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Antimicrobial Drug Synthesis from Submerge Cultures of *Pleurotus florida* in Different Agro-waste Extract Compositions

Majolagbe O.N[1*]Adebayo E. A[1] Aina D.A[2], Oniyide O.M[1]Adenuga I.A[1]
[1.]*Microbiology Unit, Department of Pure and Applied Biology, Ladoke Akintola University of Technology, P. M. B. 4000, Ogbomoso, Nigeria.*
[2.]*Department of Biosciences and Biotechnology, School of Basic and Applied Sciences, Babcock University, Ilisan-remo, Ogun State, Nigeria.*

Abstract: *The quest for new antimicrobial agents and improvement on the existing ones keep increasing all over the globe. Antimicrobial activities of exopolysaccharides of Pleurotus florida was improved by culturing the strain in medium supplemented with agro-wastes extracts. Aqueous solution of the extract was used in the preparation of the medium. The agro-waste extract include Mango leaf extract (MLE), Rice straw extract (RSE), Paper extract (PPE), Banana leaf extract (BLE) and Saw-dust extract (SDE). Phytochemical screening of the extracts revealed that, there were variations in their phytochemical constituents. P. florida was cultured in submerge fermentation for 21 days for exopolysaccaharide production. Antibacterial activity of the exopolysaccharides of P. florida obtained from each of the medium extract was determined using microorganisms from different sources.There were variations in the zones of Inhibition (ZI) for each of the tested isolates. MLE and PPE inhibited only B. megaterium at 4.83±0.27mm and 5.27±1.12mm respectively, BLE showed inhibition against only B. alvei (6.42±0.98mm) while SDE inhibited both B. subtilis and B. alvei at 5.25±1.25mm and 6.68±1.42mm respectively. However, RSE showed no inhibition against the test isolates. Also, SDE containing saponins, glycosides and especially phenolics, has the highest antimicrobial potency against Candida spp with 17.0mm zone of inhibition. In summary, it was established that many of the agro-wastes which are sources of environmental pollution still contain vital bioactive phytochemicals that if extracted could support the growth of the fungi for metabolite production which has applications in drug discovery, production and development.*
Key words*: agro-wastes, antimicrobial, drug synthesis, exopolysaccharides*

I. Introduction

The discovery of new classes of antibiotics is necessary due to the increased incidence of multiple resistance among pathogenic microorganisms to drug that are currently in clinical use [1]. Interestingly, some mushrooms and their components are target-specific in their antibiotic properties, whereas others have broader effects. Mushrooms thus provide a protective immunological shield against a variety of infectious diseases [2].

Most of the edible fungi have strong enzyme system and are capable of utilizing complex organic compounds, which occur as agricultural wastes and industrial by-product [3].It is reported on average that two or three antibiotics derived from microorganisms are launched every year and over 60% of anti-tumour and anti-infective agents that have been approved or are in late stages of clinical trials, are of natural product origin. Mushrooms are currently of interest because they are rich source of various bioactive natural products [4]. They have long been used in folk medicines and health, attracted a great deal of interest in many areas of foods and biopharmaceuticals, and are regarded as effective medicines used to treat various human diseases, such as hepatitis, gastric cancer, etc [5].

One of the major rationales of antimicrobial compounds from fungi is that humans share common microbial pathogens (e.g *E. coli*, and *S. aureus*) with fungi, hence, humans benefit from defense strategies used by fungi against microorganisms [6]. The best known drugs obtained are lentinan from *Lentinus edodes*, grifolin from *Grifola frondosa*, and krestin from *Coriolus versicolor*. These compounds are protein-bound polysaccharides or a long chain of glucose, found in cell wall, and function as anti-tumour or immunomodulatory drugs [7, 8].

The use of filamentous fungi as source of bioactive compounds have some advantages over the use of plants, in that (i) the fruiting body of fungi can be produced in much less time, (ii) the mycelium can be rapidly produced in liquid culture, which can be manipulated to produce optimal quantities of bioactive products [9].

II. Materials and Methods

Test Organisms

Characterized bacteria and fungi which were of environmental and clinical origins were used as test organisms against the exopolysaccharides of the different agro-waste extracts medium. The *Bacillus spp*. used as test organisms were isolated from underground water samples. They were characterized and identified using Bergy's Manual of Bacteriology. While the clinical isolates were collected from culture bank of BOWEN

University Teaching Hospital, Ogbomoso, Oyo State, Nigeria. The environmental isolates include *Bacillus subtilis, B. megaterium, B. alvei* while the clinical isolates include *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella spp* and *Candida spp*. All bacteria and fungi used as test organisms were maintained on Nutrient Agar (NA) and Potato Dextrose Agar (PDA) respectively at 4^0C.

Preparation of Agro-waste extracts and phytochemical screening

Agro-waste extracts was prepared from different sources and designated as: Mango leaves extract (MLE), Rice straw extract (RSE), Paper extract (PPE), Banana leaves extract (BLE) and Saw-dust extract (SDE). Each of the aqueous extract was used in the preparation of Nutrient broth used in the submerge fermentation of the *P. florida* for the production of the exopolysaccharide. The extract was further screened for its phytochemical composition.

Medium formulation

For 100 ml of each of the agro-waste extract, 2g of glucose, 0.25g of peptone, 0.25g of yeast extract, 0.2g of KH_2PO_4, 0.1g of $MgSO_4.7H_20$ and 0.1g of $CaCl_2.2H_2O$ were dissolved.

Collection and culture of *Pleurotus florida*

P. florida was collected from the culture bank of the Department of Pure and Applied Biology. It was sub-cultured onto a fresh PDA. Incubation was done at room temperature in a dark cupboard until there was a complete ramification of the mycelium. Ramification rate was observed till the 21st day of the cultivation. The mycelium was transferred into the agro-waste supplemented nutrient broth. The culture was observed for eps production over a period of days.

Extraction of exopolysaccharide (eps)

Content of the fermentation flask was sieved to obtain mycelia mats. Wet and dry weight of the residue was measured and recorded using the digital weighing balance. To 50 ml of each extract filtrate, 100 ml of acetone was added (ratio 2:1) to precipitate the polysaccharide. The mixture was then kept in the fridge at 4^0C for 24 hrs. Centrifugation was carried out and the polysaccharide was obtained by decanting. The acetone was removed from the eps by dryness and kept in freezer.

Preparation of aqueous exopolysaccharide (eps)

The extracted crude eps was concentrated and quantified. It was diluted with 10ml sterile distilled water and stored in Empendorof tubes for antimicrobial assessment. Aliquot of the sample was standardized (gml^{-1})

Phytochemical screening of agro-waste extracts.

Phytochemical screening was carried out to detect the presence of some bioactive compounds (plant constituents) such as alkaloids, tannins, saponins, phenolics, phyllobatannins, flavanoids and glycosides [10] as follows:

Test for Saponins

Exactly 2 ml of each aqueous extracts was transferred into different test-tubes and vigorously agitated for 2 mins. Presence of saponins was indicated by foaming which persisted on shaking.

Test for Alkaloids

5 ml of 1% HCl was added to 2 ml of the aqueous extracts in test-tubes, and stirred gently in a water bath. The solution obtained was cooled and then filtered. Few drops of Mayer's reagent were also added to the filtrate. Presence of alkaloids was indicated by a cream precipitate.

Test of Phenolics

Two drops of 5% Ferric chloride was added to 5ml of the aqueous extracts in a test-tube. A greenish precipitate indicated presence of phenolics.

Test for Tannins

A volume of freshly prepared 10% KOH was added to 1ml of the aqueous extracts in a test-tube. A dirty white precipitate indicated presence of tannins.

Test for Steroids

To 1 ml of the aqueous extracts in a test-tube, 5 drops of concentrated H_2SO_4 was added. Red coloration indicated presence of steroids.

Test for Phylobatannins

To 1 ml of the aqueous extracts in a test-tube, 1% HCl acid was added. A red precipitate indicated presence of phylobatannins.

Test for Flavonoids

To 3ml of the aqueous extracts in a test-tube, 1 ml of 10% NaOH was added. A yellow colouration indicated the presence of flavonoids.

Test for Glycosides

To 3ml of aqueous extract in a test-tube, 2ml of chloroform was added. Also, H_2SO_4 acid was carefully added to form a lower layer. A reddish brown colour at interface indicated the presence of glycosides.

Antibacterial test of the eps

Disc diffusion method was used for the antimicrobial assay as follows: Whatman filter paper was cut into small circle (about 6mm) and sterilized in an oven for 170^0C for 2hours. The cut paper was dipped into each of the EPS extract for 2 hours for its absorption. Fresh culture of each of the bacteria isolates were then inoculated onto a freshly prepared NA in a 9-mm Petri-dishes. Sterile forceps was used to transfer the disc containing extract onto the inoculated medium and then incubated for 18-24 hours at 37^0C. The diameters of the zones of inhibition around the discs were measured in milliliters (mm).

III. Results and Discussion

Secondary compounds, which include tannins, saponins, glycosides and alkaloids have been reported to be present in plants, especially agro wastes [11].Results of the preliminary phytochemical screening revealed the presence of some of these compounds in the agro-waste extract such as banana leaf, rice straw, saw-dust, mango leaf and paper extract. Our results showed that the agro-waste extracts contains at least one of the following phytochemicals such as: saponin, alkaloids, phenolics, steroids, flavonoids and glycosides (Table 1) which when combined with various salt compounds such as glucose, yeast extract, and peptone could have enhanced the growth of *Pleurotus florida* in submerged fermentation (Plate 1). Only tannins and phylobatannins were absent in all the agro-wastes extracts screened.

Table 1: Phytochemical Screening of Agro-Waste Extracts

Phytochemical	MLE	RSE	PPE	BLE	SDE
Saponin	-	-	+	+	+
Alkaloids	-	+	+	-	-
Phenolics	+	+	-	-	+
Tannins	-	-	-	-	-
Steroids	-	-	+	-	-
Phylobatannins	-	-	-	-	-
Flavonoids	+	+	-	-	-
Glycosides	+	-	-	-	+

*MLE = Mango leaf extract; *RSE = Rice straw extract; *WPE = Waste paper extract; *BLE = Banana leaf extract; *SDE = Saw-dust extract

Plate 1: Submerged fermentation of *P. florida* in five agro-waste extracts medium composition

There is evidence that substrate composition can influence the chemical compositions of mushroom [12] and there has been improved knowledge about its nutritional value. Mycelia growth in saw dust extract (6.8g) can be attributed to the absence or presence of one or more nutritional compounds required for fungal growth or to the presence of substance that inhibited the fungal growth and also could be as a result of maintaining this fungus under the laboratory conditions. Mycelia transference over a long period of time can cause physiological and/or morphological changes in the fungus. The growth rate of *P. florida* in submerge fermentation over a period of 21 days was monitored with dense mycelial growth rate noticeable in BLE (Table 4). In day 1-3, there was no growth yet, which is indicative of the microbial growth pattern as the organism was at the Lag growth phase. At this phase, the organism was getting adapted to the substrate environment. Growth was observed at Day 4 in BLE, day 5 in PPE, Day 6 in RSE, Day 7 in SDE and MLE. The delayed in growth may be due to variations in the phytochemical contents present in each of the different media for cultivation. BLE gave the highest growth rate as from the 4th day till the 21st day in which highly dense growth had occurred. In addition, BLE gave the highest biomass wet weight (bww) of *P. florida* (13.6±2.14) g while other extract also showed variations in growth as seen in Figure 1. In contrast, SDE did not show growth until the 7th day and less dense growth till the 21st.

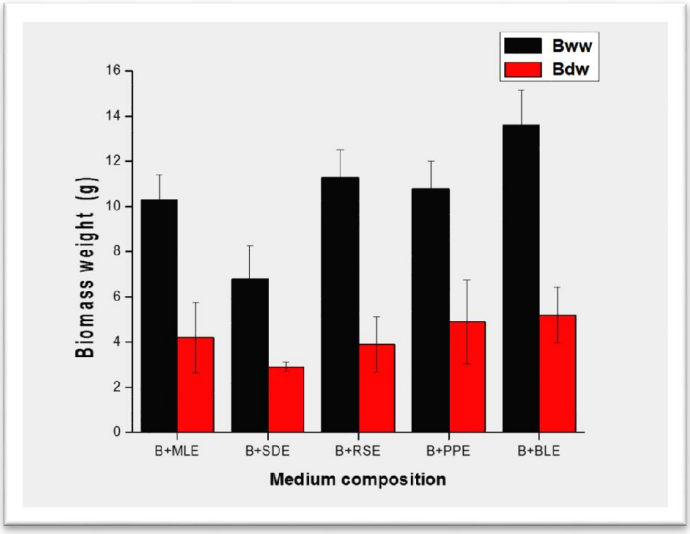

Figure 1: Biomass weight of *P. florida* cultured in the five agro-waste medium composition

Antibacterial activity of the exopolysaccharides of *P. florida* obtained from each of the medium extract was assessed by using three *Bacillus species* (*Bacillus subtilis, B. megaterium* and *B. alvei*) from polluted water source and clinical samples from various sources. Zones of Inhibition (ZI) for each of the extract determined for each of the isolates showed that MLE and PPE inhibited only *B. megaterium* at 4.83±0.27mm and 5.27±1.12mm respectively, BLE showed inhibition against only *B. alvei* (6.42±0.98mm) while SDE inhibited both *B. subtilis* and *B. alvei* at 5.25±1.25mm and 6.68±1.42mm respectively. Only RSE showed no inhibition against the test isolates as seen in Table 2.

Table 2: Antibacterial sensitivity of exopolysaccharide of *P. florida* against *Bacillus spp*. isolated from ground water sources showing Zones of Inhibition (mm)

Isolate	MLE	RSE	PPE	BLE	SDE
B. subtilis	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	5.52±1.25
B. megaterium	4.83±0.27	0.00±0.00	5.27±1.12	0.00±0.00	0.00±0.00
B. alvei	0.00±0.00	0.00±0.00	0.00±0.00	6.42±0.98	6.68±1.42

*MLE = Mango leaf extract; *RSE = Rice straw extract; *WPE = Waste paper extract; *BLE = Banana leaf extract; *SDE = Saw-dust extract;

Table 3: Antimicrobial sensitivity of exopolysaccharides of *P. florida* against clinical isolates from different sources showing Zones of Inhibition (mm)

Isolates	Sources	EXTRACTS				
		MLE	MRSE	PPE	BLE	SDE
E. coli	Urine	R	R	6.0	R	15.0
E. coli	HVS	R	8.0	7.0	R	12.0
E. coli	Wound	7.0	R	R	8.0	R
E. coli	Blood	R	R	7.0	R	R
E. coli	Stool.	10.0	R	R	R	R
E. coli	Eye swab	10.0	R	R	R	R
P. aeruginosa	Urine	R	6.0	R	R	14.0
P. aeruginosa	Wound	R	R	10.0	15.0	R
P. aeruginosa	HVS	R	6.0	6.0	5.0	15.0
Klebsiella spp.	Wound	R	R	R	R	R
Klebsiella spp	HVS	6.0	R	R	6.0	6.0
Klebsiella spp	Urine	R	R	6.0	R	15.0
Klebsiella spp.	Sputum	R	R	R	R	R
Klebsiella spp.	Semen	R	R	R	R	R
Klebsiella spp	Catheter	R	R	R	R	R
S. aureus	Abscesses	R	R	R	R	R
Proteus mirabilis	Wound	R	R	R	R	R
Candida spp	Blood	R	R	R	R	R
Candida spp.	C. swab	10	R	15.0	12.0	17.0
Candida spp.	HVS	12.0	15.0	8.0	11.0	13.0

Table 4: Growth rate of *Pleurotus florida* on submerged fermentation

Medium Composition	Growth rate (days)																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
NB + MLE	-	-	-	-	-	-	+	+	+	+	+	+	+	+	++	++	++	++	+++	+++	++
													+	+				+			
NB + SDE	-	-	-	-	-	-	+	+	+	+	+	+	+	+	++	++	++	++	++	++	++
														+							
NB + RSE	-	-	-	-	-	+	+	+	+	+	+	+	+	+	++	++	++	++	+++	+++	++
												+	+	+				+			+
NB + PPE	-	-	-	-	+	+	+	+	+	+	+	+	+	+	++	++	++	++	+++	+++	++
													+	+							+
NB + BLE	-	-	-	+	+	+	+	+	+	++	+	+	+	+	++	++	++	++	+++	+++	++
										+	+	+	+	+	+	+	+	+	+	+	+

*NB = Nutrient broth; *MLE = Mango leaf extract; *RSE = Rice straw extract; *WPE = Waste paper extract; *BLE = Banana leaf extract; *SDE = Saw-dust extract; Pf (*Pleurotus florida*; (-) No growth; (+) Little growth; (++) high growth; (+++) very high growth

A study by Lavi *et al* (2006) [13] shows that the majority of active substances in mushroom are polysaccharides and polysaccharide complexes, active hexose-correlated compounds (AHCC), polysaccharide peptides, nucleosides, complex starches and other metabolites. This is in agreement with the result of this study with the detection of alkaloids and other phytochemicals in the eps extracts. These metabolites elicit antimicrobial properties by cell membrane lysis, inhibition of protein synthesis, proteolytic enzymes and microbial adhesion which is similar to the action of standard antibiotics from pharmaceuticals. The confirmed clinical isolates used in this work obtained from different sources include: *E. coli, P. aeruginosa, Klebsiella spp. Proteus mirabilis, S. aureus,* and *Candida spp*. There were variations in response of the exopolysaccharides to all the test clinical isolates as seen in Table 3. *Proteus mirabilis* and some species of *Klebsiella* showed high degree of resistance to the exopolysaccharides, while *P. aeruginosa* and *E. coli*, were susceptible, while *Candida spp*. showed the highest degree of susceptibility to the exopolysaccharide. This is in alignment with the work of Prassad *et al* (2009) [14] who reported that some of the polysaccharide and polysaccharide peptides present in mushroom contribute to the antimicrobial property along with the phenolic components. This also correlates with the sensitivity result (Table 3.0) which indicates that SDE containing saponins, glycosides and especially phenolics, has the highest antimicrobial potency against *Candida spp*. with 17.0 mm zone of inhibition. The resistance of the isolates to the metabolite as seen in this work may be due to the possession of resistance gene (yet to be confirmed) by some of the isolates, although the susceptibility to some of the isolates recorded indicates that the exopolysaccharides has potentials for treatment of infectious agents and could be used in drug discovery. Also, extracts from mushroom species have been used in traditional Asian medicine to stimulate the immune system and treat chronic wasting diseases such as cancer tuberculosis, hepatitis and AIDS. Common to all mushrooms are phytochemicals called glycans, proteoglycans, beta-glucans and polysaccharides which are forms of glucose, an important energy source. Similarly to plants, mushroom extract can potentiate the acts of antibiotics extensively used in clinical practice for Gram positive or Gram negative bacteria, with positive action even against multi-resistant bacteria. Mushroom's exopolysaccharides could decrease therapeutic doses of standard antibiotics and reduce microorganism's resistance to drugs [15].

IV. Conclusion

In conclusion, the extracts of the mushroom used in this study inhibited the growth of some microorganisms which suggests that they are potential sources of new antimicrobial drug noting that extracts and derivatives from mushrooms hold great promise for novel medicines in modern times.

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