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Evaluation of antibacterial activity of *Lentinula edodes* gemmotherapic extract

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Abstract In this paper, it is presented the investigation of the antibacterial activity of a gemmotherapic extract of *Lentinula edodes* (Berk) Pegler, against 5 species of bacteria, three Gram-positive bacteria: *Bacillus subtilis, Bacillus cereus* var. *mycoides* and *Streptococcus faecalis* and two Gram-negative bacteria: *Pseudomonas aeruginosa* and *Serratia marcescens* using the well diffusion assay. The extract used was made from young shoots of *L. edodes*, in according to the classic gemmotherapic principles. The result revealed that the extract had a very strong inhibitory activity against *Bacillus subtilis* and *Bacillus cereus* var. *mycoides* but little or no effect on the other species at a concentration of 50mg/mL and 5mg/mL. The results indicate that *L. edodes* active compounds can be used to develop antibacterial agents against *Bacillus subtilis.*

Key words

antibacterial activity, *Lentinula edodes*, gemmotherapic extract, well diffusion assay

Infectious diseases caused by microbial agents are one of the most important problem in the last decade. The overuse of antibiotics has led to a development and increase of resistance of bacteria and fungi to antibiotics. Classic antibiotics had also other side effects such as immunosuppression among others.

As a consequence, a new promising area of research gained importance, to develop a new type of antibiotics which would be plant and fungal based. For this purpose, many plant species were screened for antimicrobial properties [5, 7].

Among different techniques used to extract new compounds, the gemmotherapy has been used with moderate success.

Gemmotherapy originally used only buds ("gemma" meaning bud) but in recent years, it has extended in using a wide array of embryonic tissues and thus changing its name to Plant Stem Cell Therapy. In the same way as Gemmotherapy, it uses a wide variety of embryonic plant parts, collected in the spring at a critical stage in the plants growth when much of the plants energy is directed to the growing areas. As a technique, is an important subsection of phytotherapy [10].

Recent analysis with HPTLC on compounds extracted from vegetal meristematic tissues showed that the content in active compounds is higher [8].

These findinds confirm older studies which showed that gemmotherapic extracts are rich in active compounds [15].

Classic gemmotherapic extracts are prepared according to gemmotherapic principles from the French Pharmacopoeia, (as cited in [8]), which consists in maceration of plant buds with equal thirds of water, alcohol and glycerin.

In the search for new compounds, macrofungi have also been screened with good results. They have long been used as a valuable food source and as traditional medicines around the world since ancient times, especially in Japan and China. Amongst the sources of natural compounds, the fungi, especially the basidiomycetes, have stimulated interest from investigators.

Shiitake mushroom, the common Japanese name for *Lentinula edodes* (Berk) Pegler derives from the mushroom associated with the shii tree (Castanopsis cuspidate Schottky) and take, the Japanese word for mushroom. Japan is the world leader in production of this type of mushroom, is one of the five most cultivated edible mushrooms in the world.

L. edodes has been shown to produce a variety of degradative extracellular enzymes, including cellulases, manganese peroxidases [16]. This has interested researchers due to its medicinal properties, and several biologically active compounds have been isolated and purified from the mushrooms, mycelia and aqueous extract [2].

These compounds exhibit antitumor, antifungal, antibacterial, hypoglycemic and antioxidant properties [6]. Recent studies have also confirmed the antiviral properties [14]. Among these compounds, some of the best characterized are lentinan and lentin. The antimicrobial activity of *L. edodes* has been confirmed against the Gram-positive and Gram-negative Bacteria, [2].

In this study, we will test a gemmotherapic extract from young parts of *L. edodes* based on the principle

that gemmotherapic extracts have a more intense inhibitory activity compared with the traditional extracts. This is a novel approach and to the author's knowledge, has never been used on *L. edodes*.

Materials and Methods

Fungal material collection

Strains of *L. edodes* were cultivated in a greenhouse at the University of Agricultural Sciences in Timisoara. Young parts were collected from very young mushrooms and put immediately in ethanol of 96% concentration.

Preparation of gemmotherapic extracts

The solutions were made with equal thirds of alcohol, glycerol and distilled water. 5 g of fresh buds were collected, cleaned, washed with distilled water and grounded. The obtained material was subjected to maceration for 4-5 days in 25-50 g of ethanol of 90^0 concentration. Equal amounts of water and glycerol were then added, in a ratio of 1:1 until a final ratio of 1:20 between the mushroom tissue and solvent was reached [4]. The process of extraction took place for three weeks in a dark place at 10^0C, using an orbital shaker. The extract was then filtered, concentrated by using a rotavap and weighted. The dry material was diluted for the tests and filtered through a sterile membrane filter. Two concentrations were tested: one of 50 mg/mL and the second, of 1:10 dilution factor solution.

Bacteria counting

The bacteria were counted using a Burker chamber. The values are shown in (Table 1).

Table 1

The Burker chamber count data

Bacteria species	Number of cells/ml
Pseudomonas aeruginosa	$1.6x10^8$
Serratia marcescens	$1.7x10^8$
Bacillus subtilis	$8x10^7$
Bacillus cereus var. *mycoides*	$15x10^5$
Streptococcus faecalis	$2.2x10^8$

Microorganisms

The bacteria used in this experiment are three Gram-positive bacteria: *Bacillus subtilis*, *Bacillus cereus* var. *mycoides* and *Streptococcus faecalis* and two Gram-negative bacteria: *Pseudomonas aeruginosa* and *Serratia marcescens.* The bacterial cultures were grown in Mueller Hinton Agar (Merck) and Mueller Hinton Broth (Merck).

Well diffusion assay and antibacterial activity

The antibacterial activity was determined using the hole in plate assay procedure [11]. All bacterial cultures were maintained on nutrient agar slants at temperature of 4^0C and sub cultured onto nutrient agar broth for 24 hours prior to testing. The pure cultures of the microorganisms were inoculated onto Muller-Hilton nutrient broth incubated at temperature of 37^0C for 24 hours. 25 ml of nutrient agar was poured into the 100 mm plate, with an even depth of 4 mm on a level surface shaken and allowed to cool.

The nutrient agar plates were seeded with 0.1 ml of standardized inoculums of each of the five test organisms. The inoculum was spread evenly over plate with a sterile glass spreader. Using a sterile cork-borer of 5 mm diameter, three holes were made into the Petri dishes seeded with bacterial culture. The bottoms of the holes were sealed with agar to avoid seepage. 50µl of extracts were introduced in the wells, using a micro liter syringe. Concentrations of 5 and 50 mg/mL extracts were reconstituted in distilled water and transferred into the wells. The plates were kept for 30 min at room temperature to allow diffusion of the extract, and then were incubated at temperature of 37^0C for 24 hours. After the incubation period, the zones of inhibition were measured using a digital caliper. In this study, the measurement is taken including the 5 mm diameter of the hole. The diameter of the zone of inhibition was measured at three different angles and the mean of those measurements was taken. Antibacterial activity was recorded when the zone of inhibition was greater than 6 mm.

Studies were performed in triplicates and the mean value was calculated. A solution of only alcohol, glycerol and water in equal ratios was used as a negative reference.

Statistical analysis

Data were averages of three results ± Standard Deviations (SD) by using Microsoft Excel.

Results and Discussions

In (Table 2) are shown the mean zones of inhibition measured after 24 hours and in the (Figure 1) is shown the graph with the measurements. The 1:10 dilution factor solution presents little or no visible inhibitory effect, most likely because the concentration here is too low as shown in the (Figure 2). All values were expressed as means ± standard error means.

From the measurements we obtained, it can be observed that *B. subtilis* and *Bacillus cereus* var. *mycoides* presented the highest sensitivity, the lowest being the *Pseudomonas aeruginosa*. Almost all bacteria species tested are susceptible to the extract, at 50 mg/mL concentration and intermediate at 5 mg/mL. The diameter of the zones of inhibition approximately

doubles at a tenfold concentration in some cases as it can be seen from Figure 2.
These results are similar with those reported by Hearst [3] and Rao [13]. Their results using disk diffusion method showed that Shiitake mushroom extract had extensive antimicrobial activity against 85% of the organisms it was tested on, including 50% of the yeast and mold species tested.

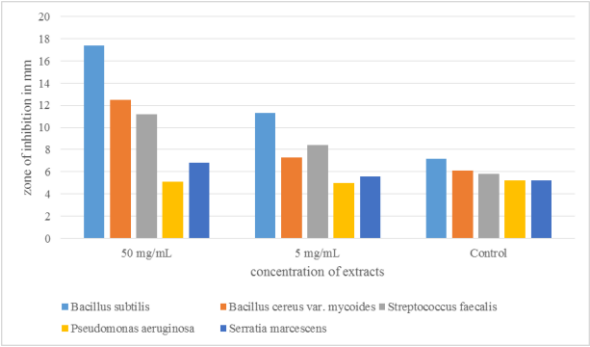


Fig. 1. Antimicrobial activity of *Lentinula edodes* solutions and the control, zone of inhibition in mm

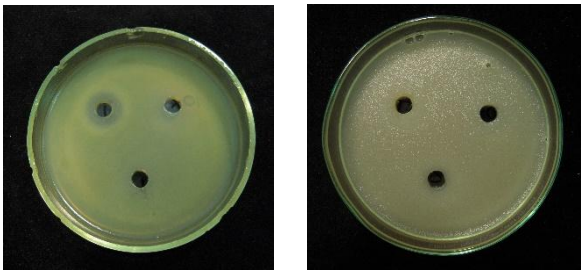
Casaril, Kasuya, & Vanetti, in [1] also showed that aqueous extracts from *L. edodes* inhibit Gram-positive and Gram-negative bacteria including *B. subtilis*.
Although similar tests were performed with slightly different types of cork borers with diameter ranging from 5 to 8 mm, different extract volumes and different concentrations, the results from the present study, confirms previous findings made on crude alcoholic and water extracts of *L. edodes*.

Table 2

Antimicrobial activity of *L. edodes* by well diffusion method after 24 hours. Measurement taken including the 5 mm diameter of the hole

Bacteria/Zone of inhibition in mm*	50mg/ml	5mg/ml	Control
Bacillus subtilis	17.4±0.2	11.3±0.2	7.2±0.2
Bacillus cereus var. *mycoides*	12.5±0.3	7.3±0.2	6.1±0.2
Streptococcus faecalis	11.2±0.2	8.4±0.4	5.8±0.4
Pseudomonas aeruginosa	5.1±0.3	5.0±0.2	5.2±0.2
Serratia marcescens	6.8±0.2	5.6±0.2	5.2±0.2

In another study, Kitzberger, in [6] tested polar and non-polar solvent supercritical extracts of *L. edodes* on bacteria and fungi using the agar diffusion method and determined the MIC through the microdilution method. Through the agar diffusion test, with a 7 mm diameter holes, he obtained a 12 mm halo inhibition zone for *B. cereus*. The MIC value obtained was 0.25 mg/mL for *B. cereus*. The data in this study, indicate that Gram-positive bacteria were more susceptible to inhibition as compared to Gram-negative bacteria. This finding confirms numerous previous similar reports regarding this aspect [17, 12].
The use of antibiotics has reduced the incidence of infectious diseases but their extensive uses in therapy, has led to the appearance of drug-resistant bacteria [9], which is a major public health issue worldwide. For this purpose, numerous plant and fungal extracts were screened for antimicrobial properties that could protect people from microbial infections.
The fungal extracts can also be used in combination with traditional antibiotics. In the literature, there are reports regarding the use of fungal crude extracts in combination with fewer amounts of antibiotics for anti-bacterial activities, especially for antibiotic-resistant bacteria, compared to antibiotics alone.



Pseudomonas aeruginosa *Serratia marcescens*

Bacillus subtilis *B. cereus var. mycoides*

Figure 2. The zones of inhibition for the extracts of *M. charantia* at 50 mg/ml (left), 1:10 dilution (right) and control (bottom), for *Pseudomonas aeruginosa*, *Serratia marcescens*, *Bacillus subtilis* and *Bacillus cereus* var. *mycoides*.

Conclusions

In according with our experimental results, it can be concluded that the gemmotherapic extract from *Lentinula edodes* has a great potential in the development of more potent and efficient antimicrobial agents.

Also, the gemmotherapic extraction technique presents several advantages, most important being the ability to extract a broader range of active compounds. Their biological activity is further enhanced by possible synergistic interactions.
Further studies are required in order to identify the active components from *L. edodes* and to study the possible synergistic interactions.

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