrated efficient antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Salmonella* sp. [23] Broad-spectrum inhibition of the mushroom extracts against different groups of bacteria might concern the physicochemical properties of the bioactive compounds in the solvents used [26]. However, the variation in these antibacterial activities might be due to the differences in their bioactive compositions or concentrations, methods of extraction, and the susceptibility of the different bacterial strains tested [27].

ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen donating antioxidants and of chain-breaking antioxidant [28]. This method is often used in evaluating total antioxidant power of single compounds and complex mixtures of various mushrooms [29]. In recent years, multiple drug resistance for human pathogenic microorganisms has been developed due to indiscriminate use of commercial antibacterial drugs commonly used in the treatment of infectious diseases. The increasing demand for natural bioactive compounds in the pharmaceutical and food industries have risen in recent years. Since the mushroom contains a significant amount of vitamins, fibers, phenolic compounds and carotenoids, research interests have focused on the determination of antioxidant capacity. Antioxidant capacity of mushroom extracts is determined by the amount of phenolic compounds and their quantity influences the inhibition capacity of the free radicals. In the case of *Ganoderma lucidum*, *Pleurotus pulmonarius* and *Flammulina velutipes* extracts, they had good values of the content of total phenolics with antioxidant properties. Antioxidant activity of the cultivated mushrooms has significant importance because this activity greatly contributes to their nutraceutical properties which enhance nutritional value or functional food. Phenolic compounds such as phenolic acids and tannins are known as major components of

antioxidant in mushrooms. *Gannoderma* sp. and *Pleurotus* sp. contain several types of phenolic compounds such as vanillic acid [30], myricetin, naringin, homogentistic acid, 5-o-caffeoylquinic acid, chrysin, rutin, gentistic acid, gallic acid, protocatechuic acid, caffeid acid, tannic acid, syringic acid and p-coumaric acid [31]. The most antioxidant properties of mushrooms are in the form of phenolic acids and flavonoids, followed by tocopherols, ascorbic acid and carotenoids [32]. The IC_{50} value, defined as the concentration of antioxidant required for 50% scavenging of ABTS radicals is a parameter used to measure antioxidant activity; a smaller IC_{50} value corresponds to a higher antioxidant activity of the mushroom extract. IC_{50} value of the mushroom crude extract was 2.81 mg/ml to 10.57 mg/ml (Table 5). ABTS radical scavenging activity of aqueous extract of mycelia indicates its ability to scavenge free radicals by preventing lipid oxidation via a chain-breaking reaction. These values showed that cultivated mushroom has higher antioxidant activity compared to vitamin E standard (Table 5).

Our research indicated that *Ganoderma lucidum*, *Pleurotus pulmonarius* and *Flammulina velutipes* had antioxidant and antibacterial properties (Table 3, Table 4 and Table 5). Our results agreed with Mondal *et al.* (2013) [33] who reported that Reishi and certain mushrooms had antioxidant, antibacterial and antifungal properties which were much effective against *Staphylococcus aureus* and *Escherichia coli* through agar-well diffusion method. Radical scavenging and antioxidant activities of *Ganoderma lucidum* extracts were higher than those of *Pleurotus pulmonarius* and *Flammulina velutipes*. All mushrooms used in this study were found to have various degrees of antibacterial effects against tested bacteria. The major constituents of the aqueous extract from *Pleurotus pulmonarius* appeared to be polysaccharides, which supported the presumption that antibacterial activity could be due to the presence of polysaccharides [25]. Use of synthetic bacteriocides to control food spoilage bacteria has been discouraged due to their effects on food, acute residual toxicity, long-term degradation and other side-effect in human [23]. The major problems related to the use of chemicals were the resistant of pathogenic bacteria. Use of higher concentration of chemical causes microbial resistance and enhances high level of toxic residues in products. Further, bioactive compounds are biodegradable and are nearly non-toxic residues in nature and safety to develop for commercial purposes with lower cost.

In conclusion, the mushroom mycelium contains many different bioactive compounds with diverse biological activities. *Pleurotus pulmonarus*, *Ganoderma lucidum* and *Flammulina velutipes* mycelium extracts have a strong inhibiting effect which linked with phenolic compounds, and other beneficial or therapeutic health effects in addition to the prevention of some food-borne disease. Results showed that the mushroom mycelium extracts could be used as a rich source of antibacterial and antioxidant in pharmaceutical-type products. It could be suggested that the aqueous extract of potential cultivation mushroom contains potential antibacterial compounds, antioxidant activity and may be useful for evaluating substances of interest. Further research could be structural elucidation of the bioactivecompounds of the polysaccharide substance derived from aqueous extracts of *Ganoderma lucidum*, *Pleurotus pulmonarius* and *Flammulina velutipes*.

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Comparative antioxidant and antimicrobial properties of *Lentinula edodes* Donko and Koshin varieties against priority multidrug-resistant pathogens

Juliana Garcia [1,*,#], Ana Afonso [1,2,#], Conceição Fernandes [2], Fernando M. Nunes [3], Guilhermina Marques [1], Maria José Saavedra [1,*]

[1] *CITAB – Centro de Investigação e Tecnologias Agroambientais e Biológicas, Universidade de Trás-os-Montes e Alto Douro, 5001- 801 Vila Real, Portugal*
[2] *CIMO – Centro de Investigação de Montanha, Instituto Politécnico de Bragança 5300-253 Bragança, Portugal*
[3] *CQ – Centro de Química, Universidade de Trás- os-Montes e Alto Douro, 5001-801 Vila Real, Portugal*

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ABSTRACT

The problematic increase in multidrug-resistant bacteria translates into the urgent need to discover novel and effective antimicrobial substances. Herein, mushrooms could be a promising alternative of natural source of new antimicrobials. The present work aimed to compare the phytochemical composition and antimicrobial activity of methanol and aqueous crude extracts of *Lentinula edodes* var. Koshin and Donko. Disk diffusion method was used to screen the antimicrobial activity and to assess the synergistic effect of the mushroom extracts. Microdilution method was used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). Phytochemical characterization of mushrooms extracts was achieved by analysis of total phenols, *ortho*-diphenols content and its antioxidant activity. The results noticed a positive relation between phenolic compounds content, antioxidant activity, and antimicrobial capacity of the mushroom's extracts. The *L. edodes* var. Koshin aqueous extracts, which contained the highest amount of total phenolic compounds, exhibited the highest scavenging capacity of ABTS which, in turns, exhibited the highest antimicrobial efficacy in inhibiting the growth of methicillin-resistant *Staphylococcus aureus*. Moreover, the combination between mushrooms extracts and commercial antibiotics showed favorable synergistic effects against tested bacteria. These results suggest that *L. edodes* var. Koshin may represent an important and valuable therapeutic source of compounds to be used against multidrug-resistant bacteria.

1. Introduction

Nowadays, the world is witnessing a dramatic increase related to multidrug-resistant bacteria. The indiscriminate use of antibiotics, among other factors, has been contributed to the development of resistant organisms, leading to easily curable diseases becoming a serious problem (McAdam *et al.*, 2012).

In 2014, the World Health Organization advised that antibiotic resistance reveals serious worldwide threat to Public Health, highlighting the risks associated to the absence of alternative therapies against multidrug-resistant microorganisms (WHO, 2014). In 2015 the European Centre for Disease Prevention and Control estimated 671,689 infections in the EU and European Economic Area caused by multidrug-resistant bacteria, resulting in 33,110 deaths, most of them acquired in healthcare settings (Weist and Högberg, 2016). In 2017, WHO published its first list of antibiotic-resistant "priority pathogens" – a catalogue of 12 families of bacteria which pose the greatest threat to human health. This list was drawn up in a bid to guide and promote research and development of new antibiotics. It is divided into three categories according to the urgency of need for new antibiotics: *critical, high* and *medium priority* (WHO, 2017).

One of the most troubling problems with antibiotic resistance is healthcare associated infections (HCAIs). HCAIs are associated with prolonged hospital stay, which in turns leads, to an increasedcosts, morbidity and mortality (WHO, 2011). Hence, notwithstanding the impossibility to prevent bacteria evolution, it is important to discover novel, natural, and effective antimicrobial substances against pathogenic microorganisms resistant to conventional treatments. Naturally produced antimicrobials have gained an increase of popularity and, among them, mushrooms could be a promising alternative as source of

* Authors to whom correspondence should be addressed.
E-mail addresses: jugarcia_18@hotmail.com (J. Garcia), saavedra@utad.pt (M.J. Saavedra).
# Both of these authors contributed equally to this work

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new antimicrobials (Gyawali and Ibrahim, 2014; Zdenka *et al.*, 2016). In fact, mushroom species release several bioactive compounds, such as terpenoids, flavonoids, tannins, alkaloids, and polysaccharides which could be used as novel antibiotics. Herein, *Lentinula edodes* (shiitake mushrooms) has been attracted particular attention concerning its antimicrobial activity (Bender *et al.*, 2001). Therefore, in the present work, the *in vitro* antimicrobial activity of *Lentinula edodes* var. Donko and Koshin aqueous and methanolic extracts was screened against several clinical isolates of carbapenem-resistant *Klebsiella pneumoniae* and methicillin-resistant *Staphylococcus aureus* that belong to the WHO *critical* and *high priority* agent groups. Those clinical isolates are from CHTMAD – Hospital Center of Trás-os-Montes and Alto Douro, Portugal. The comparison between the two mushroom varieties was taken into account since there are substantial differences in morphology and growing conditions which may interfere on the content of bioactive compounds and biological activities. In fact, the var. Donko grows slowly at low temperatures, and present a thick cap, short stalk and dark brown color (Figure 1A). On the other hand, var. Koshin develops rapidly at mild temperatures and it has a thin cap and a pale coloration (Figure 1B) (Miles and Chang, 2004).

Considering the results of the antimicrobial activity, the most promising species extracts were combined with commercial antibiotics against different clinical isolates, including multidrug-resistant microorganisms. Moreover, for the first time, the two varieties of *L. edodes* were compared regarding the antimicrobial activity and phytochemical composition.

2. Material and Methods

2.1. Chemicals and drugs

Methanol was of analytical grade purity from Lab-Scan (Lisbon, Portugal). The culture media Brain Heart Infusion agar (BHIA), Mueller Hinton broth (MHB), Mueller Hinton agar (MHA) and all antibiotics were obtained from Oxoid (Humpshire, UK). The saline solution was prepared with NaCl from Merck (Darmstadt, Germany). The dye resazurin was purchased from Sigma–Aldrich (St Louis, MO, USA) to be used as microbial growth indicator.

2.2. Mushrooms material

Lentinula edodes var. Donko and Koshin mushrooms, produced in Floresta Viva company (Amarante, Portugal), were stored at − 20°C, freeze-dried (Dura Dry TM μP, −41°C and 500 mTorr) and grounded to a fine powder.

2.3. Lentinula edodes extracts

Mushrooms extracts were obtained by two different extrac methods: (1) Exhaustive aqueous extracts: 5g of dried mushrooms terial was added to 150 mL of distilled water. The mixture was agita at room temperature (orbital shaker, one hour, 150 rpm), then centrifuged. The supernatant was filtered (Whatman no. 4 filter paper), and again 100 mL of distilled water was added to the pellet. The whole procedure was repeated to a total of 4 times. The total extracted was stored at −20°C before lyophilization to obtain the final extract; (2) Exhaustive methanolic extracts: 5g of dried mushrooms material was added to 150 mL of a 80% methanol solution (methanol/distilled water v/v). The mixture was agitated at room temperature (orbital shaker, one hour, 150 rpm), then centrifuged. The supernatant was filtered and again 100 mL of the previous solution was added to the pellet. The whole procedure was repeated to a total of 4 times. The total extracted volume was concentrated in a vacuum rotary evaporator at 38°C to remove methanol and stored at −20°C before lyophilization to obtain the final extract.

2.4. Microorganisms and culture media

The microorganisms used were clinical isolates from patients hospitalized in various departments of Hospital Centre of Trás-os-Montes and Alto Douro (CHTMAD) - these are located in the cities of Lamego, Peso da Régua, Chaves, and Vila Real, Portuguese north province of Trás-os-Montes and Alto Douro. Ethical approval for this study was granted by the Ethics Committee of Hospital Vila Real (CHTMAD), according to a research collaboration protocol established in 2004. These strains belong to MJH and MJMC collections and are stored at −70°C in aliquots of BHI (Brain Heart Infusion) medium with 15% (v/v) glycerol, in the Microbiology Laboratory of the Veterinarian Science Department at UTAD. Six Gram-negative bacteria, *Klebsiella pneumonia* isolated from biological fluids (MJH 513, MJH 569, MJH 579, MJH 599, MJH 602, MJH 640, MJH 662), and thirteen Gram-positive bacteria, methicillin-sensitive *Staphylococcus aureus* (MSSA) (MJMC 018, MJMC 026, MJMC 109, MJMC 110, MJMC 511) and methicillin-resistant *S. aureus* (MRSA) (MJMC 025, MJMC 027, MJMC 102, MJMC 111, MJMC 507, MJMC 534 B, MJMC 539, MJMC 545, MJMC 552), isolated from wound exudates, were used to screen the antimicrobial activity of the mushroom extracts. All strains were identified by morphological and biochemical tests (morphological identification of colonies, Gram staining, conventional biochemical identification methods and MicroScan WalkAway identification panels), followed by Kirby-Bauer antibiotic sensitivity assays, using different antibiotics (10 μg).

Escherichia coli (CETC 434) and *Staphylococcus aureus* (CETC 976) strains were obtained from *Spanish Type Culture Collection (CETC)*. Ethics approval for this study was granted by the Ethics Committee of Hospital

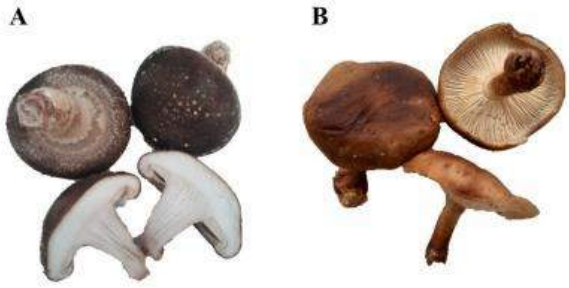

Figure 1. *Lentinula edodes* Donko (A) and Koshin (B) varieties

Vila Real.

2.5. Antimicrobial activity

Briefly, bacterial suspension with the turbidity adjusted to 0.5 McFarland standard units, were spread with a sterile cotton swab onto Petri dishes (90mm of diameter) containing 4 mm of Mueller-Hinton agar. Six-millimeter diameter sterile paper discs were dispensed on the seeded agar plates and imprinted with 12 µL of 1000 µg.mL^{-1} extracts solution (in Dimethyl Sulfoxide (DMSO) 10%). Incubation for 24 h at 37°C, followed by diameter measurement (mm) of the clear inhibitory zones around the discs imprinted with the extracts. In all experiments, a negative control (12 µL DMSO) and a positive control [standard commercial antibiotic gentamicin (10 µg)] were included.

The antimicrobial activity was classified according to the following scheme: noneffective (-): inhibition halo = 0; moderate efficacy (+): 0 < inhibition halo < antibiotic inhibition halo; good efficacy (++): antibiotic inhibition halo < inhibition halo < 2x inhibition halo; strong efficacy (+++): inhibition halo > 2x antibiotic inhibition halo (Aires *et al.*, 2009).

Minimum inhibitory concentration (MIC, lowest concentration of mushroom extract able to inhibit bacterial growth) was evaluated by a resazurin microdilution assay (Sarker *et al.*, 2007). Bacteria tested were picked from overnight cultures in BHIA. A small portion of bacteria was transferred into a bottle with 50 mL of MHB, capped and placed in an incubator overnight at 37°C. After 16 h of incubation, bacterial suspension was adjusted to an optical density of 0.5 measured at OD500 nm. The resazurin solution (3.4 mg.mL^{-1}) was prepared in sterile distilled water. A 96-wells sterilized microplate was used and a volume of 100 µL of MHB was used in each well, together with 200 µL of extract solution, or positive control. From the first well (belonging to the first horizontal line) 100 µL was taken and added to the next well and then this step was repeated to each of the following wells in the vertical line, allowing a serial fold dilution of decreasing concentration (range of 1000 µg.mL^{-1} to 7.81 µg.mL^{-1}). In addition, 20 µL of bacterial suspension and 20 µL of resazurin solution was added to each well. Microplates were incubated at 37°C for 18–24 h. All tests were performed in triplicate and MIC was then assessed visually by the color change of resazurin in each well (blue to pink in the presence of bacteria growth). For the determination of minimum bactericidal concentration (MBC, the lowest concentration of mushroom extract at which bacterial growth by at least 99.0%), the content from each well without changes in color was plated on Mueller-Hinton Agar and incubated at 37 °C for 24 h. The lowest concentration that yielded no growth after this subculturing was taken as the MBC.

2.5.1. Synergistic effect

The screening of synergistic effects was performed by the disk diffusion method in solid medium. Taking into account the antimicrobial results, the synergistic effect was only evaluated for *L. edodes* var. Koshin. Two discs were dispensed into the plates by antibiotic and one of them was impregnated with 12 µL of the mushroom extract solution. Seven antibiotics were used: gentamicin (CN), amoxicillin/clavulanic acid (AMC), ciprofloxacin (CIP), vancomycin (VA), imipenem (IPM), ertapenem (ETP) and meropenem (MEM).

2.6. Determination of total phenolic compounds

The total phenolic compounds in the extracts were determined by the Folin–Ciocalteu method as previous described (Gouvinhas *et al.*, 2017), with some modification. Briefly, 10 µL of mushrooms extract at a concentration of 1 mg.mL^{-1} or gallic acid standards (0.01 to 1.0 mg.mL^{-1} in methanol) were mixed with 185 µL of distilled water in a 96-well plate followed by the addition of 25 µL of Folin-Ciocalteu reagent. After an incubation period of 5 min, sodium carbonate (75 µL of 7% Na_2CO_3) was added and further incubated for 2 h in the dark and at room temperature. The absorbance was then measured at 725 nm against a blank on a Biotek Powerwave XS2 plate reader (BioTek Instruments, Inc. USA) at 25 °C. The phenolic content was expressed as mg gallic acid equivalents *per* gram of extract (mg GAE/g dry weight).

2.7. Ortho-diphenols content

For the analysis of the ortho-diphenols content, a colorimetric method, based on a complex reaction with sodium molybdate dehydrate, was applied (Ferreira *et al.*, 2020). Briefly, extract aliquots (60 µL at a concentration of 1 mg.mL^{-1}) or gallic acid standards (0.01 to 1.0 mg.mL^{-1} in methanol) were reacted for 25 min with 200 µL of a sodium molybdate dihydrate solution (5% prepared in ethanol/water, 1:1 v/v). The absorbance of the samples and standards was measured at 370 nm against to blank (ethanol/water 1:1, v/v) on a plate reader at 25 °C. The results were expressed as mg of gallic acid equivalents *per* gram of extract (mg GAE/g dry weight).

2.8. Radical Scavenging Activity on ABTS radical

The radical scavenging activity of both mushroom extracts on ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation was measured using a spectrophotometric methodology, according to previously described procedure (Ferreira *et al.*, 2020). Briefly, an ABT radical cation stock solution (7.4 mM) was prepared by reacting equ amounts of ABTS and potassium persulfate (2.6 mM) and allowing t stand 16 h in the dark and at room temperature. The radical solution wa then diluted with methanol to an absorbance of 0.70 (±0.02) at 734 nm at 25 °C. The samples (10 µL ranging from 0.0625 to 1 mg.mL^{-1}) were mixed with 190 µL of the radical solution and after 6 min incubation the absorbance was measured. The results were expressed as Trolox equivalent antioxidant capacity (mM Trolox / g dry weight).

2.9. Statistical Analysis

For the evaluation of antimicrobial activity, all assays were carried out in duplicate and the results are expressed as mean ± standard deviation. Regarding the chemical characterization of the extracts, all assays were performed in triplicate. Data are expressed as mean ± standard deviation and were statistically analyzed by one-way analysis of variance (one-way ANOVA), followed by Holm-Sidak's multiple comparison test. Statistical analyzes were performed using GraphPad Prim for Windows (Version 7) and differences were considered significant when $p < 0.05$ (95% significance).

3. Results

3.1. Antimicrobial susceptibility of clinical isolates

Concerning the assessment of antibiotic susceptibility, the results revealed that some of the clinical isolates used are multidrug-resistant bacteria (Tables 1 and 2) being a problem due to the insufficient alternatives of effective antibiotics. As seen in Table 1, in Gram-positive clinical bacteria isolated from wound infections, the methicillin, ciprofloxacin and levofloxacin had the highest antibiotics resistance. The clinical isolates MJMC 534 B, MJMC 539 and MJMC 552 are resistant to 7, 8 and 9 antibiotics, respectively, being the most multidrug-resistant bacteria. Gentamicin and vancomycin are the most effective antibiotics, as thirteen of the fourteen isolates are sensitive to those antibiotics.

Concerning to Gram-negative clinical bacteria isolated from different types of biological samples (Table 2), all microorganisms were resistant to amoxicillin / clavulanic acid and ertapenem, a carbapenem used as a last resort in the treatment of Extended-Spectrum β-lactamase (ESBL) -producing bacterial infections. The clinical isolates MJH 569 and MJH 599 have the highest number of antibiotic resistances, being evident the

Table 1
Susceptibility of different antibiotics against Gram-positive bacteria.

	MJMC018	MJMC025	MJMC026	MJMC027	MJMC102	MJMC109	MJMC110	MJMC111	MJMC507	MJMC511	MJMC 534 B	MJMC539	MJMC545	MJMC552
Penicillin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	R	R	R
Methicillin	S	R	S	R	R	S	S	R	R	S	R	R	R	R
Ampicillin	nd	nd	nd	nd	nd	nd	nd	R	nd	R	nd	nd	nd	R
Amoxicillin/ Cavulanic acid	R	S	R	S	R	S	S	R	R	S	R	R	S	R
Oxacillin	nd	nd	nd	nd	nd	nd	nd	R	R	S	R	nd	R	R
Clindamycin	nd	nd	nd	nd	nd	nd	nd	I	I	R	R	R	nd	R
Daptomycin	nd	nd	nd	nd	nd	nd	nd	nd	nd	S	S	S	nd	nd
Erythromycin	nd	nd	nd	nd	nd	nd	nd	nd	nd	S	R	R	nd	R
Gentamicin	S	S	S	S	S	S	S	S	S	S	S	R	S	S
Ciprofloxacin	R	S	R	R	R	S	S	R	R	S	R	R	S	R
Levofloxacin	R	S	R	R	R	S	S	R	R	S	R	R	S	R
Linezolid	nd	nd	nd	nd	nd	nd	nd	nd	S	nd	S	S	S	S
Trimethoprim/ Sulfamethoxazole	nd	nd	nd	nd	nd	nd	nd	nd	S	S	S	S	S	S
Vancomycin	S	S	S	S	S	S	S	S	S	nd	S	S	S	S

S- Susceptible; I- Intermediate; R- Resistant (this classification was made according to the interpretative breakpoints suggested by Clinical and Laboratory Standards Institute-CLSI); nd- not determined.

low sensitivity to the β-lactam antibiotic group.

3.2. Antimicrobial activity evaluation of extracts

The *L. edodes* extracts were tested at concentration of 500 μg.mL^{-1} and it was observed that by increasing the concentration to 1000 μg.mL^{-1}, the size of the inhibition halos also increased. Therefore, only the results obtained with the concentration of 1000 μg.mL^{-1} are presented in this work. Data obtained on screening of antimicrobial activity of the two varieties of *L. edodes* extracts, against Gram-positive and Gram-negative bacteria, are shown in Table 3. Concerning to aqueous extracts, the bacterial inhibition halos varied between 7 – 11 mm and between 7 – 16 mm for var. Donko and var. Koshin, respectively (Figure 2 and Table 3). Methanolic extracts of both varieties were not effective against either isolate tested. Also, Gram-negative isolates were resistant to all extracts tested.

Therefore, and according to the classification scheme proposed by Aires et al. (2009), the aqueous extract was "moderately effective" against all Gram-positive isolates, and "noneffective" against all Gram-negative isolates. The methanolic extract was showed to be "noneffective" for the 23 isolates tested (Table 3).

Since the most promising results were obtained with *L. edodes* var. Koshin mushrooms extracts, the MIC of both extracts (aqueous and methanolic) was only determined for this variety in Gram-positive clinical isolates (Table 4). In aqueous extract, the MIC values ranged from 15.63 to 250 μg.mL^{-1}, and in methanolic extract, the values ranged from 31.25 to 1000 μg.mL^{-1}. The best antimicrobial activity was achieved for MJMC 102 and MJMC 534 B clinical wound infection isolates and, in the MIC assay, they were the most sensitive. Noteworthy, the methanolic extract, that was not effective in the disc diffusion method, was efficient against all strains tested and the MIC values ranged from 500 to 1000 μg.mL^{-1}. Regarding to MBC, the lowest concentration with bactericidal effect was 500 μg.mL^{-1}, to MJMC 111 and MJMC 552 with aqueous extract, and to MJMC 534 B with methanolic, all MRSA isolates.

3.3. Synergistic effect

Data obtained in the assessment of synergistic effect between *L. edodes* var. Koshin aqueous and methanolic extracts and commercial antibiotics are shown in Figure 3 and 3. Regarding to Gram-positive bacteria, both extracts exhibiting favourable synergistic effects with gentamicin (CN), vancomicyn (VA) and ciprofloxacin (CIP). No change in the "Resistant" to "Sensitive" profile was observed in the antibiotics tested, however the disk diffusion method showed an increase in halos size, in some cases approaching the value stipulated by the EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines to change from" Resistant" to "Sensitive", such as the combination of aqueous extract + amoxicillin / clavulanic acid (AMC) in the MJMC 534 B ulcer clinical isolate (increased from 11 mm to 19 mm). Antagonistic effects were also observed with the combination of extracts and amoxicillin/clavulanic acid (AMC) antibiotic.

Gram-negative bacteria (Figure 4) did not showed synergistic effects between extracts and the tested antibiotics. On the other hand, for meropenem (MEM) a decrease of ± 10 mm in halos size was observed, which indicated antagonism effect, and even a change in susceptibility profile (from "Sensitive" to "Resistant").

3.4. Total phenolic compounds and ortho-diphenols components

The total phenolic compounds content in the extracts varied significantly for the two *L. edodes* varieties and extraction solvent used (Figure 5A). Comparing the methanolic and aqueous extracts from var. Koshin (2.07 ± 0.12 and 4.09 ± 0.59 mg GAE/g dry weight, respectively), the results showed that there were significant differences concerning the extraction solvent used, with the aqueous solvent presenting a higher total phenolic compounds content than methanol. Noteworthy,

Table 2
Susceptibility of different antibiotics against Gram-negative bacteria.

	MJH 513	MJH 569	MJH 579	MJH 599	MJH 602	MJH 640	MJH 662
Ampicillin	nd	R	nd	R	nd	R	nd
Amoxicillin/ Clavulanic acid	R	R	R	R	R	R	R
Piperacillin/ Tazobactam	R	R	nd	R	R	R	R
Imipenem	I	I	I	I	I	I	I
Ertapenem	R	R	R	R	R	R	R
Meropenem	R	R	I	S	I	R	I
Cefuroxim	nd	R	nd	R	nd	R	nd
Cefoxitin	R	R	nd	S	R	nd	nd
Cefotaxime	R	ESBL	nd	ESBL	R	R	R
Ceftazidime	nd	ESBL	nd	ESBL	nd	R	R
Ciprofloxacin	R	R	nd	R	R	S	S
Fosfomycin	nd	S	nd	S	nd	S	nd
Nitrofurantoin	nd	R	nd	nd	nd	S	nd
Norfloxacin	nd	R	nd	R	nd	S	nd
Levofloxacin	nd	R	nd	R	nd	S	nd
Gentamicin	S	S	S	S	S	S	I
Trimethoprim/ Sulfamethoxazole	R	R	nd	R	nd	S	R
Tobramycin	R	R	nd	R	nd	S	nd
Amikacin	I	I	nd	I	I	S	I
Colistin	S	nd	nd	nd	S	S	S
Tigecycline	S	nd	nd	nd	S	nd	S

S- Susceptible; I- Intermediate; R- Resistant; ESBL- Extended-spectrum beta-lactamases (this classification was made according to the interpretative breakpoints suggested by Clinical and Laboratory Standards Institute-CLSI); nd- not determined.

Table 3
In vitro antimicrobial activity of positive control and aqueous and methanolic extracts of *L. edodes* var. Donko and Koshin (1000 μg.mL^{-1}), determined by the diameter of inhibition zones (mm).

		AQUEOUS EXTRAC		METHANOLIC EXTRAC		CONTROL	
	Isolate	Donko	Koshin	Donko	Koshin	CN	DMSO
GRAM +	MJMC 018	7 ± 0.0 (+)	10 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{24 \pm 0.2}$	6 ± 0.0
	MJMC 025	8 ± 0.0 (+)	8 ± 0.0 (+)	6 ± 0,0 (-)	6 ± 0,0 (-)	S $^{22 \pm 0.2}$	
	MJMC 026	8 ± 0.0 (+)	7 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{22 \pm 0.0}$	
	MJMC 027	8 ± 0.0 (+)	7 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{22 \pm 0.05}$	
	MJMC102	8 ± 0.0 (+)	12 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{25 \pm 0.5}$	
	MJMC109	9 ± 0.0 (+)	11 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{25 \pm 0.5}$	
	MJMC110	9 ± 0.0 (+)	11 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{25 \pm 0.5}$	
	MJMC111	9 ± 0.0 (+)	10 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{24 \pm 1.0}$	
	MJMC507	10 ± 0.0 (+)	10 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{25 \pm 0.0}$	
	MJMC511	10 ± 0.0 (+)	10 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{26 \pm 0.5}$	
	MJMC534 B	11 ± 0.0 (+)	16 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{27 \pm 0.0}$	
	MJMC 539	9 ± 0.0 (+)	9 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	R $^{15 \pm 0.15}$	
	MJMC 545	7 ± 0.0 (+)	8 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{23 \pm 0.25}$	
	MJMC 552	9 ± 0.0 (+)	9 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{25 \pm 0.2}$	
	CETC976	7 ± 0.0 (+)	7 ± 0.0 (+)	6 ± 0.0 (-)	6 ± 0,0 (-)	S $^{22 \pm 0.0}$	
GRAM -	MJH513	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	S $^{17 \pm 0.0}$	6 ± 0.0
	MJH569	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	S $^{21 \pm 0.0}$	
	MJH579	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	S $^{19 \pm 0.5}$	
	MJH599	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	S $^{18 \pm 0.0}$	
	MJH602	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	R $^{22 \pm 0.5}$	
	MJH640	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	S $^{20 \pm 0.0}$	
	MJH662	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	R $^{13 \pm 0.0}$	
	CETC434	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	6 ± 0.0 (-)	S $^{20 \pm 0.0}$	

Results are expressed as mean ± SD (standard deviation) of 2 replicates. Note: 6 mm value corresponds to disc diameter.

the amount of total phenolic compounds content in aqueous var. Koshin extract (4.09 ± 0.59 mg GAE/g dry weight) was significantly higher than in the aqueous extract from var. Donko extract (2.12 ± 0.49 mg GAE/g dry weight), suggesting that *L. edodes* varieties may differ on the content of phenolics compounds.

The overall trend observed for the total phenolic compounds (Figure 5A) content was also observed for the *ortho*-diphenols content (Figure 5B), i.e. there were significant differences between varieties of *L. edodes* and between the solvents used for extraction. As observed for the total phenolic compounds content, the content of *ortho*-diphenols in aqueous extracts from var. Koshin (0.11 ± 0.02 mg GAE/g dry weight) was significantly higher than in methanolic extract (0.04 ± 0.01 mg GAE/g dry weight), indicating that there were significant differences between the solvents used for extraction. Likewise, the content of *ortho*-diphenols in aqueous of var. Koshin extract (0.11 ± 0.02 mg GAE/g dry weight) was significantly higher than *ortho*-diphenols in aqueous var. Donko extract (0.08 ± 0.01mg GAE/g dry weight) in line with previously described for the total phenolic compounds content of the extracts.

3.5. *In vitro* Antioxidant activity

As shown in Figure 6, a higher ABTS•+ radical scavenging activity was observed for the aqueous extract of var. Koshin (1.53 ± 0.13 mmol Trolox / g dry weight) when compared with the methanolic extract of var. Koshin (0.77 ± 0.10 mmol Trolox/ g dry weight). Noteworthy, there was observed a significant difference between the aqueous extract of var. Koshin (1.53 ± 0.13 mmol Trolox / g dry weight) and aqueous extract of var. Donko (1.17 ± 0.10 mmol Trolox / g dry weight).

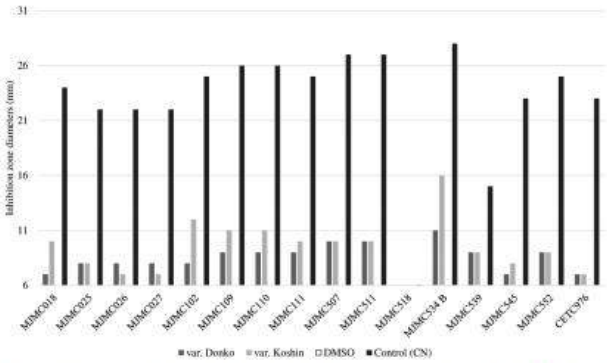

Figure 2. *In vitro* antimicrobial activity of positive control (Gentamicin) and aqueous extracts of *L. edodes* var. Donko and Koshin (1000 μg.mL^{-1}) against Gram-positive isolates, determined by the diameter of inhibition zones (mm).

Table 4
Minimum Inhibitory Concentration (μg.mL^{-1}) and Minimum Bactericidal Concentration for aqueous and methanolic extracts of *L. edodes* var Koshin.

		AQUEOUS EXTRACT		METHANOLIC EXTRACT		CONTROL
	Isolate	CMI	CMB	CMI	CMB	CMI
MRSA	MJMC 025	250	>1000	1000	>1000	125
	MJMC 027	125	>1000	500	>1000	125
	MJMC102	62.5	>1000	250	1000	7.81
	MJMC111	62.5	500	500	>1000	7.81
	MJMC507	125	>1000	250	1000	7.81
	MJMC534 B	62.5	1000	500	500	7.81
	MJMC 539	125	>1000	500	>1000	62.5
	MJMC 545	125	>1000	500	>1000	125
	MJMC 552	250	500	500	1000	125
MSSA	MJMC 018	62.5	>1000	500	>1000	7.81
	MJMC 026	125	>1000	1000	1000	125
	MJMC109	125	1000	1000	>1000	125
	MJMC110	62.5	1000	500	1000	62.5
	MJMC511	62.5	1000	500	1000	7.81
	CETC976	15.63	n.d.	31.25	n.d.	7.81

Moreover, the ABTS•+ radical scavenging activity of the aqueous extract of var. Donko (1.17 ± 0.10 mmol Trolox / g dry weight) was significantly higher than the methanolic extract of var. Koshin (0.77 ± 0.10 mmol Trolox/ g dry weight) (Figure 6). These results are in accordance with the results obtained for the total phenolic compounds and *ortho*-diphenols contents of both methanolic and aqueous extracts. On the other hand, no differences in the ABTS+. scavenging activity were observed between the methanolic extracts of var. Koshin and Donko.

4. Discussion

The rapid emergence of resistant bacteria is universal compromising the efficacy of the existing available antibiotics. Currently, the most notorious multidrug-resistant bacteria have been identified as the so-called "ESKAPE" which includes *S. aureus*, and *K. pneumoniae* being responsible for significant high morbidity and mortality (Li and Webster, 2018).

According to the OECD's projections, by 2050 Italy, Greece and Portugal "will have the highest mortality due to antimicrobial resistance" among EU members (OECD, 2018). Hence, there is an urgent need for the development of new and effective drugs against current multidrug-resistant pathogens (Zaman *et al.*, 2017). Natural resources have been widely exploited, being fungal species an important potential source of bioactive compounds with exceptional therapeutic value (Poucheret *et al.*, 2006). Herein, the two varieties of *Lentinula edodes* were assessed as an alternative to fight antibiotics resistance. Accordingly, the present study aimed to evaluate the antimicrobial properties of *L. edodes* var. Koshin and Donko aqueous and methanolic extracts and its synergistic effect with current clinical antimicrobials in multidrug resistant bacteria isolates obtained from a Portuguese hospital. Noteworthy, for the first time, the two varieties of *L. edodes* were compared concerning to phytochemical composition and antimicrobial activity.

The evaluation of antimicrobial activity, by disc diffusion method, revealed that both aqueous extracts were effective against Gram-positive bacteria (MSSA and MRSA), and both methanolic extracts were not effective against either isolates. Aqueous extracts from the *L. edodes* var. Koshin showed the best results, since the highest antimicrobial activity was obtained against to MRSA MJMC 534B. This is a result of great importance since the incidence of MRSA, especially in Portuguese hospitals, is one of the most important in Europe (45%) (ECDC, 2017). Noteworthy, it should be pointed out that MRSA MJMC 534B is clinical wound infection isolate causing high morbidity and mortality; longer hospital stays, delay in wound healing, increase economic burden and discomfort. Therefore, these results obtained with aqueous extracts on Gram-positive isolates are an important step towards to search for new effective agents against *S. aureus* in infected wounds. These results are in accordance with previous reports, in which different extracts from 48 mushroom species including *L. edodes*, were evaluated and it was observed that Gram-positive bacteria were more sensitive than Gram-negative bacteria. Among all extracts, the aqueous extracts of *Clitocybe geotropa* and *Lentinula edodes* showed the highest antimicrobial activity against all strains tested (Venturini *et al.*, 2008). In agreement, in another study, the aqueous *L. edodes* demonstrated notable antimicrobial activity against MRSA (Hearst *et al.*, 2009). The antimicrobial differences between the two *L. edodes* varieties and two solvents (aqueous and methanol) may be due to the total phenols and *ortho*-diphenols contents present, which was higher for the aqueous extract of the Koshin variety. In fact, several authors have previously associated the antimicrobial activity of different natural sources to phenolic compounds (Barros *et al.*, 2008; Alves *et al.*, 2012). Usually, the differences observed between varieties can be explained by several factors, namely, genetic, physiological and morphologic characteristics, agroclimatic conditions and ripening stage and in this case, the differences in phenols content, as well as antioxidant activity are probably,

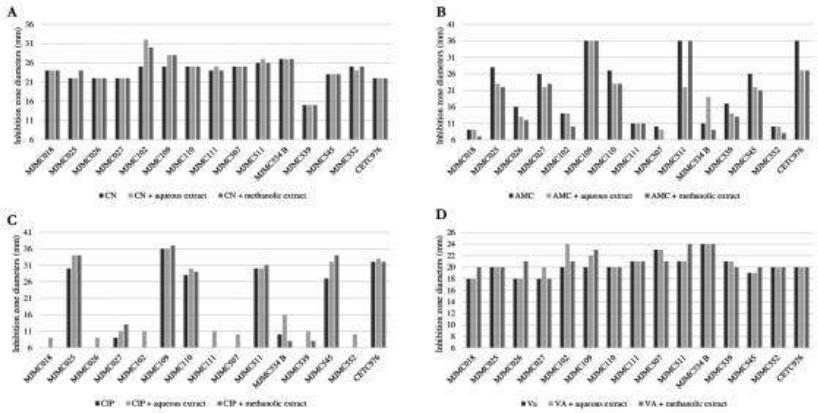

Figure 3. Inhibition zone diameter (mm) in the synergistic effect assessment with combination of *L. edodes* var Koshin aqueous/methanolic extracts and (A) Gentamicin, (B) Amoxicilin /clavulanic acid, (C) Ciprofloxacin, (D) Vancomycin on Gram-negative isolates.
Guidelines EUCAST/CLSI for *Staphylococcus aureus*: CN S ≥18 mm; AMC) ≥20; CIP S ≥21 mm; VA S ≥12 mm.

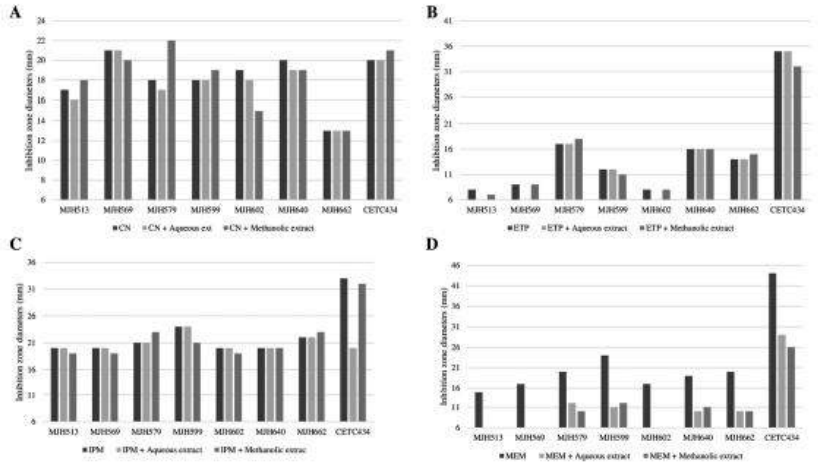

Figure 4. Inhibition zone diameter (mm) in the synergistic effect assessment with combination of *L. edodes* var Koshin aqueous/methanolic extracts and (A) Gentamicin, (B) Ertapenem, (C) Imipenem, (D) Meropenem on Gram-negative isolates.
Guidelines EUCAST/CLSI for *Klebsiella pneumoniae*: CN S ≥14 mm; ETP S ≥25 mm; IPM S ≥17 mm; AMC S ≥19 mm; MEM S ≥16 mm.

mainly due to genetic, morphologic characteristics and growing conditions.

It should be highlighted that more studies should be performed in order to elucidate the mechanism of bacteriostatic or bactericide effect and the specific phenolic/ *ortho*-diphenols compounds found in these extracts should be tested against selected bacteria in order to identify molecules responsible for the mushrooms bioactivity.

In the MIC determination, methanolic extracts also had antimicrobial activity, however with higher MIC values. These results do not match those obtained in disc diffusion method. However, the absence of an inhibition zone does not necessarily mean that the extract is not effective against that microorganisms, although rather that the diffusion was not complete, especially for the less polar compounds that diffuse more slowly into the culture medium (Moreno *et al.*, 2006).

Regarding the evaluation of synergistic effect, this is a pioneer study between *L. edodes* different extracts and commercial antibiotics and constitutes an important step, since the current available data are mainly correlated with plant extracts and not with mushroom extracts

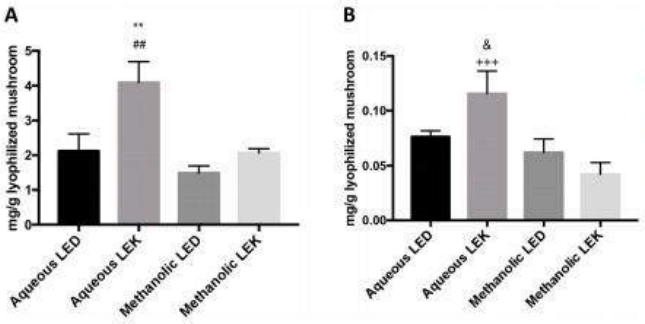

Figure 5. Total phenols (A) and ortho-diphenols (B) composition in aqueous and methanolic extracts from *L. edodes* var. Donko and Koshin.
Results are presented as mean ± standard deviation (n =3). Statistical comparisons were made using the one-way ANOVA, followed by the Holm-Sidak's multiple comparisons test, (**$p < 0.01$ *vs.* aqueous var. Donko extract; $^{\#\#}p < 0.01$ *vs.* methanolic var. Koshin extract; $^{\&}p < 0.05$ *vs.* aqueous var. Donko extract; $^{+++}p < 0.001$ *vs.* methanolic var. Koshin extract).

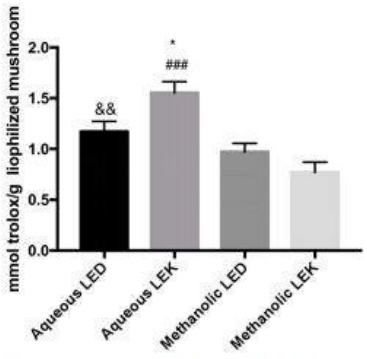

Figure 6. Antioxidant properties of aqueous and methanolic extracts from *L. edodes* var. Donko and Koshin expressed as Trolox equivalent antioxidant capacity.
Results are presented as mean ± standard deviation (n =3). Statistical comparisons were made using the one-way ANOVA, followed by the Holm-Sidak's multiple comparisons test, (*$p < 0.05$ *vs.* aqueous var. Donko extract; $^{\#\#\#}p < 0.001$ *vs.* methanolic var. Koshin extract; $^{\&\&}p < 0.01$ *vs.* methanolic var. Koshin extract).

(Alves *et al.*, 2014). The synergistic effect between mushrooms extracts and antibiotic may constitute a strategy employed for protection against increasing microorganism's resistance. The results obtained showed that there was a synergistic effect between both mushrooms extracts and some standard antibiotics for Gram-positives bacteria namely gentamicin, vancomicyn, and ciprofloxacin. No change in the "Resistant" to "Sensitive" profile were observed, however, in some cases, the halo value size approached the value determined by EUCAST to change from "Resistant" to "Sensitive". Similar to this result, Alves *et al.* (2014) reported synergistic effect against MRSA using methanol and aqueous extracts from different wild mushroom species, and synergistic effects were observed for two quinolones (ciprofloxacin and levofloxacin) and for some β-lactams (penicillin, ampicillin and cefoxitin). Other authors have also reported synergistic effects of plant extracts and quinolones (ciprofloxacin and levofloxacin) against MRSA (An *et al.*, 2011).

Antagonistic effects were also observed with extracts + amoxicillin/clavulanic acid combination, which may be explained by the amount of metabolites present in the extracts that may lead to decrease of the active substances action, through competitiveness by the same action target and / or through inhibition of antibiotic active principle. Gram-negative isolates showed antagonism with the addition of extracts to meropenem. The carbapenem group represents a last line in the fight against multidrug resistant bacterial infections, due to the broad spectrum of action against Gram-negative and Gram-positive bacteria, including ESBL-producing microorganisms. Carbapenems act by inhibiting bacterial cell wall synthesis by binding and inactivating PBPs (Penicillin-binding proteins). The intensity of binding to PBPs depends the type of carbapenem. Meropenem has a high affinity for PBP 3 relative to imipenem and ertapenem (Meroueh *et al.*, 2006). Here, the possibility that some compounds, present in the mushroom extract, will first bind to PBP 3 may justify this antagonistic effect.

These extracts, as well as specific compounds, should be evaluated for further conclusions. Moreover, more studies are required in order to clarify the mechanisms of action that support the observed effects upon each antibiotic and microorganism.

Given the problem associated with antibiotic resistance worldwide, *Lentinula edodes* is a potential source of antibacterial compounds that can be used prophylactically to prevent the risk of infection and may be used in combination with antibiotics, to reduce the time of infection and the possible occurrence of resistance phenomena.

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Author Contributions

J.G. and A.A. carried out the chemical and the antimicrobial experiments and have equally contributed to the realization of the research. J. G., A.A., C.F., F.M.N., G.M. and M.J.S. wrote the manuscript. C.F., G.M. and M.J.S. proposed the subject, designed and supervised the antimicrobial study. F.M.N. designed and supervised the chemical study. All authors reviewed and contributed to the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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