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Antibacterial activity of bioactive metabolites from Pleurotus pulmonarius against selected isolates

Sunday Ademola Laba[1]*, Francis Chukwumma Omeonu[2], Musa Olusegun Arekemase[1], Muinat Olanike Kazeem[1], Ismael Babatunde Onajobi[3]

[1]*Department of Microbiology, University of Ilorin, Ilorin, Nigeria*

[2]*Department of Botany, University of Ibadan, Ibadan, Nigeria*

[3]*Department of Microbiology, Olabisi Onabanjo University, Ago Iwoye, Nigeria*

ABSTRACT

Submerged fermentation of *Pleurotus pulmonarius* was carried out for over a period of 7 days in order to obtain phytotoxic metabolites. Separation of the metabolites was carried out using standard methods (mycelia, aqueous, and organic extracts). The antibacterial activity of the metabolites from the three extracts was investigated against *Escherichia coli* ST2747, *Klebsiella pneumonia* strain PMK1, *Salmonella enterica* subsp. *enterica sero-var typhimurium* strain 08-1736, and *Pseudomonas aeruginosa* VRFPA04 using agar-well diffusion technique. Phytochemical analysis was determined qualitatively and minimum inhibitory and minimum bactericidal concentrations were determined by plate assays. Standard antibiotics were used to compare the antibacterial activity of metabolites using the disk diffusion methods. The antimicrobial assay revealed that only the mycelial extract exhibited activity against the selected test strains. Phytochemical screening showed the presences of three phytoconstituents which include alkaloids, flavonoids, saponins, tannin, steroids, glycosides, phenols, and anthraquinones at varying concentrations in the mycelia extract. The highest zone of inhibition of the test organism was recorded in *K. pneumoniae*, while the least was detected in *P. aeruginosa.* Phytotoxic metabolites obtained through submerged fermentation have been discovered to be antagonistic against pathogenic organisms which could be carried out to develop antimicrobial drugs to combat the menace of drug resistance as witnessed in some synthetic drugs.

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1. Introduction

The application of natural resources for novel bioactive agents may provide solutions for drug discovery and development (Newman and Cragg, 2007). Mushrooms are members of phyla Basidiomycota and Ascomycota. They are traditionally believed to be remedies of many diseases, as well as prolific producers of bioactive metabolites. They are known for their phytotoxic, cytostatic, antibacterial, antiviral, antifungal, and other pharmacological activities (Jeong et al., 2011; Jonathan et al., 2012). Their products, such as functional food supplements, are available in the market with claimed biological activities and potential benefits on human health (Jeong et al., 2011). Many bioactive metabolites produced by mushrooms have shown therapeutic benefits because of the presence of biologically active

* **Corresponding author** Sunday Ademola Laba ✉ labademola2@gmail.com ✉ Department of Microbiology, University of Ilorin, Ilorin, Nigeria.

compounds (Bao et al., 2001). These active metabolites include high molecular weight compounds like proteins, lipids polysaccharides, and a series of complex low molecular weight metabolites with different chemical compositions, such as alkaloids, polyketides, terpenoids, and bioactive metabolites, gotten from non-ribosomal peptide synthesis (Ferreira et al., 2010).

Submerged fermentation is believed to be a promising alternative in the production of diverse bioactive compounds on a large scale (Enman et al., 2012). Nevertheless, it should not be taken for granted that bioactive metabolites which are readily obtained from mushroom fruit bodies are also found in their corresponding cultures of the same species or strain (Enman et al., 2012). Biological active compounds extracted from fruiting bodies may function as reverse transcriptase inhibitors which is an antimicrobial agent that can prevent the growth of fungi, bacteria, and protozoans. Mushrooms are natural antibiotics whose cell wall glucans are known for their immunomodulatory properties, as well as their secondary metabolites, from their mycelium that combat bacteria (Wang et al., 2007).

Antimicrobial resistance has been discovered to be a serious challenge to human existence in this generation. Application of convectional synthetic drugs in remediating the existing human, animal, and plants diseases therapy has proved unsuccessful (Zepeda-Bastida et al., 2016). Pathogens are increasingly resistant to many such drugs presently on sale in the market. As a result, naturally produced antimicrobials are gaining widespread popularity and acceptance (Roy et al., 2016). This study aimed at utilizing the antimicrobial potential of mushroom extracts as bioactive metabolites on selected pathogenic bacteria.

2. Materials and Methods

2.1. Collection and maintenance of test microorganisms

The fungus (*Pleurotus pulmonarius)* and bacteria (*Escherichia coli* ST2747, *Salmonella enterica* subsp. *enterica serovar typhimurium* strain 08-1736, *Klebsiella pneumoniae* strain PMK1, and *Pseudomonas aeruginosa* VRFPA04) used in this study were obtained from the culture collection unit of the Department of Microbiology, University of Ilorin, Nigeria. They were routinely sub-cultured and maintained and stored in the refrigerator at 4°C.

2.2. Fungus fermentation

Five mycelial agar plugs (7 mm) from a 6-day-old culture were inoculated into 100 ml of mushroom complete medium which was prepared by adding peptone (5 g), KH_2PO_4 (0.5 g), $MgSO_4$ (0.5 g), ascorbic acid (0.05 g), maltose (10 g), and streptomycin (0.1 g) in a 250-ml flask. The pH of initial substrates was adjusted to pH 9 through addition of 1 M NaOH. This was carried out in triplicates and incubated at 28°C in a Gallenkhamp rotary shaker at revolution of 120 rpm for 7 days

2.3. Metabolite extraction

The phytotoxic metabolite secreted during fermentation was collected every 24 hours by withdrawing the whole flask for the fermentation time frame (7 days). Fungal cultures were filtered using Whatman No. 1 filter paper. Mycelia of the fungal culture was harvested on filter paper, dried in oven at 40°C, and ground in a mortar with ethyl acetate solvent (1:1 vol). The filtrate was partitioned in a separating funnel with ethyl acetate solvent (1:1) and allowed to stand for at least 24 hours (Das et al., 2010) (El-Naggar et al., 2007). Both organic and aqueous extracts were concentrated using a water bath at 40°C. These were kept in the refrigerator for subsequent use.

2.4. Phytochemical screening of extract

The extract was subjected to qualitative and quantitative screening of phytochemicals using standard methods (Ghani, 2003; Harborne, 1973; Raman, 2006).

2.5. Antibacterial susceptibility

Antibacterial activity of the mushroom mycelia was assayed. The activities of the extract against the selected organisms were determined by modified agar-well diffusion assay method as described by Perez et al. (1990). Mueller-Hinton agar (MHA) plates were inoculated with 0.5 McFarland standard of the test organism by dropping a loop full of the broth on the solidified agar plate and using a sterile cotton swab to uniformly spread the inoculum on the agar plate. The plates were left for 30 minutes for the organism to diffuse into the agar. Agar wells of 7-mm diameter were bored on agar plates using sterile cork borer. The agar wells were then filled with the concentrated extracts using sterile micropipette. The plates were then incubated at 37°C for 24 hours. The zones of inhibition on the plates were measured and recorded.

2.6. Determination of minimum inhibitory concentration (MIC)

Extract concentrations at 200, 175, 150, 125, and 100 mg/ml were prepared. The MIC activity was conducted by streaking 0.1 ml of 24-hour-old inoculums

Table 1. Phytochemical analysis of mycelial extract of *P. pulmonarius.*

Phytoconstituents	Biochemical test	Result
Alkaloids	Mayer's test	+++
Phenols	$FeCl_3$ test	+++
Saponins	Frothing's test	++
Flavonoids	Alkaline test	++
Tannins	$FeCl_3$ test	+
Glycosides	Legal's test	++
Steroids	Libermann–Burchard's test	++
Anthraquinones	NH_4OH test	++

+++: highly present, ++: moderately present, +: faintly present.

of each test isolates separately onto prepared sterile MHA plates. Agar wells were made using sterile corks at the center of an agar plate for each test strain. The prepared extract at different concentration was introduced into each well and allowed to diffuse at room temperature and incubated at 37°C for 24 hours. Plates were observed for the presence of clear zones around the agar wells which were measured.

2.7. Determination of minimum bactericidal concentration (MBC)

The MIC test tubes that showed no visible growth after being incubated were sub-cultured on sterile MHA and incubated at 37°C for 18 hours. The concentrations which showed no growth after incubation were considered to have MBC (Abalaka et al., 2012).

2.8. Activity of standard antibiotics on test organism

This was carried out using standard antibiotic disks (Abtek R) and sterile MHA was inoculated with 0.5 McFarland standard of the test organisms. Antibiotic disks were aseptically placed on already inoculated agar plates using sterile forceps. The plates were incubated at 37°C for 24 hours and zones of inhibition were measured.

2.9. Statistical analysis

Data from this study were analyzed using analysis of variance to detect differences among the treatments. Means were separated using Duncan's multiple test ($p = 0.05$) the analyses were carried out using the SAS statistical software.

3. Results and Discussion

Medicinal mushrooms are of great importance to the health of individuals and communities. From this present study, submerged fermentation was carried out on *P. pulmonarius* and it resulted in the production of efficient metabolites of interest. The observation from this study is in consonance with the findings of other researchers (Jonathan et al., 2008; Stamets, 2007).

Determination of the phytochemicals present in the aqueous mycelia extract revealed the presence of alkaloids, flavonoids, steroids, tannins, saponins, glycosides, phenols, and anthraquinones at varying concentrations (Table 1). The presence of these metabolites has proved it to be of therapeutic importance. The results are in agreements with the findings of other researchers (Akinyele et al., 2012; Okwu, 2001; Okwulehie and Nosike, 2015; Unekwu et al. 2014).

The antibacterial activity of metabolites' extracts from the fermentation of *P. pulmonarius* was determined against some selected bacteria (*E. coli* ST2747, *S. enterica* subsp. *enterica serovar typhimurium* strain 08-1736, *K. pneumoniae* strain PMK1, and *P. aeruginosa* VRFPA04. The highest inhibition zones were recorded against *K. pneumoniae* (28.00 mm), followed by *S. enterica*, while the least was recorded in *P. aeruginosa* (Table 2). The results of this finding agree with the findings of other researchers that metabolites from medicinal mushrooms are antagonistic to many bacteria, except *P. aeruginosa* (Adebayo et al., 2012; Jumbo et al., 2008). The sensitivity pattern of the test bacteria to reference antibiotics in comparison to phytotoxic metabolite is presented in Table 3. Generally, the antibiotics produce higher activity than the phytotoxic metabolite. Ciprofloxacin showed the highest activity against *P. aeruginosa* VRFPA04 (33.00 mm), followed by ofloxacin activity against *E. coli* (29.50 mm), while all the test organism are resistant to cefuroxime, cefixime, and ceftazidime (Table 3). The comparative analysis of antibacterial activity of reference antibiotics with mycelia extract revealed that reference antibiotics were better in activity than the mycelia extract. This may be as a result of the fact that standard antibiotics are well purified and it possesses a definite line

Table 2. Diameter of the zones of inhibition (mm) of metabolites produced by *P. pulmonarius* on the test organisms.

Organism	Zones of inhibition (mm)						
	Fermentation time (days)						
	1	2	3	4	5	6	7
S. typhimurium	5.00	8.00	11.00	15.50	18.50	19.00	25.00
K. pneumonia	7.00	9.00	10.40	18.00	22.00	25.00	28.50
E. coli	0.00	5.00	8.00	9.50	11.60	13.50	16.50
P. aeruginosa	0.00	0.00	0.00	4.00	4.50	4.50	5.00

Table 3. Antibacterial susceptibility pattern of test strains to reference antibiotics.

Antibiotics	Test strains			
	Diameter zone of inhibition (mm)			
	A	B	C	D
Cefuroxine (5 µg)	-	-	-	-
Gentamicin (10 µg)	19.50	19.60	15.45	15.00
Cefixime (5 µg)	-	-	-	-
Ofloxacin (5 µg)	20.15	18.50	29.50	22.50
Augmentin (30 µg)	-	-	19.33	-
Amphicillin (5 µg)	16.50	15.45	17.00	-
Ceftazidine (30 µg)	-	-	-	-
Ciprofloxacin (5 µg)	22.00	26.50	24.45	33.00

A: *S. enterica* subsp. *enterica serovar typhimurium strain* 08-1736, B: *Klebseilla pnemoniae* strain PMK1, C: *E. coli* ST2747, D: *P. aeruginosa.*

Table 4. MIC and MBCs of mycelia extracts of *P. pulmonarius* on *P. aeruginosa* VRFPA04.

MIC (mg/ml)	Fermentation time (days)							MBC (mg/ml)
	1	2	3	4	5	6	7	
200	–	–	–	–	+	+	+	+
175	–	–	–	–	–	–	–	+
150	–	–	–	–	–	–	–	ND
125	–	–	–	–	–	–	–	ND

(–) = No activity at that concentration, (+) = activity at that concentration, (++) = bactericidal activity, ND = not determined.

Table 5. MIC and MBCs of mycelia extracts of *P. pulmonarius* on *K. pneumoniae* strain PMK1.

MIC (mg/ml)	Fermentation time (days)							MBC (mg/ml)
	1	2	3	4	5	6	7	
200	+	+	+	+	+	+	+	++
175	–	–	–	–	+	+	–	++
150	–	–	–	–	–	–	–	ND
125	–	–	–	–	–	–	–	ND

(–) = no activity, (+) = activity, (++) = bactericidal activity, ND = not determined.

Table 6. MIC and MBCs extracts against *E. coli* ST2747.

MIC (mg/ml)	Fermentation time (days)							MBC (mg/ml)
	1	2	3	4	5	6	7	
200	–	–	+	+	+	+	+	– –
175	–	–	–	–	–	+	–	ND
150	–	–	–	–	–	–	–	ND
125	–	–	–	–	–	–	–	ND

(–) = no activity, (+) = activity, (– –) = no bactericidal activity, ND = not determined.

Table 7. MIC and MBCs of mycelia extracts against *S. enterica* subsp. *enterica serovar typhinurium* strain 08-1736.

MIC (mg/ml)	Fermentation time (days)							MBC (mg/ml)
	1	2	3	4	5	6	7	
200	–	+	+	+	+	-	+	– –
175	–	–	–	–	–	–	–	ND
150	–	–	–	–	–	–	–	ND
125	–	–	–	–	–	–	–	ND

(–) = no activity, (+) = activity, (– –) = no bactericidal activity, ND = not determined.

of action to microorganisms unlike their crude metabolites which contain a combination of active and inert components, some of which can militate against antimicrobial action. This is similar to observations made by Shittu et al. (2005).

The results of the MIC and MBC are shown in Tables 4–7. The results revealed that the metabolites have more effects on the test organisms at highest concentration (200 ml/g).

4. Conclusion

From this study, it is evident that the mycelia of *P. pulmonarius* possesses certain metabolites which are phytotoxic to certain bacteria and submerged fermentation has proven to be better in terms of metabolite recovery and reduced risk of contamination compared to solid-state fermentation. This could serve as a means of producing alternative remedies to the cure of microbial infections which have become highly resistant to the available medications. Since there are edible mushrooms which are readily available and affordable, it would reduce the cost of healthcare delivery, especially in rural communities, where these mushrooms are readily available.

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Conflict of interest

The authors declare no divergence of interest in this study.

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