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Macrofungal Extracts on the Bacteria Inhibition of *Bacillus subtilis*

Amruta Ponugupati
Mills E. Godwin High School, USA
2101 Pump Road, 23238, Henrico, Virginia, USA

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Macrofungus has been utilized as a medicinal remedy since ancient times. There has not been abundant research on its medicinal uses in comparison to other species of edible mushrooms, but studying its effects could lead to a global contribution regarding its therapeutic application. The purpose of this study was to determine the varying effect of mushroom extracts on antimicrobial properties against *Bacillus subtilis*. It was hypothesized if different edible mushroom extracts are treated to *B. subtilis*, then *Lentinula edodes* extract will show the greatest antibacterial activity with the largest clear zone diameter in millimeters. Determining which mushroom species exhibits the greatest inhibitory effect on bacteria can lead to the identification of alternative compounds to substitute the current ineffective antibacterial products. Prepared extracts from *Agaricus bisporus*, *Plureotus ostreatus*, and *Lentinus edodes* species were placed into wells of Luria Broth agar petri dishes that had been incubated with 5 day old *Bacillus subtilis*. After a 48 hour incubation period, the research hypothesis was supported as *L. edodes* had the largest mean clear zone diameter. Statistical analysis of the results revealed the calculated t-value for *L. edodes* vs. *P. ostreatus*, *L. edodes vs. A. bisporus,* and the mushroom levels vs. the control were all are significant, except for *P. ostreatus* vs. *A. bisporus*. It is believed that the presence of lenthionine, eritadenine, and lentinan, gave *L. edodes* its superior antibacterial properties. Unidentified protein content may have contributed to other mushroom species' antimicrobial activity. The unknown compounds from the mushrooms could be isolated and, with further study, be of benefit for humans with the development of new antibiotics to combat bacterial resistance to medication.

Keywords: mushrooms, bacteria, antibiotic resistance, antibacterial

INTRODUCTION

Macrofungi has been utilized as a medicinal remedy in tradition since ancient times. Several edible mushrooms, having been already used with herbal folk medicine, have also demonstrated anti-proliferative, immunomodulatory, antiviral, antifungal, and antibacterial effects. While the notion of mushrooms carrying antimicrobial properties has been recognized, there has not been abundant research on its medicinal uses in comparison to other species of edible mushrooms. By studying the globally popular edible mushrooms for antibacterial activity, a contribution of potential therapeutic applications could be supported. Mor

specifically, the antibacterial properties of various mushroom extracts could lead to progressions in understanding the compounds that enable such activity. The field of applied biotechnology identifies edible macrofungus as having a great potential to agriculture and medical advancements. (Bisen, 2010).

Edible mushroom extracts have been seen as a competitor to common synthetic medicines, in respect to their potential antibacterial properties. (Tehrani, 2012).The natural antibiotics produced by mushrooms have been the subject of many recent studies that have been testing their efficacy on gram negative and gram positive bacteria. Society can benefit from acknowledging the utility of mushrooms as a natural source of antibacterial agents, due to the increasing number of bacteria becoming resistant to commercial antibiotics. There is very little information about the antimicrobial and antioxidant activities of wild edible mushrooms. By studying the antimicrobial effects of an edible mushroom, compounds can be isolated from the fruiting body to determine the origination of the mushroom's antibacterial activities. Determining which mushroom species exhibits the greatest inhibitory effect on bacteria can lead to the identification of specific and alternative compounds to substitute the current antibacterial products, which have been ineffective by bacterial resistance (Sonawane, 2012). This may lead to progressions in understanding antimicrobial properties and assist with the implementation of antibiotics medically.

Wild mushrooms are rich in carbohydrates and protein. Proteins found in these fungi have shown several biological activities that prevent health disorders. Edible mushrooms are low in fat, have a high percentage of polyunsaturated fatty acids and also contain many vitamins and minerals. In relevance to their role in medicine, antibiotics in mushrooms are not as well documented, so the discovery of new antimicrobial agents is still highly possible (Thallaimaharani, 2013). Most mushroom extracts have exhibited antibacterial activity to multiple strains of bacteria and have been used medicinally for centuries. *Lentinula edodes* has proven pharmacological properties, including an anti-tumor effect, antimicrobial properties, improved liver function and a reduction of viremia chronic hepatitis B patients, and the inhibition of human immunodeficiency virus infection in *vitro* (Hirasawa, 1999). *Pluerotus ostreatus* and *Lentinula edodes* have been recognized as the most active species in regard to antimicrobial activity. (Bisen, 2010). Studies have shown that the isolated proteins from the mushroom, *Agaricus bisporus* was effective against both gram negative and gram positive bacteria, such as Staphylococcus aureus (Tehrani, 2012).

As a gram positive bacteria, *B. subtilis* is known for its strong endospore which gives the organism the ability to sustain in extreme environments. The dependent variable will be measured by the diameter of the inhibition zone in millimeters around each well filled with a mushroom extract. In *vitro,* the agar diffusion method can determine antibacterial activity.

The purpose of this experiment is to determine which aqueous extract of edible mushroom will exhibit the greatest antimicrobial effects when treated against *Bacillus subtilis*. The independent variable in this experiment are the types of edible mushroom extracts, *Lentinula edodes*, *Agaricus bisporus*, and *Pluerotus ostreatus*. All of these mushroom extracts have shown antibacterial properties, but to varying degrees (Sonawane, 2012). The dependent variable in this experiment will be the antibacterial activity of each mushroom extract, as observed by measuring the zone of inhibition in millimeters. It is hypothesized that if different edible mushroom extracts are treated to *B. subtilis*, then *Lentinula edodes* will show the greatest antibacterial activity with the largest clear zone diameter in millimeters. Scientific investigations have led to isolation of many compounds from *L. edodes* having health promotion activities. Specifically, *L. edodes* contains the polysaccharide Lentinan, which is crucial for the biological and pharmacological activity of the mushroom.

Based on previous studies, *L. edodes* has been observed to have higher antibacterial effectiveness because of this substance. There is no control for the experiment as the sole purpose is to determine which macrofungal extract is the most effective against the already grown bacteria. Without a mushroom extract or bacteria, there will be no inhibition to observe.

PROCEDURE

While wearing safety gloves, 500 mL of liquid *Bacillus subtilis* culture was brought to the Vertical Laminar fume hood for inoculation onto already prepared Luria broth agar plates. One mL of *B. subtilis* was taken from the beaker with a micropipette and was poured and spread onto one large agar plate. The plate was then allowed to sit in an incubator for five days. This step was repeated four other times for a total of five LB agar plates cultured with bacteria. In the meantime, other preparations regarding mushroom extracts were pursued.

For mushroom extract preparation, all 2.5 kilograms of *Pluerotus ostreatus* were washed with distilled water and cut into small pieces at a biosafety level 2 research laboratory. The pieces were then placed in an Omega VRT400HDS Juice extractor to successfully separate the pulp from the extract of the mushroom. As the extract was being made, it was poured into one large 250 mL beaker stored in 1 kg of ice until the juicer was done creating the extract. The final extract, in liquid form, was then transferred into several plastic test tubes to be filtered and sterilized. A disposable syringe with a filtration mechanism was then used to transfer the mushroom extract from the large plastic test tubes into 15 smaller test tubes that held a maximum of 2 mL by simply extracting the liquid with the syringe with a built in filter, and placing it in the other small test tubes. Because not all of the extract was needed to fill fifteen 2 mL test tubes, the remainder of the extract from the original plastic test tubes were stored in a freezer of -20 * C for future or potential use.

After the 15 sample extracts of *Pluerotus ostreatus* were sterilized and placed into the 2 mL sample test tubes, they were brought over to the vertical laminar fume hood for inoculation onto the bacterial plates. All five bacterial plates, which had been resting after 5 days of bacterial growth, had 12 wells punctured into them by a hole puncher. Then using another micropipette, 250 micro-milliliters had been taken from the small test tubes, and inserted into one hole of the agar plate. A total of only four holes were filled on each agar plate until moving onto the other 4 petri dishes. These steps were repeated for the remaining mushroom levels of *L. edodes* and *A.bisporus*. After all mushroom extracts were placed in their correct wells on each agar plate, the petri dishes were sealed and placed in an incubator for 48 hours before being taken out to measure the inhibition zone. The data was then recorded and analyzed for statistical significance.

RESULTS

The effects of various mushroom extracts on its antibacterial properties were studied within petri dishes grown with *Bacillus subtilis* and the final results of the inhibition zone diameter in millimeters can be seen in table 1 and 2 along with graph 1. The research hypothesis formulated stated that the *Lentinula edodes* mushroom extract would have the greatest antimicrobial effect against *B. subtilis* by having the largest clear zone diameter. The mean of each mushroom extract's clear zone was determined with *Lentinus edodes* having the largest mean clear zone diameter of 2.0 mm. *Pluerotus ostreatus* had the second largest mean diameter (1.6 mm) and *Agaricus bisporus* had the smallest (1.8 mm). The control group of no extract had no observed inhibition (0 mm).The comparisons between each type of mushroom extract on their antimicrobial properties revealed that there was an

effect on inhibiting *B. subtilis*. Because of this, the research hypothesis was supported as *Lentinus edodes* had the largest zone of inhibition, but it was also noted that all macrofungal extracts had an effect on inhibiting microbial activity. The variance and standard deviation, calculated from all sets, were very low for all levels of the independent variable which indicated a close set of data. There were outliers present in the data, as every mushroom extract had data points outside the calculated 2SD range. The minimum value of 1.4 mm for *Lentinus edodes* was an outlier, and 2.6 mm was also an outlier as the maximum value for both *Pluerotus ostreatus* and *Agaricus bisporus.*

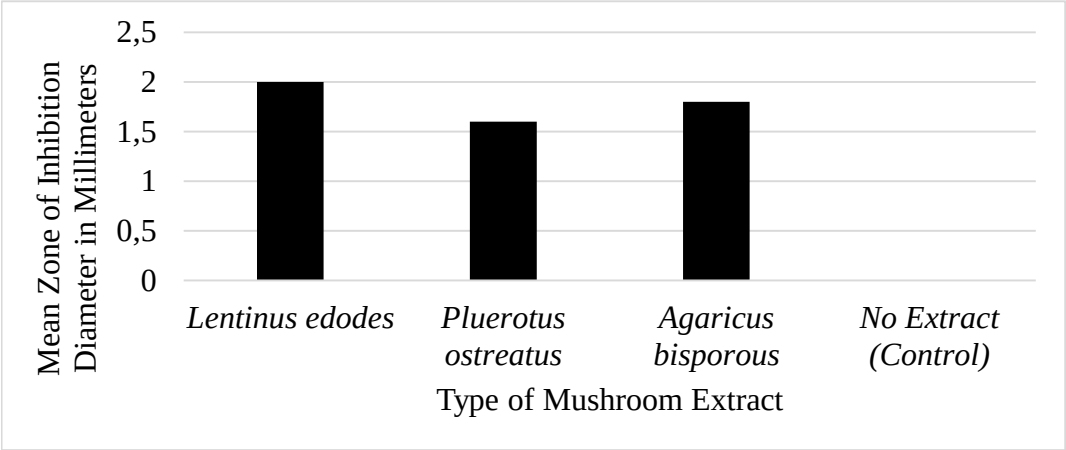
Multiple t-tests were performed on the data, with a level of significance of 0.05 and degrees of freedom of 38. The null hypothesis stated that there would be no significant difference between each type of mushroom extract and its antibacterial activity. The calculated t-value for *L. edodes* vs. *P. ostreatus* of 4.276 and the calculated t value for *L. edodes vs. A. bisporus* of 2.965 were both greater than the critical t-value of 2.024, ($p < 0.05$) meaning the results were significant and that the null hypothesis should be rejected. The t-test performed between the control and *L. edodes* had a calculated t-value of 36.214 ($p < 0.05$), and was greater than the critical, indicating that it was also significant. No Extract vs. *Pluerotus ostreatus* produced a value of 21.193 ($p < 0.05$) and data was also statistically significant.

No Extract vs. *Agaricus bisporus* t-test produced a value of 20.647 ($p <0.05$). The null hypothesis was rejected and the data indicated that the probability of the results being due to chance or error was less than 1 in 20 for all of these t-tests. The t test *P. ostreatus* vs. *A. bisporus* produced a value of 1.734 which was less than the critical t-value ($p > 0.05$), indicating that the null hypothesis should not be rejected, and that the probability of the results being due to chance or error is greater than 1 in 20. The results were significant for all of the independent variable data sets except for *Agaricus bisporus* vs. *Pluerotus ostreatus*. Overall, the data results were mostly all statistically significant, except for *Agaricus bisporus* vs. *Pluerotus ostreatus*, which implies that the type of mushroom extract does have a significant effect on clear zone diameter against *B. subtilis* in comparison to another extract and the control.

Table 1. The effect of type of mushroom extract on diameter of zone of inhibition in millimeters

Trial	**Diameter of Clear Zone in Millimeters (mm)**		
	Lentinus edodes	*Pluerotus ostreatus*	*Agaricus bisporus*
1	1.7	1.6	1.8
2	1.9	1.8	1.6
3	1.7	1.6	1.3
4	1.8	1.2	1.8
5	2	1.2	1.7
6	1.9	0.9	1.9
7	2	1.5	1.7
8	2.2	1.6	2.3
9	2.2	1.6	1.4
10	1.8	1.9	2
11	2.1	1.5	1.4
12	2.2	1.4	1.1
13	2.4	1.7	2.2

14	1.9	1.6	1.8
15	2.1	1.4	2
16	1.4	1.5	1.5
17	1.8	2.6	2.6
18	2.1	1.3	2
19	2.2	1.6	1.7
20	1.6	1.8	2.5
Mean	2.0	1.6	1.8



Graph 1. The effect of type of mushroom extract on the zone of inhibition diameter against *B. subtilis* in millimeters

Table 2. Statistical analysis on the effect of mushroom extract on diameter of inhibition zone against *B. subtilis* in millimeters

Descriptive Information	**Type of Mushroom Extract**			
	Lentinus edodes	*Pluerotus ostreatus*	*Agaricus bisporus*	No Extract
Mean	2.0 mm	1.6 mm	1.8 mm	0 mm
Range	1.0 mm	1.5 mm	1.5 mm	0 mm
Maximum	2.4 mm	2.6 mm	2.6 mm	0 mm
Minimum	1.4 mm	0.9 mm	1.1 mm	0 mm
Variance	0.061	0.114	0.152	0 mm
Standard Deviation	0.264	0.338	0.390	0 mm
1SD	1.736- 2.264	1.262- 1.938	1.410- 2.190	0.000-0.000
2SD	1.472- 2.528	0.924-2.276	1.020-2.580	0.000-0.000
3SD	1.208- 2.792	0.586- 2.614	0.630-2.970	0.000-0.000
Number	20	20	20	20
Results of t-test	*Lentinus edodes* vs. *Pluerotus ostreatus* t= 4.276 p < 0.05 *Lentinus edodes* vs. *Agaricus bisporus* t = 2.965 p < 0.05 *Agaricus bisporus* vs. *Pluerotus ostreatus* t = 1.734 p> 0.05 No Extract vs. *Lentinus edodes* t = 36.214 p < 0.05 No Extract vs. *Pluerotus ostreatus* t = 21.193 p < 0.05 No Extract vs. *Agaricus bisporus* t= 20.647 p < 0.05 At df= 38, a = 0 .05; t = 2.024 for significance			

CONCLUSIONS

The purpose of this experiment was to determine the effects of various fungal extracts on antibacterial properties against *Bacillus subtilis* using the disk diffusion testing method. To study the potential pharmacological activities of macrofungi, three different extracts were produced from *Lentinus edodes, Pluerotus ostreatus,* and *Agaricus bisporus.* These extracts were placed into well holes from petri dishes already grown with *B. subtilis* and incubated for 48 hours to observe the zone of inhibition, in addition to a control group of petri dishes with no extract added. The formulated research hypothesis stated if *L. edodes* extract was used against the growth of *B. subtilis*, it would have the greatest antimicrobial effect. The *Lentinus edodes* extracts had the largest mean clear zone diameter (2.0 mm) and *Pluerotus ostreatus* had the smallest clear zone (1.6 mm). These results supported the research hypothesis as *L. edodes* exhibited the greatest antibacterial properties, and demonstrated that different mushroom extracts have an effect on bacterial resistance.

After performing statistical analysis, the data was revealed to be significant for all of the independent levels except those regarding *Agaricus bisporus* vs. *Pluerotus ostreatus*. The t-tests performed for *Lentinus edodes* vs*. Pluerotus ostreatus, Lentinus edodes* vs*. Agaricus bisporus* had a 1 in 20 probability that the results were most likely due to the independent variable ($p < 0.05$). The t-tests between the control and all mushroom extracts were also significant, indicating the 1 in 20 probability of the results being due to the independent variable, and that these levels have an antibacterial effect. The t-test for *Agaricus bisporus vs. Pluerotus ostreatus* indicated the data to be insignificant, thus the 1 in 20 chance the results were due to chance or error. ($p > 0.05$). Overall, the results denote that the type of mushroom extract has a significant effect on the antibacterial activity against *B. subtilis*.

Other experiments have investigated the effects of various macrofungal extracts on their potential antibiotic properties. *Lentinus edodes*, or the Shiitake mushroom, exhibited a greater antimicrobial effect against gram positive than gram negative bacteria (Bisen, 2012), with *Bacillus subtilis* and *Staphylococcus aureus* as the most highly inhibited bacteria, along with *E. coli* K-12 (Casaril, 2011). Other experiments studied the antibacterial effects of *L. edodes* versus *Pluerotus ostreatus*. Data quantitatively showed that the strong Shiitake mushroom extract had extensive antimicrobial activity against 85% of the organisms it was tested on, which included 29 bacterial and 10 fungal pathogens for the demonstration of microbial inhibition. This compared favorably with the results from both the positive control Ciprofloxacin, a commercial antibiotic, and *P. ostreatus*, in terms of the number of species inhibited by the activity of the metabolites integral to the Shiitake mushroom (Hearst, 2009). When culture extracts of *Agaricus bisporus* were assayed *in-vitro* for the antibacterial effects, inhibitory action on gram and gram negative bacterial growth was achieved. In the experiment, *E.coli*, *Enterobacter aerogens*, *Klebsiella pneumoniae* were the most sensitive bacteria amongst the tested microorganisms by *A. bisporus.* When the protein concentration of the mushroom was reduced, bacterial growth increased in all subjects except for *B. subtilis* (Tehrani, 2012). One experiment showed that the most active species was *P. ostreatus*, as it demonstrated broad- spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria. In addition, *P. ostreatus* demonstrated strong activity against *Mycobacterium aurum, Staphylococcus aureus, Streptococcus Sp., Acinetobacter calcoaceticus* and *Klebsiella oxytoca.*

The results between the type of macrofungal extract and bactericidal properties were predominantly statistically significant, as there was observed inhibition on resting the *B. subtilis* cultures, most prominently by the *Lentinus edodes* extract.

Different mushroom species possess different constituents and in varied concentration, which accounts for the distinction in antimicrobial effects. In other experiments, microbial analyses revealed that the chloroform and ethylacetate extracts of Shiitake mushroom showed stronger inhibitory activity compared to the aqueous extract. The main components of chloroform, ethylacetate, and aqueous extracts of *L. edodes* may be lenthionine, disulphide derivative, eritadenine, and lentinan, which indicates that the presence of these substances resulted in its inhibitory activity. Lentinan is an activator for macrophage T-lymphocytes and other immune effector cells and may be responsible for *L. edodes'* observable, yet indirect antitumour and antimicrobial properties (Rao, 2008). Antiproliferative properties of *A. bisporus* was also due to lectin, and studies show that at least a portion of the antimicrobial properties belongs to its protein contents. Because *MRSA,* Methicillin-resistant *S. aureus*, showed inhibitory action against *A. bisporus*, this mushroom also represents a medically prominent standpoint for new identification of antibacterial compounds (Tehrani, 2012). *P. ostreatus* also demonstrated inhibitory action against most bacteria, but there is little information about the mycelia of the species, which could eventually be attained with the purification of the biological active components from the mycelia crude extracts of Pluerotus spp. (Kalyoncu, 2010). It is known that several macrofungi such as *Ganoderma, Lentinus, and Pleurotus*, produce bioactive metabolites, but the biologic activity of most species have not been previously studied. Overall, while it is not specifically known which substances in each mushroom provide its antibacterial properties, it is widely acknowledged that both the fruiting body and mycelium of each mushroom contain compounds with wide-ranging antimicrobial activity. Their compounds could be isolated from many edible mushrooms species and, with further study, could be of benefit for humans. By doing so, alternative compounds may substitute the current antibacterial products which are ineffective by the bacterial resistance.

Continued study could involve the direct isolation and identification of active compounds in mushrooms to determine which substance possesses the antibacterial property.

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APPENDIX

EDD

Title: The Effect of Macrofungal Extract on Antimicrobial Properties against *Bacillus subtilis*

Hypothesis: If different edible mushroom extracts are treated to B. subtilis, then *Lentinula edodes* will show the greatest antibacterial activity with the largest clear zone diameter in millimeters.

Independent Variable: Type of Mushroom Extract		
Lentinus edodes	*Pluerotus ostreatus*	*Agaricus bisporus*
20	20	20

Dependent Variable: Diameter of Inhibition Zone on Petri dish

Constants: Type of bacteria used, amount of extract placed in each well in petri dish, time for incubation growth, amount of bacteria spread on petri dish, size of wells/holes in petri dish, size of petri dish, number of holes on each size of petri dish, incubation temperature and location.

