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Research Article

Antibacterial Properties of Ethanolic Extract of Mushrooms Sold in Malaysian Local Market

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Abstract: Mushroom contains a number of bioactive compounds that act as antibacterial agents, which can inhibit the pathogenic microorganisms. The antibacterial properties in the mushroom could be an alternative to the existing antibacterial medication. Different types of mushrooms have different bioactive compounds. Thus, the antibacterial properties of different types of mushroom against selected bacteria were tested. The types of mushrooms that were used in this experiment were *Agaricus bisporus*, *Flammulina velutipes*, *Lentinula edodes* and *Pleurotus ostreatus*. The mushrooms were extracted by using 95% ethanol. The ethanolic extracts of the four types of mushroom were tested against Gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and Gram-negative bacteria (*Salmonella typhimurium* and *Escherichia coli*). The antibacterial activity was determined by microdilution assay. The results showed that the ethanolic extract of *F. velutipes* had the highest inhibitory activity against all tested bacteria while the least active was the ethanolic extract of *P. ostreatus*. The MIC value for all ethanolic extracts ranged from 500 mg/ml to 1000 mg/ml. It was also found that *S. aureus* was the most susceptible bacteria when being tested with ethanolic extracts of mushroom. The achieved results showed that the antibacterial activities of ethanolic mushroom extracts depend on the type of mushroom and the type of bacteria tested.

Keywords: Mushrooms, antibacterial activity, ethanolic extract, microdilution assay, Minimum Inhibitory Concentration (MIC).

INTRODUCTION

Mushrooms have been widely consumed as delicacy and food. There are edible and inedible mushrooms, based on their toxicity. Edible mushrooms are widely consumed by different countries since a long time ago. One of the most common edible mushrooms is white button mushroom (*Agaricus bisporus*). Other examples of mushrooms that can be consumed are oyster mushroom (*Pleurotus ostreatus*), enoki mushroom (*Flammulina velutipes*), shiitake mushroom (*Lentinula edodes*), and more. Beside their distinct flavour, mushrooms are also eaten because of their nutritional value. Rezaeian and Pourianfar (2016) had stated that edible mushrooms contain good nutrients such as essential oils, fiber, protein, minerals, vitamins, lectins, and important bioactive compounds. The bioactive compounds found in mushroom include terpenoids, phenols, steroids, nucleotides with their derivatives, polysaccharides, and glycoproteins (Miles & Chang, 1997). These bioactive compounds or metabolites available in plants are claimed to have health promoting properties such as antimicrobial, antioxidant, anticancer, cholesterol lowering and immunostimulatory in certain mushrooms (Kosanic *et al.*, 2013).

As mushroom can are easily grown, they need antibacterial and antifungal properties to survive (Yamac & Bilgili, 2008). A study done by Mirfat *et al.*, (2014) showed that mushroom extract has antibacterial activity which able prevent the growth of wide range of bacteria. In their study, bioactive compounds present in *Schizophyllum commune*, a type of mushroom, were extracted separately using four different solvents, which were methanol, ethyl acetate, dichloromethane and water. The compounds obtained were then used to observe their inhibitory effect on the growth of Gram-positive bacteria (*Bacillus cereus*, *B. subtilis*, *Enterobacter faecalis* and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Plesiomonas*

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shigelloides, *Pseudomonas aeuroginosa*, *Proteus vulgaris*, *Salmonella* sp., *S. typhi*, *Shigella* sp., *S. flexneri*, *Streptococcus mitis*, *S. mutans*, and *S. sanguis*). Their results showed the dichloromethane extract of *S. commune* had higher antibacterial activity than other extracts. The study also described that Gram-positive bacteria were more susceptible to the bioactive compound resulting in larger inhibition area compared to Gram-negative bacteria.

MATERIALS AND METHODS

Preparation of Media

Mueller-Hinton Agar (MHA) was prepared by dissolving 38.0 g of MHA powder (OXOID) in 1 L of distilled water, followed by autoclaving at 121 ºC for 30 minutes. The sterilized agar liquid was set to warm before pouring into sterile petri dishes. The agar was then allowed to solidify and stored inversely at 4 ºC until further use.

For Mueller-Hinton Broth (MHB) preparation, 21.0 g of MHB powder (OXOID) was dissolved in 1 L of distilled water, followed by autoclaving at 121 ºC for 30 minutes. The broth was then stored at 4 ºC until further use.

BACTERIAL CULTURE

Two Gram-positive bacteria, which were *Staphylococcus aureus* and *Bacillus cereus*, and two Gram-negative bacteria, *Escherichia coli*, and *Salmonella typhimurium* were used in this study. The bacteria were obtained from Microbiology Laboratory of Faculty of Science and Technology in Universiti Sains Islam Malaysia (USIM), Nilai. Each bacterial strain was grown from stock culture by streaking them on nutrient agar (NA) and incubated at 37 ºC for 16 to 18 hours (overnight). Then, a single colony of each bacteria was cultured overnight in Mueller-Hinton broth (MHB) at 37 ºC.

SAMPLE PREPARATION

In this study, four types of edible mushroom were used, which were *A. bisporus*, *F. velutipes*, *L. edodes*, and *P. ostreatus*. The mushroom samples were bought at hypermarket in Nilai, Negeri Sembilan. The mushrooms were then washed thoroughly with distilled water. Each sample were cut into small pieces and dried in a drying oven at 60 ºC for 24 hours. The dried mushrooms were grounded into fine powder by using a heavy duty grinder prior to extraction.

SAMPLE EXTRACTION

Mushrooms were extracted by using method described by Hleba *et al.*, (2016) with some modifications. About 40 g of powdered mushrooms were soaked in 400 ml of ethanol (1:10; w/v) in a conical flask respectively. The solutions were left at room temperature for 48 hours. After 48 hours, the samples were filtered by using Whatman No. 1 filter paper through Buchner funnel and the filtrates were evaporated by using rotary evaporator at 50 ºC to get the crude extract. The crude extracts were dissolved in dimethyl sulfoxide (DMSO) as stock solution and stored at 4 ºC in refrigerator until further use. The stock solutions were prepared at the concentration of 1000 mg/ml, 3000 mg/ml, and 5000 mg/ml.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) *VIA* MICRODILUTION METHOD

MIC of the mushroom extracts were determined by microdilution method where sterile 96-well microtitre plates were used (Sarker *et al.*, 2007). A 50 µl of Mueller Hinton broth (MHB) were added first into each well of microtitre plate. Next, 50 µl of test samples with different concentrations (5000 mg/ml, 3000 mg/ml, and 1000 mg/ml) were loaded into each well. A 50 µl of streptomycin was used asPositive control while 50 µl of blank MHB as negative control. Then, 50 µl of overnight bacterial inoculums, which were adjusted to the concentration of 0.5 McFarland, were added into each well. The microtitre plates were then incubated at 37 ºC for 24 hours. Table 3.5 was included to help in visualizing the method. Bacterial growth was indicated by the turbidity of the solution. Then, the MIC was determined by observing the lowest concentration that is non-turbid, which indicates no bacterial growth. The observation of turbidity or bacterial growth was done in two ways; visual assessment and spectrophotometric assessment. Visual assessment was done with the aid of Thiazolyl Blue Tetrazolium (MTT), a yellow tetrazole dye, which converted into purple when come in contact with living cells. The MIC was determined observing the changing of colour of the sample in the wells. Spectrophotomeric assessment was done by reading the optical density (OD) at 600 nm using ELx800 absorbance microplate reader (Biotek). Percentage of inhibition was then calculated to determine the MIC value of the samples (Salauddin *et al.*, 2015).

$$\text{Percentage of inhibition \%} = \frac{\text{OD of negative control} - \text{OD of sample}}{\text{OD of negative control}} \times 100$$

Determination of Minimum Bactericidal Concentration (Mbc)

MBC of the mushroom extracts were determined by streaking a loopful of each test samples from the 96-well microtitre plate that shows no turbidity onto MHA plates. The MHA plates were then incubated at 37 ºC for 24 hours. MBC value was determined by the lowest concentration of test samples that shows no growth of bacteria on the agar.

RESULTS AND DISCUSSION

Minimum Inhibitory Concentration (MIC) Of Mushroom Extracts

MIC was done to determine the lowest concentration of test sample that can inhibit the bacterial growth. The antibacterial activity of four types

of ethanolic mushroom extracts were tested against two species of Gram-positive bacteria (*B. cereus* and *S. aureus*) and two species of Gram-negative bacteria (*E. coli* and *S. typhimurium*). Determination of MIC was done in microdilution method, where 96-well microtitre plates were used. Concentration of ethanolic mushroom extracts ranging from 5000 mg/ml to 7.81 mg/ml were tested. MIC value of a sample was recorded by observing the turbidity or bacterial growth in each well. This step was done in two ways, which were by visual assessment and spectrophotometric assessment.

DETERMINING MIC BY VISUAL ASSESSMENT

The microtiter plates were observed for the turbidity in each well. The turbidity indicates the growth of bacteria while the non-turbid well shows that there is an inhibition. The well with the lowest concentration of ethanolic mushroom extracts to have an inhibition was considered as MIC value. However, visual assessment of bacterial growth may lead to false data as it lacks precision. Therefore, MTT was used as an indicator of the bacterial growth. MIC was indicated by the unchanged colour of the MTT after loaded into each well. Table 1 shows the result of mushroom extracts against the Gram-positive and Gram-negative bacteria.

Results showed that ethanolic extracts of mushroom have antibacterial activity against the selected Gram-positive and Gram-negative bacteria. The inhibitory effects range from 500 mg/ml and above. *F. velutipes* and *A. bisporus* are noticeably effective among the other mushrooms starting from 500 mg/ml and above, which followed by *L. edodes* with the MIC value of 1000 mg/ml against *E. coli* and 500 mg/ml against the other bacteria (*B. cereus*, *S. aureus*, and *S. typhimurium*). *P. ostreatus* only showed inhibition against all four bacteria at 1000 mg/ml.

Determining Mic by Spectrophotometric Assessment

Depending only on visual assessment lacks precision and may lead to false data. Therefore, the observation of the bacterial growth was also done spectrophotometrically by measuring the optical density (OD) at 600 nm of each well to strengthen the previous results. The reading of OD was observed after 24 hours of incubation. Then, the percentage of inhibition of each well was calculated by comparing the average OD of test sample with average OD of control.

Figure 1 shows the percentage of inhibition of *P. ostreatus* against tested bacteria. The inhibition of *P. ostreatus* extract against all four bacteria (*B. cereus*, *S. aureus*, *E. coli*, and *S. typhimurium*) reach 75% to 95% at the concentration of 1000 mg/ml and above. Thus, the MIC values recorded for *P. ostreatus* for all tested bacteria are at 1000 mg/ml (BC: 77.72%, SA: 76.12%, EC: 90.63%, ST: 80.82%).

Table 1.Minimum Inhibitory Concentration (MIC) of ethanolic extract of mushrooms on B. cereus, S. aureus, E. coli, and S. typhimurium

Type of mushroom	Bacteria	The concentration of ethanolic extracts of mushroom (mg/ml)					Positive control	MIC (mg/ml)
		250	500	1000	3000	5000		
P. ostreatus (oyster)	*B. cereus*	+	+	-	-	-	-	1000
	S. aureus	+	+	-	-	-	-	500
	E. coli	+	+	-	-	-	-	500
	S. typhimurium	+	+	-	-	-	-	1000
A. bisporus (white button)	*B. cereus*	+	-	-	-	-	-	500
	S. aureus	+	-	-	-	-	-	500
	E. coli	+	-	-	-	-	-	500
	S. typhimurium	+	-	-	-	-	-	500
L. edodes (shiitake)	*B. cereus*	+	-	-	-	-	-	500
	S. aureus	+	-	-	-	-	-	500
	E. coli	+	+	-	-	-	-	1000
	S. typhimurium	+	-	-	-	-	-	500
F. velutipes (enoki)	*B. cereus*	+	-	-	-	-	-	500
	S. aureus	+	-	-	-	-	-	500
	E. coli	+	-	-	-	-	-	500
	S. typhimurium	+	-	-	-	-	-	500

Notes: + : presence of growth; - : no growth

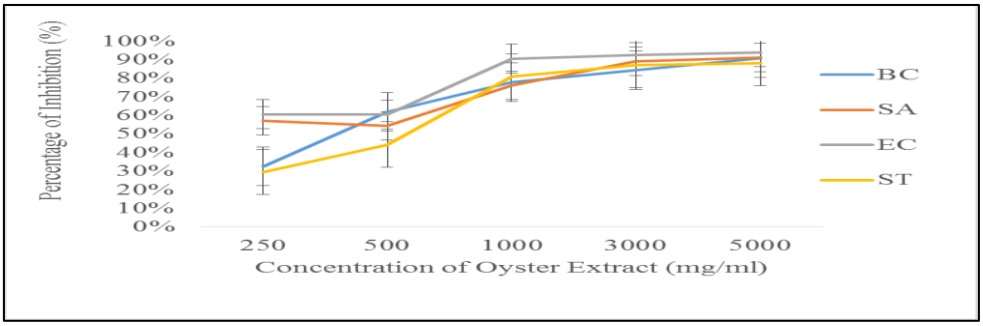

Fig. 1Percentage of Inhibition of *P. ostreatus* (Oyster) Extract against Selected Bacteria

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From Figure 2, the data indicates the percentage of inhibition of most A. bisporus extracts reach 75% at 500 mg/ml. This extract gave MIC value of 500 mg/ml against B. cereus (86.12%), E. coli (89.28%), and S. typhimurium (88.27%). When treated with S. aureus, the MIC value achieved is at 1000 mg/ml with percentage of inhibition of 82.29%.

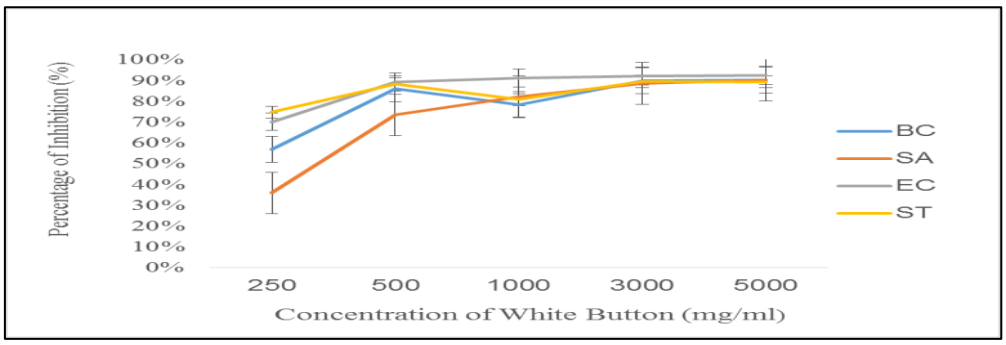

Fig. 2.Percentage of Inhibition of *A. bisporus* (White Button) Extract against Selected Bacteria
Notes: BC: *B. cereus*, SA: *S. aureus*, EC: *E. coli*, ST: *S.*

Figure 3 shows the percentage of inhibition of *L. edodes* extract against bacteria tested reach 75% and above mostly starting from 1000 mg/ml. This extract gave the MIC value of 500 mg/ml when tested with *E. coli* (77.49%) and 1000 mg/ml with the rest of bacteria (BC: 80.44%, SA: 76.26%, ST: 77.66%).

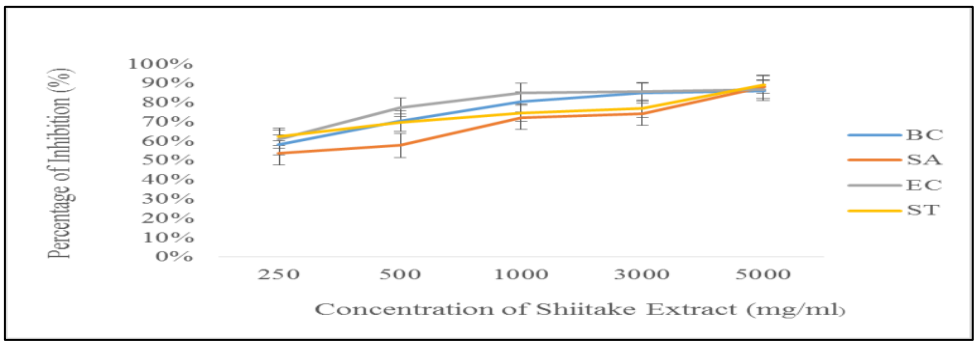

Fig. 3Percentage of Inhibition of L. edodes (Shiitake) Extract against Selected Bacteria
Notes: BC: *B. cereus*, SA: *S. aureus*, EC: *E. coli*, ST: *S. typhimurium*

From Figure 4, it visualizes that 75% of inhibition is achieved mostly at 500 mg/ml of *F. velutipes* extract against tested bacteria. The MIC value recorded are 500 mg/ml for all bacteria (SA: 78.84%, EC: 80.05%, ST: 79.21%) except for *B. cereus*, which shows inhibition starts from 1000 mg/ml (73.33%).

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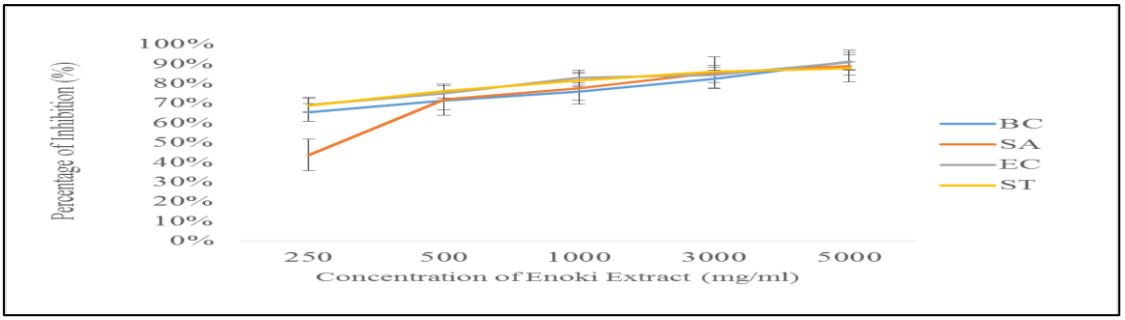

Fig. 4.Percentage of Inhibition of *F. velutipes* (Enoki) Extract against Selected Bacteria

One-way ANOVA analysis was done to observe the percentage of inhibition against the type of mushroom. The data is shown as in Appendix C. The type of mushrooms was classified into four types mushroom that were *P. ostreatus* (oyster), *A. bisporus* (white button), *L.* edodes, and *F. velutipes*. Results showed that F = 3.92 while *p*-value is 0.009. This result indicates that there was a significant difference between the means of the mushrooms.

Based on one-way ANOVA analysis on the percentage of inhibition against the type of bacteria which consist of *B. cereus* (BC), *S. aureus* (SA), *E. coli* (EC), and *S. typhimurium* (ST), the data showed there was a significant difference between the mean of type of bacteria (Appendix D, *p*-value = 0.016)

Hence, the percentage of inhibition is highly depending on both type of mushroom and type of bacteria.

MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF MUSHROOM EXTRACTS

The results of MBC for mushroom extracts as in Table 2. The MBC value for the mushroom extracts range from 500 mg/ml and above. Most of the extracts showed their bactericidal activity starting from 1000 mg/ml. However, it is found that all of the mushroom extracts tested do not give bactericidal effect against *B. cereus*. The extract that gives the highest MBC value, which is at 500 mg/ml is *A. bisporus* when tested with *S. aureus* and *E. coli*. However, this extract is not able to kill *S. typhimurium* and *B. cereus* as. On the other hand, the other mushroom extracts (*P. ostreatus*, *L. edodes*, and *F. velutipes*) showed MBC value at 1000 mg/ml against *S. aureus*, *E. coli*, and *S. typhimurium.*

Based on this study, it was found that ethanolic mushroom extracts exhibit inhibitory effect against both Gram-positive and Gram-negative bacteria tested. According to Reid *et al.*, (2016), mushroom that was extracted with ethanol gave the highest inhibition of bacterial growth compared to acetone and methanol.

Table 2.MBC of ethanolic mushroom extracts against tested bacteria

Type of mushroom	Bacteria	The concentration of ethanolic extracts of mushrooms (mg/ml)					Positive control	MBC (mg/ml)
		250	500	1000	3000	5000		
P. ostreatus (oyster)	*B. cereus*	+	+	+	+	+	-	NB
	S. aureus	+	+	-	-	-	-	1000
	E. coli	+	+	-	-	-	-	1000
	S. typhimurium	+	+	-	-	-	-	1000
A. bisporus (white button)	*B. cereus*	+	+	+	+	+	-	NB
	S. aureus	+	-	-	-	-	-	500
	E. coli	+	-	-	-	-	-	500
	S. typhimurium	+	+	+	+	+	-	NB
L. edodes (shiitake)	*B. cereus*	+	+	+	+	+	-	NB
	S. aureus	+	+	-	-	-	-	1000
	E. coli	+	+	-	-	-	-	1000
	S. typhimurium	+	+	-	-	-	-	1000
F. velutipes (enoki)	*B. cereus*	+	+	+	+	+	-	NB
	S. aureus	+	+	-	-	-	-	1000
	E. coli	+	+	-	-	-	-	1000
	S. typhimurium	+	+	-	-	-	-	1000

+ : presence of growth; - : no growth; NB : not bactericidal

There are other factors affecting the antibacterial activity such as type of mushrooms, presence of secondary metabolites including phenolic compounds (Kosanic *et al.*, 2013; Reid *et al.*, 2016). In an antimicrobial study done by Yahya (2017) using different varieties of rocket salad extract against *E. coli* (strain K12) also showed similar results where abundance of *E.coli* K12 was reduced when the bacteria

were cultured in extract of leaves obtained from rocket varieties containing high concentration of glucosinolate which a secondary metabolites compounds available in cruciferous vegetables. These compounds are said to act as defence against herbivores, pests and pathogens (Bell *et al.,* 2017).

According to the achieved results, it was found that the extract of *F. velutipes* has the highest antibacterial activity among the other extracts, which requires a minimum of 500 mg/ml to inhibit all tested bacteria. The data aligned with Kamra and Bhatt (2012) which stated low MIC value indicates stronger capability of an extract in antibacterial activity. The next highest antibacterial activity extract was *A. bisporus* extract where it able to kill *S. aureus* and *E. coli* at the concentration of 500 mg/ml. *L. edodes* showed better antibacterial activity than *P. ostreatus* as the *L. edodes* extract requires lower concentration to inhibit bacterial growth compared to *P. ostreatus* extract. Chowdhury *et al.,*(2015) had recorded the data similar to the achieved result in this study. Thus, the least effective mushroom extract is *P. ostreatus* as it requires a higher concentration (averagely 833.33 mg/ml and above) to inhibit the bacterial growth. Venturini *et al.,* (2008) had also reported that *P. ostreatus* showed no inhibition when being tested with *B. cereus* and *S. aureus*.

From the conducted experiment, data showed said Gram-negative *E. coli* was the most susceptible bacteria when treated with the mushroom extracts. A higher percentage of inhibition was shown when the mushroom extracts were tested with *E. coli*. The average range of inhibition had already reached 60 % starting from 250 mg/ml of all mushroom extracts. In contrast, Gezer *et al.,* (2006) recorded the achieved result whereby the mushroom extract exhibits a better inhibition against Gram-positive compared to Gram-negative. The reason is Gram-negative bacteria contains cell wall, which the outer membrane is made up from lipopolysaccharides that can prevent certain substances from invading the cell wall. However, the susceptibility of mushroom extracts against Gram-negative bacteria is might due to the extract affecting not only the cell wall of the Gram-negative bacteria but including the mechanisms of cell growth as well such as bacterial DNA replication and transcription (Reid *et al.,* 2016). There is not much difference of inhibition between *S. aureus* and *S. typhimurium,* whereby both were still susceptible to mushroom extracts but in higher concentrations (500 mg/ml to 1000 mg/ml).

Contrarily, the Gram-positive *B. cereus* seems to be quite resistant towards the mushroom extracts. The extracts are able to inhibit the bacteria within the range from 500 mg/ml and above but they are not bactericidal to kill the bacteria. This can be related to a study by Salauddin *et al.,*(2015), which shows similar result when treating the mushroom extracts against *B. cereus.* In his study, *P. ostreatus* was treated against *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Salmonella paratyphi*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus megaterium*. The antimicrobial activity was determined by agar disc diffusion method. It was found that *P. ostreatus* showed the greatest inhibition zone against *B. megaterium* and the lowest inhibition zone against *S. paratyphi*. However, the extract showed no zone of inhibition against *V. parahaemolyticus* and *B. cereus*. Only small inhibition zone was showed when treating with standard kanamycin, which means that both bacteria may be resistant to the mushroom extract.

CONCLUSION

In this study, the ethanolic extract of all mushrooms tested had exhibited antibacterial activity against Gram-positive *B. cereus* and *S. aureus*, and Gram-negative *E. coli* and *S. typhimurium*. The antibacterial activity of ethanolic mushroom extract depends on both type of mushroom and bacteria tested, which gave different susceptibility when being treated with the ethanolic mushroom extracts.

The highest antibacterial activity was exhibited by *F. velutipes* at 500 mg/ml against all bacteria tested, followed by *A. bisporus* extract, which showed bactericidal effect against *S. aureus* and *E. coli* at 500 mg/ml. *L. edodes* showed higher antibacterial activity than *P. ostreatus* as lower concentration of *L. edodes* was required to inhibit the bacterial growth. As for *P. ostreatus* extract, most inhibition only exhibited at 1000 mg/ml onwards. The most sensitive bacteria when treated with the mushroom extracts was *E. coli.* Most of the mushroom extracts were able to inhibit the bacterial growth of *E. coli* and reached 60 % of inhibition starting from 250 mg/ml. As for *S. aureus* and *S. typhimurium,* both can be inhibited but only a slightly higher concentration of ethanolic mushroom extract were required. For *B. cereus*, although the mushroom extracts showed inhibitory activity, the results showed that the extracts were not bactericidal against the bacteria.

The extract of *F. velutipes* showed the highest antibacterial activity. This extract possessed a good antibacterial property and could be the possible substitution of antibacterial agent used in pharmaceutical industry. However, further research is required in determining the exact minimum and maximum concentration that is enough to exhibit a good antibacterial activity, as too much concentration may also bring harm to human health. Isolation and characterization of new bioactive compounds that are responsible in exhibiting the antibacterial activity could also be conducted. It is also recommended to identify the effect if two or more mushroom extracts are combined against bacteria.

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