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In vitro antimicrobial activity of *Armillaria mellea* against pathogenic organisms

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Abstract
The rampant multi-drug resistance among human pathogenic microorganisms has necessitated a continuous search for new and potent antimicrobial substances, especially among plants. Also, the importance of herbal plants as sources of alternative medicine is documented worldwide. In this study, antimicrobial activities of extracts of fruit bodies of *Armillaria mellea* against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, *Candida albicans* and *Candida glabrata* were investigated. Antimicrobial components from the mushrooms were extracted using ethanol, methanol and water and examined by agar well diffusion method. The Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) were evaluated for each extract of the mushroom. Results of *Armillaria mellea*, have shown promising antimicrobial activities against the tested organisms. *B. cereus, S. aureus, C. albicans and C. glabrata* were the most susceptible to aqueous extract of *Armillaria mellea* while ethanolic and methanolic extract showed different susceptible to the test organisms. The phytochemical analysis revealed the presence of varying levels of bioactive compounds. Saponins, protein and carbohydrate were detected in all the extracts while glycosides, alkaloids, flavonoids and tannins were found in some. The results obtained from this study suggest that *Armillaria mellea* has broad-spectrum of activity against microbial isolates used.

Keywords: *Armillaria mellea*, antimicrobial, agar well diffusion method, phytochemicals

Introduction
In many countries of the world, Nigeria inclusive, edible mushrooms are good for food and are used in medicine to protect against free radicals and infections [1, 2]. The use of mushrooms as food as well as medicine is gaining popularity in recent times [3]. The nutritive and properties of many mushrooms have been documented [4-6]. Mushrooms have been found to contain all the essential amino acids [3, 7]. Edible mushrooms are attractive because of their flavor, taste and delicacy [8]. Many species of edible mushrooms exist in nature, less than 20 species are used as food and only 8-10 species are regularly cultivated in significant extent [9]. Mushrooms characteristically contain many different bioactive compounds such as polysaccharides, glycosides, sesquiterpenes etc with diverse biological activities such as anticancer, antibacterial, antifungal and antiviral agent [10].
Infectious diseases remain one of the major threats to human health. Although numerous antibiotics have been used against pathogens, antimicrobial resistance is an increasing public health problem [11]. The antibiotics in mushrooms are less well documented in the discovery of new antimicrobial agents with different structural types. Mushrooms need antibacterial and antifungal compounds to survive in their natural environments. Therefore, antimicrobial compounds could be isolated from many mushroom species and could be of beneficial for humans [12]. Most of the medicinal extracts from mushrooms are different forms of polysaccharides, and all of them are strengtheners of the immune system with few or no side effects [13].
Armillaria mellea, commonly known as honey fungus, is a basidiomycete fungus in the genus *Armillaria*. It is a plant pathogen and part of a cryptic species complex of closely related and morphologically similar species. It causes *Armillaria* root rot in many plant species and produces mushrooms around the base of trees it has infected. The symptoms of infection appear in the crowns of infected trees as discoloured foliage, reduced growth, dieback of the branches and death. The mushrooms are edible but some people may be intolerant to them.
The species was originally named *Agaricus melleus* by Danish-Norwegian botanist Martin Vahl in 1790; it was transferred to the genus *Armillaria* in 1871 by Paul Kummer [14]. The basidiocarp of each has a smooth cap 3 to 15 cm in diameter, convex at first but becoming flattened with age often with a central raised umbo, later becoming somewhat dish-shaped. The margins of the cap are often arched at maturity and the surface is sticky when wet. Though typically honey-coloured, this fungus is rather variable in appearance and sometimes has a few dark, hairy scales near the centre somewhat radially arranged. The gills are white at first, sometimes becoming pinkish-yellow or discoloured with age, broad and fairly distant, attached to the stipe at right angles or

are slightly decurrent. The spore print is white. The stipe is of variable length, up to about 20 cm long and 3.5 cm in diameter. It is fibrillose and of a firm spongy consistency at first but later becomes hollow. It is cylindrical and tapers to a point at its base where it is fused to the stipes of other mushrooms in the clump. It is whitish at the upper end and brownish-yellow below, often with a very dark-coloured base.

Mushrooms need antibacterial and antifungal compounds to survive in their natural environment. Therefore, antimicrobial compounds could be isolated from many mushroom species and could be of benefit for humans. This study was designed to evaluate the antimicrobial activity of *Armillaria mellea* mushroom extracts on bacterial and fungal isolates. The study aimed to determine their minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) and the phytochemical properties of the mushroom so as to offer informed recommendation on its use for the treatment of problem of antibiotic resistance.

Materials and Methods

Collection and Identification of Materials

Armillaria mellea was collected from different sources of Umuahia North Local Government area, Abia state and identified by a botanist in the Department of botany, University of Nigeria, Nsukka.

Test Organisms Used

Pure cultures of *Escherichia coli* JCM 20135 and *Bacillus cereus* IFO 13804 were obtained from Department of Microbiology, University of Nigeria Nsukka while pure cultures of *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231, *Pseudomonas aeruginosa* ATCC 25783 and *Candida glabrata* ATCC 22018 were obtained from Spectramedics Laboratories No. 2 Adebayo close, Araromi Offiri Ikenne Road, Sagamu, Ogun State.

Standard Antimicrobials

Tetracycline (5 µg/ml), Amplicillin (5 µg/ml), Oxacillin (5 µg/ml) and Nystatin (20 µg/ml) oxoid discs were used as positive standards.

Sample Preparation and Extraction

Fresh *Armillaria mellea* mushrooms were thoroughly washed with distilled water, cut into pieces, air-dried at room temperature and pulverized using manual grinder. Fifty grams of each of the ground samples was soaked in 500 ml ethanol, cold water, and methanol for 24 hours with intermittent shaking. Each sample was filtered using Whatman №1 filter paper. The filtrate was dried with a rotary evaporator in order to obtain the extract which was scooped and poured into well-labeled sample bottles and stored at 4ºC [15, 16, 17,18, 2].

Inoculum Preparation

Inoculum was prepared by emulsifying overnight colonies from an agar medium. A 0.5 McFarland standard (equivalent to approximately 10^8 cfu/ml) was used. Media plates were inoculated within 30 minutes of standardizing the inoculum to avoid changes in inoculums density.

Determination of Antimicrobial Activity of *Armillaria mellea* Extracts

Antimicrobial activity of mushroom extracts was determined according to the National Committee of Clinical Laboratory Standards [19]. Agar disc diffusion method on SDA and Muller-Hinton agar were used for fungi and bacteria respectively. A micropipette was used to introduce 100 µL of the inoculum onto the agar plate, and spread with glass rod spreader under sterile conditions. The paper discs of 6 mm diameter soaked in 10 µL of different concentrations of the extracts (500, 250, 125, 62.5, 31.25, 15.63 and 7.81 mg/mL) was applied on the agar plate, paper discs of 6 mm with dilute dimethylsulfoxide were used as negative control and antibiotics discs of tetracycline (10 µg/mL) and ampicillin (10 µg/mL) were used for Gram negative bacteria isolates, oxacillin (5 µg/mL) was used for Gram positive bacteria isolates whereas antifungi disc of nystatin (20 µg/mL) oxoid disc was used as positive control. This procedure was carried out in triplicate for the entire test organisms and allowed to stand for 30 min on the bench after which they were incubated for 24 h at 37 ± 2ºC for bacteria and 72 at 28 ± 2ºC for yeast. After incubation, the inhibition zone diameters produced by the different concentrations of the crude extracts were measured (in millimeter) using transparent meter rule.

Determination of Minimum Inhibitory Concentrations (MICs) of *Armillaria mellea* Extracts

The MIC of the extracts was determined for the test organisms in triplicates at varying concentrations (250, 125, 62.5, 31.25, 15.62, 7.80 and 3.90 mg/ml). To obtain these concentrations, 1.0 ml of varying concentrations of the extracts with double strength (500, 250, 125, 62.5, 31.25, 15.62 and 7.80 mg/ml) were constituted in different test tubes. About 1.0 ml of

Mueller-Hinton broth (for bacteria) and Sabouraud dextrose broth (for fungi) was added and then a loopful of the test organism, previously diluted to 0.5 McFarland turbidity standard, was introduced. Controls of Mueller-Hinton broth and Sabouraud dextrose broth without the mushroom extract were set up. All the bacterial cultures were incubated at 37 ± 2ºC for 24 hours and yeast culture incubated at 28 ± 2ºC for 72 hours. After incubation

each tube was examined for microbial growth. The lowest concentration of the extract that inhibited the growth of the test organisms as detected by lack of visual turbidity was designated the MIC [19].

Determination of Minimum Bactericidal Concentrations (MBCs) and Minimum Fungicidal Concentrations (MFCs) of *Armillaria mellea* Extracts

MBC was determined by selecting tubes that showed no bacterial growth during the MIC determination. A loopful from each of the tubes was sub-cultured on the Mueller Hinton Agar and incubated for 24 hours at 37 ± 2ºC. MFC was determined by selecting tubes that showed no fungal growth during MIC determination. A loopful from each of the test tubes was subcultured on Potato Dextrose agar. The plates were incubated for 72 hours at 28 ± 2ºC [19].

Statistical Analysis

Experimental values were given as means ± standard deviation (SD). Statistical significance of data were analyzed at $P \leq 0.05$ (Independent-Samples T Test) using statistical package for social sciences (SPSS, Armonk, NY, USA) version 20.

Results and Discussion

Figure 1 shows the antimicrobial activity of *Armellaria mellea* methanol extract on the test microorganisms. *S. aureus* was most susceptible to the extract followed by *C. glabrata* and *E.coli* while *B. cereus, E.coli* and *C. albicans* were not inhibited even at the highest concentration of 500 mg/ml. However, inhibition of the antibacterial and antifungal control for the test organisms were significantly higher ($p < 0.05$) than that of the extract.

Figure 2 shows the antimicrobial activity of *Armellaria mellea* ethanol extract on the test microorganisms. *P.aeruginosa, C. albicans* and *B. cereus* were inhibited at concentrations of 500mg/ml, 250 mg/ml and 125 mg/ml while *S. aureus* was inhibited at concentrations of 500 mg/ml and 250 mg/ml. *E. coli* and *C. glabrata* were not inhibited even at the highest concentration of 500 mg/ml. Statistically, inhibition of the antibacterial and antifungal control for the test organisms were significantly higher ($p < 0.05$) than that of the extract.

Figure 3 presents the antimicrobial activity of *Armellaria mellea* aqueous extract on the test microorganisms. The different test organisms showed varied susceptibility to the test extract. *C. glabrata* and *C. albicans* were well inhibited by the extract. *B. cereus* and *S. aureus* were only inhibited at concentrations of 500 mg/ml, 250 mg/ml and 125 mg/ml while *E. coli and P. aeruginosa* were not inhibited even at the highest concentration of 500 mg/ml. Statistically, inhibition of the antibacterial and antifungal control for the test organisms were significantly higher ($p < 0.05$) than that of the extract.

Table 1 shows the result of the average MIC and MBC of the ethanolic, methanolic and aqueous extracts of *A. mellea* on test organisms. The MIC of ethanolic extract of *A. mellea* varied between 62.50 and 250 mg/ml with MBC of 62.50 to 250 mg/ml. The MIC of methanolic extract of *A. mellea* varied between 62.5 and 125 mg/ml for *B. cereus* and *S. aureus* with MBC of 125 mg/ml where as the aqueous extract of *A. mellea* showed MIC of 125 mg/ml for *B. cereus* and *S. aureus* with MBC of 125mg/ml for *B. cereus* and *S. aureus* while other organisms showed no activity.

Table 2 shows the result of the average MIC and MFC of the ethanolic, methanolic and aqueous extracts of *A. mellea* on test organisms. MIC of ethanolic extract of *A. mellea* showed 62.5 mg/ml with MFC of 125 mg/ml for *C. albicans* with no activity on *C. glabrata*, the MIC of methanolic extract of *A. mellea* showed 62.5 mg/ml for *C. glabrata* with MFC of 125 mg/ml with no activity on *C. albicans* whereas the MIC of aqueous extract of *A. mellea* showed 31.25 mg/ml for *C. albicans* and 31.25 mg/ml for *C. glabrata* with MFC of 62.5 mg/ml for *C. albicans* and 31.25 mg/ml for *C. glabrata*.

Herbal plants are among the most commonly used antimicrobial agents in food and have been used traditionally for thousands of years in the control of various health complications including infectious diseases. Antimicrobial activity of the crude extract of *A. mellea* as well as phytochemical characteristics were studied. The results indicated that extracts from mushroom have antimicrobial properties as reported by [15, 16, 17, 18, 2]. It is interesting to note that the pathogenic microorganism, *Pseudomonas aeruginosa,* which is resistant to conventional synthetic antibiotic like gentamicin was found to show susceptibility to ethanol extracts of *A. mellea.* Mushrooms produce various antiviral, antifungal compounds to survive in the wild against competing or pathogenic agents [2]. Also observed in this study is that there were variations in the degree of antimicrobial activities of mushrooms. This result is in agreement with the reports of Akyuz *et al*. [20] in Turkey and that of Jaggadish *et al*. [21].

The broad spectrum activity of mushrooms was also brought to light as the extracts of mushrooms showed inhibitory effects on clinical isolates used for this investigation. This suggests that the bioactive products which are contained in mushrooms are in concentrations which exude varying degrees of antimicrobial activity. It is interesting to note from the results of this study that clinical isolates both Gram positive and Gram negative bacteria were sensitive to the extracts. This is in collaboration with the findings of Onyeagba *et al*. [22] and Desouza *et al*. [23]. The sensitivity of isolates to the mushroom extracts implies that intrinsic substance in the extracts is unknown to the microorganisms which made it impossible for them to resist. The variations in the antimicrobial activities of *A. mellea* extracts may be due to the differences in their bioactive compositions or concentrations, methods of extraction and mechanism of action of active ingredients [16, 17].

Conclusion

This research has further illuminated the medicinal value of *A. mellea*. From the present study, it can be concluded that *A. mellea* possesses quantities of compounds which have potent antimicrobial activity. Therefore, they have lots of potentials for use in the production of novel drugs and medicines, considering the lingering threat of multi-drug resistance. Furthermore, clinical evaluation of *A. mellea* through *in vivo* based research is highly recommended to achieve low cost, less side effect treatment and prevent recurrent infections.

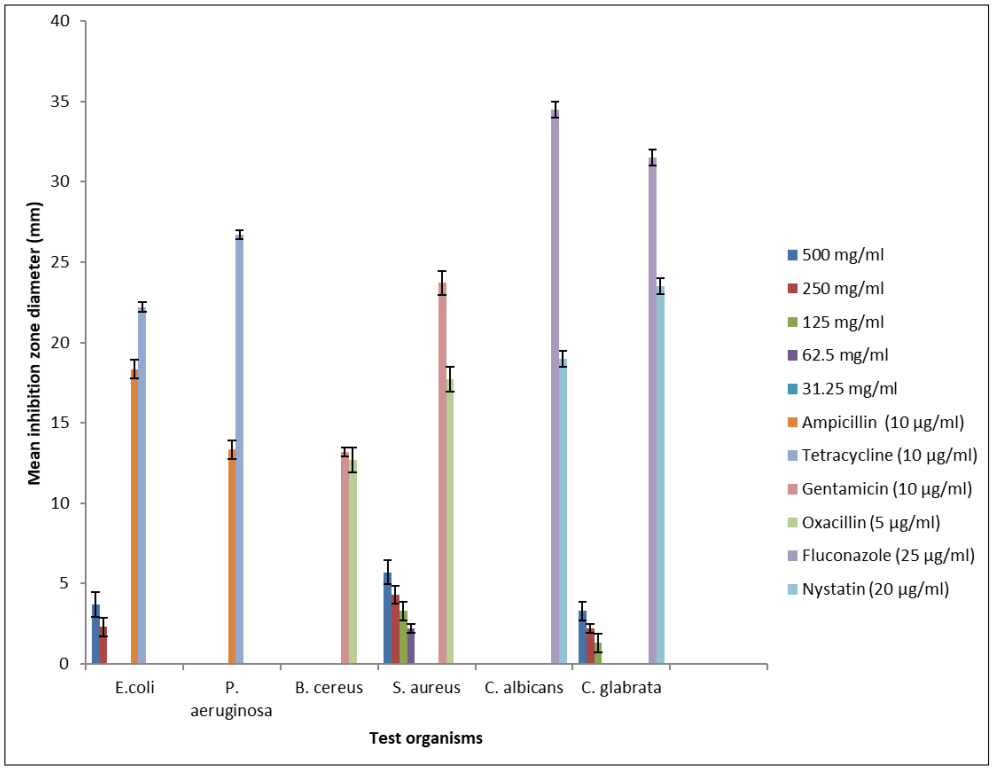

Fig 1: The antimicrobial activity of *Armillria mellea* methanol extract on the test organisms

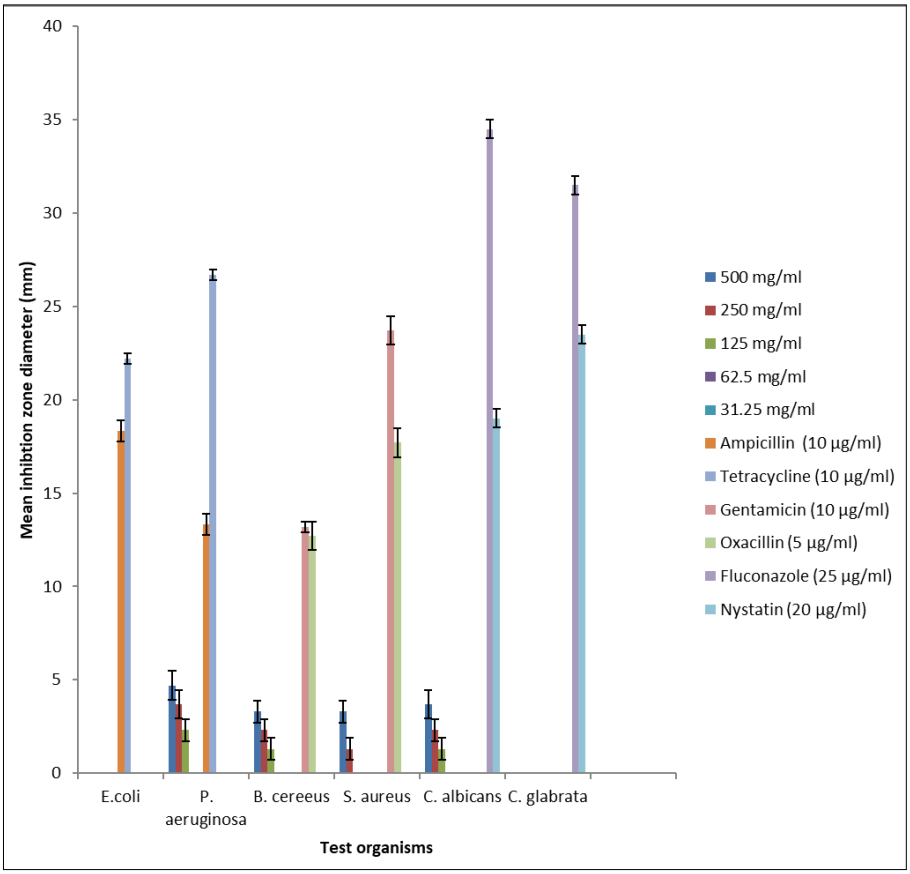

Fig 2: The antimicrobial activity of *Armillria mellea* ethanol extract on the test organisms

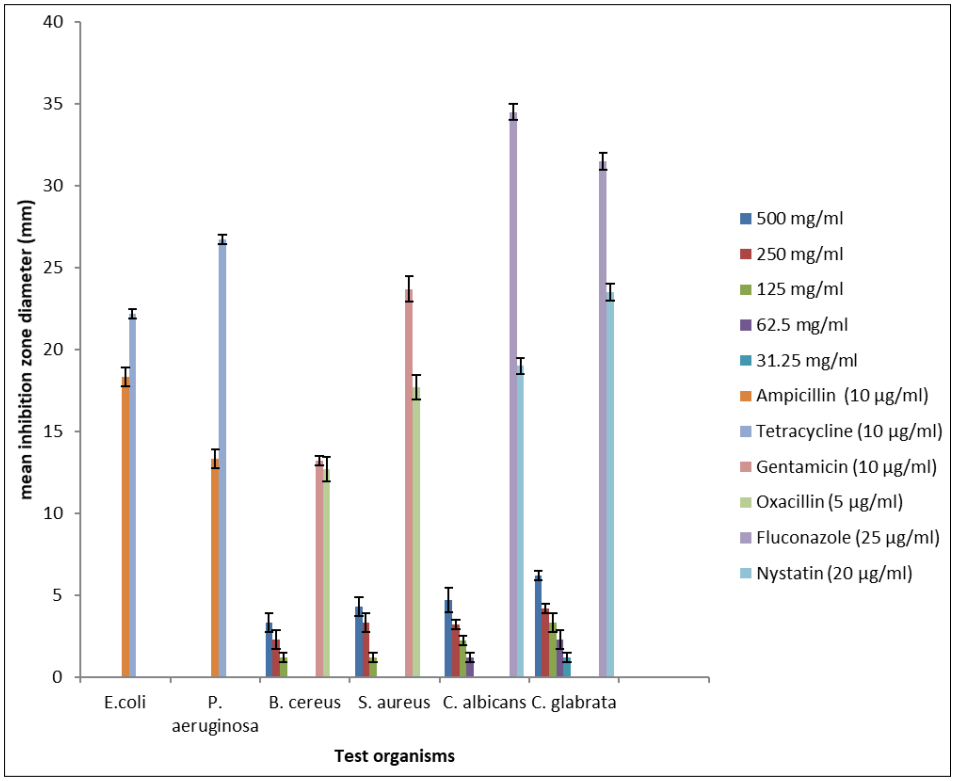

Fig 3: The antimicrobial activity of *Armillria mellea* aqueous extract on the test organisms

Table 1: The MIC and MBC of the crude extract of *A. mellea*

Extract	Test organism	MIC (mg/ml)	MBC (mg/ml)
Ethanol	*B. cereus*	ND	ND
	S.aureus	62.5	62.5
	P. aeruginosa	62.5	125
	E.coli	250	250
Methanol	*B. cereus*	62.5	125
	S.aureus	125	125
	P. aeruginosa	ND	ND
	E.coli	ND	ND
Aqueous	*B. cereus*	125	125
	S.aureus	125	125
	P. aeruginosa	ND	ND
	E.coli	ND	ND

ND = NOT Determined

Table 2: The MIC and MFC of the crude extract of *A. mellea*

Extract	Test organism	MIC (mg/ml)	MFC (mg/ml)
Ethanol	*C.albicans*	62.5	125
	C.glabata	ND	ND
Methanol	*C.albicans*	ND	ND
	C.glabata	62.5	125
Aqueous	*C.albicans*	31.25	62.5
	C.glabata	31.25	31.25

ND = Not Determined

Table 3: Phytochemical analysis of *Armillria mellea* in different Solvent

Mushroom name	Solvents	Gly	Tan	Sap	Fla	Car	Pro	Alk
Armillria mellea	Ethanol	++	-	++	+	+	++	+
	Methanol	+	++	++	-	++	+	+
	Aqueous	++	-	+	+	+	+	-

- = not present, + = present in low concentration, ++ = moderate, +++ = present in high concentration, Gly = Glycoside, Tan = Tannins, Sap = Saponins, Fla = Flavonoids, Car = Carbohydrates, Pro = Proteins, Alk = Alkaloids.

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