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ANTIMICROBIAL ACTIVITY OF *PLEUROTUS SQUARROSULUS* ON CLINICAL PATHOGENIC BACTERIA AND FUNGI

ABSTRACT

Aim: To evaluate the antimicrobial activities of *Pleurotus squarrosulus* mushroom extracts on bacterial and fungal isolates.

Study design: *Pleurotus squarrosulus* was obtained from different sources in Umuahia North Local Government, Abia state, Nigeria and identified in the Department of botany, University of Nigeria, Nsukka.

Place and duration of study: Antimicrobial activities of *Pleurotus squarrosulus* was carried out in the department of microbiology between January 2016 and August 2016

Methodology: *Pleurotus squarrosulus* was extracted using ethanol, methanol and aqueous. Antimicrobial susceptibility tests were carried out by agar well diffusion technique using National Committee of Clinical Laboratory Standard. Qualilative phytochemical analysis was carried out using standard methods.

Results: Methanol, ethanol and aqueous extracts of *Pleurotus squarrosulus* were tested against *E.coli, B. cereus, S. aureus, P. aeruginosa, C. albicans* and *C. glabrata.* The different test microorganisms showed varied susceptibility to the test extracts. All the test organisms were inhibited by methanol, ethanol and aqueous extract at varied concentrations ranging between 500 mg/ml and 125 mg/ml. Statistically, inhibition of the antibacterial and antifungal control for the test organisms were significantly higher ($P < 0.05$) than that of the extracts. The phytochemical analysis revealed the presence of saponin, carbohydrates, tannins, flavonoids and proteins in all the extracts while glycoside and alkaloids, were found in some.

Conclusion: The finding of this result suggest that *Pleurotus squarrosulus* possess broad-spectrum antimicrobial activity. The potential of developing antimicrobials from plants appear rewarding.

Key words: *Pleurotus squarrosulus, antimicrobial activities, phytochemicals, bacteria, yeast*

1. INTRODUCTION

Time immemorial, mushrooms have been used as a part of regular diet due to their nutritional and medicinal values [1]. Mushrooms have been found to contain minerals, vitamins and nutritive compounds, proteins, polysaccharide and a low fat content [2]. Mushrooms are also rich sources of natural antibiotics. Their cell wall glucans have been known to poses immunomodulatory properties with many of their secondary metabolites combating bacteria, fungi and viruses [3, 4, 5, 6, 7, 8, 9]. Prior to the discovery of their high medicinal value, mushrooms have been used for hundreds of years in traditional medicine for curing various types of diseases such as antimicrobial, antioxidant, antiviral, anticancer, antitumor, anti-inflammatory, cardiovascular diseases, immunomodulating, central activities [10, 11].

Besides, mushroom has been used extensively in traditional medicine for curing of various types of diseases [12, 13, 14]. For centuries, mushrooms have been prescribed for treatment of diseases such as gastro-intestinal disorder, bleeding, high blood pressure and various bacterial infections [15]. While some of the medicinal values associated with mushroom must have arisen from surperstitious beliefs and

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myths, they have provided information for curiosity research studies. Research has shown that some of these claims are not mere myth but are authentic [16, 17]. Besides medicinal and nutritional use, mushroom can be used as natural dyes for fabrics [18].

Pleurotus squarrosulus is a common edible mushroom. It was first cultivated in Germany as a subsistence measure during World War I [19] and is now grown commercially around the world for food. It is related to the similarly cultivated "king oyster mushroom". *Pleurotus squarrosulus* can also be used industrially for mycoremediation purposes. *Pleurotus squarrosulus* is one of the more commonly sought wild mushrooms, though it can also be cultivated on straw and other media. It has the bittersweet aroma of benzaldehyde (which is also characteristic of anise or almonds) [20].

The mushroom has a broad cap spanning 5–25 cm; natural specimens range from white to gray or tan to dark-brown; the margin is inrolled when young, and is smooth and often somewhat lobed or wavy. The flesh is white, firm, and varies in thickness due to stipe arrangement. The gills of the mushroom are white to cream, and descend on the stalk if present. If so, the stipe is off-center with a lateral attachment to wood. The spore print of the mushroom is white to lilac-gray, and best viewed on dark background. The mushroom's stipe is often absent. When present, it is short and thick. Due to the dearth in literature on the dual value of *Pleurotus squarrosulus* as food and its antimicrobial efficacy, this study was designed.

2. MATERIALS AND METHODS

2.1 Collection and identification of materials

Pleurotus squarrosulus 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.

2.2 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.

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2.3 Standard antimicrobials

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

2.4 Sample preparation and extraction

Fresh *Pleurotus squarrosulus* mushrooms were thoroughly washed with clean 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 300 ml ethanol, cold water, and methanol for 24 hours with intermittent shaking. Each sample was filtered using Whatman №1 filter paper. The filtrate was poured into a crucible and allowed to dry under steady air current in order to obtain the extract which was scooped and poured into well-labeled sample bottles and stored at 4ºC [21].

2.5 Inoculum preparation

Pure cultures of *Escherichia coli* JCM 20135 and *Bacillus cereus* IFO 13804 were obtained from the 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 22018were obtained from Spectramedics Laboratories, Sagamu, Ogun State, Nigeria. Inoculum was prepared by emulsifying overnight colonies from an agar medium. A 0.5 McFarland standard (equivalent to approximately 10^8cfu/ml) was used. Media plates were inoculated within 30 minutes of standardizing the inoculum to avoid changes in inoculums density.

2.6 Determination of antimicrobial activity of mushroom extracts

Antimicrobial activity of mushroom extracts was determined according to the National Committee of Clinical Laboratory Standards [22]. Agar well diffusion method on Sabouraud dextrose agar (SDA) and Muller-Hinton agar were used for fungi and bacteria respectively. Up to 100 µl of the inoculum was poured onto the agar plate and spread with glass rod under sterile conditions. Wells (6mm diameter) were bored into the agar using sterile cork-borer and 0.1 ml of different concentrations of the extracts (500, 250, 125, 62.5, 31.25, 15.63 and 7.81 mg/ml) was applied into each well. Negative control wells were filled with dilute dimethylsulfoxide while positive controls were antibiotic discs of tetracycline (10 µg/ml); ampicillin (10 µg/ml) for Gram negative bacteria isolates and oxacillin (5 µg/ml); gentamicin (10 µg/ml) for Gram positive bacteria isolates. Antifungal discs of fluconazole (25 µg/ml) and nystatin (20 µg/ml) (Oxoid, United Kingdom) were used as positive controls for fungal isolates.This procedure was done in triplicate for the entire test organisms, allowed to stand for 30 minutes on the bench and incubated for 24 hours at 37±2 ^{0}C for bacteria and 72 hours at 28±2 ^{0}Cfor yeast. After

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incubation, the inhibition zone diameters produced by the different concentrations of the crude extracts were measured (in millimeter) and recorded. Antimicrobial activities were expressed in terms of the mean value of the inhibition zone produced by the mushroom extracts.

2.7 Determination of minimum inhibitory concentrations (MICs) of the mushroom 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± 2℃ for 24 hours and yeast culture incubated at 28± 2℃ 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 [23].

2.8 Determination of minimum bactericidal concentrations (MBCs) of the mushroom 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℃± 2℃. The MBC was determined as the least concentration that showed no visible growth on the plate [23].

2.9 Determination of minimum fungicidal concentrations (MFCs) of the mushroom extracts

MFC was determined by selecting tubes that showed no fungal growth during MIC determination. A loopful from each of the test tubes was sub-cultured on Potato Dextrose agar. The plates were incubated for 72 hours at 28 ± 2℃. The MFC was determined as the least concentration that showed no visible growth on the plate [23].

2.10 Statistical analysis

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

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3. RESULTS AND DISCUSSION

Natural products not only provide valuable components but also an important source of bioactive compounds that provide lead information for developing useful synthetic compounds. Mushrooms contain a large number of biologically active components that impart health benefits and protection against degenerative diseases. They have been traditionally used in all over world for treatment of variety of chronic disease. Antimicrobial activity of the crude extract of *Pluerotus squarrosulus* as well as phytochemical characteristics were studied. Table 1 shows the result of the average MIC and MBC of the ethanolic, methanolic and aqueous extracts of *P. squarrosulus* on the test organisms. The MIC of ethanolic extract of *P. squarrosulus* varied between 15.63 and 31.25 mg/ml with MBC of 15.63 to 31.25 mg/ml. The MIC of methanolic extract of *P. squarrosulus* varied between 3.90 and 125 mg/ml with MBC of 7.81 to 125 mg/ml while the MIC of aqueous extract of *P. squarrosulus* varied between 31.25 and 62.50 mg/ml with MBC of 31.25 to 125 mg/ml.

Table 2 shows the result of the average MIC and MFC of the ethanolic, methanolic and aqueous extracts of *P. squarrosulus* on test organisms. The MIC of ethanolic extract of *P. squarrosulus* showed 15.63 mg/ml for *C. albicans* and 125 mg/ml for *C. glabrata* with MFC of 31.25 and 125 mg/ml, respectively, the MIC of methanolic extract of *P. squarrosulus* showed 250 mg/ml for *C. albicans* while *C. glabrata* showed no activity with MFC of 250 mg/ml for *C.albicans* while the MIC of aqueous extract of *P. squarrosulus* showed 7.81 mg/ml for *C. albicans* and 62.5 mg/ml for *C. glabrata* with MFC of 15.25 and 125 mg/ml, respectively.

Table 3 shows the phytochemical analysis revealed the presence of bioactive compounds which were present at varying levels. Saponins, protein and carbohydrate were detected in all the extracts while glycosides, alkaloids, tannins and flavonoids were found in some.

Figure 1 shows the antimicrobial activity of *Pleurotus squarrosulus* methanol extract on the test organisms. The mean inhibition zone diameter varied directly with increase in extract concentration. All the tested strains of *E.coli, P. aeruginosa, B. cereus, S. aureus* and *C. albicans* were inhibited at different concentrations of 500 mg/ml to 31.25 mg/ml whereas *C. glabrata* was not inhibited by the extract even at

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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 presents the antimicrobial activity of *Pleurotus squarrosulus* ethanol extract on the test organisms. The different test microorganisms showed varied susceptibility to the extract. All the test organisms were well inhibited except *C. glabrata* that was only inhibited at concentrations of 500 mg/ml and 250 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 3 shows the result obtained for the antimicrobial activity of *Pleurotus squarrosulus* aqueous extract. *B. cereus, S. aureus, C. albicans* and *C. glabrata* were well inhibited by the extract. *E. coli* and *P. aeruginosa* 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.

The results indicated that extracts from mushroom have antimicrobial properties as reported by Nwachukwu and Uzoeto [21]. Mushrooms produce various antiviral, antifungal compounds to survive in the wild against competing or pathogenic agents [24, 25]. Also observed in this study is that there were variations in the degree of antimicrobial activities of mushrooms. 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 *Pleurotus squarrosulus* extracts may be due to the differences in their bioactive compositions or concentrations, methods of extraction and mechanism of action of active ingredients [26]. The results of the present study strengthened the outcomes of earlier works done by others that showed mushrooms produced a great variety of antimicrobial agents. For instance, it is known that the extract from fruit bodies of several *Lactarius* sp. [27, 28]; *Fomitopsis* sp. [29]; *Boletus* sp. [30]; *Cortinarius* sp. [31]; *Ganoderma lucidum*, *Navesporus floccosa* and *Phellinus rimosus* [32]; *Pleurotus tuber-regium* [33]; *Amanita caesarae*, *Armillaria mellea*, *Chroogomphus rutilus*, *Clavariadelphus truncates*, *Clitocybe geotropa*, *Ganoderma* sp., *Ganoderma carnosum*, *Hydnum repandum*, *Hygrophorus agathosmus*, *Lenzites betulina*, *Leucoagaricus pudicus*, *Paxillus involutus*, *Polyporus arcularius*, *Rhizopogon roseo*, *Sarcodon imbricatus*, *Suillus collitinus*, *Trametes versicolor*, *Tricholoma auratum*, *Tricholoma fracticum* [34]; *Lactarius deliciosus*,

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Sarcodon imbricatus and *Tricholoma portentosum* [35]; *Russula delica* [36]; *Pleurotus eryngii* var. *ferulae* [37]; *Infundibulicybe geotropa*, *Lactarius controversus*, *Lactarius delicious* and *Phellinus hartigii* [38]; *Lactarius indigo* [39] and *Stereum ostrea* [40] contain a wide range of antimicrobial activity.

4.CONCLUSION

This research has further illuminated the medicinal value of *Pleurotus squarrosulus* found in Umuahia North Local Government, Abia State Nigeria. From the present study, it can be concluded that *Pleurotus squarrosulus* possesses good 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 mushrooms through *in vivo* based research is highly recommended to achieve low cost, less side effect treatment and prevent recurrent infections.

Table 1: The MIC and MBC of crude extract of *Pleurotus squarrosulus*

Extract	Test organism	MIC (mg/ml)	MBC (mg/ml)
Ethanol	*B. cereus*	15.63	15.63
	S.aureus	15.63	31.25
	P. aeruginosa	15.63	31.25
	E.coli	31.25	31.25
Methanol	*B. cereus*	3.90	7.81
	S.aureus	31.25	62.5
	P. aeruginosa	62.5	62.5
	E.coli	125	125
	B. cereus	62.5	125
	S.aureus	31.25	31.25

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Aqueous	*P. aeruginosa*	ND	ND
	E.coli	ND	ND

ND = NOT DETERMINED

Table 2: The MIC and MFC of the crude extract of *Pleurotus squarrosulus*

Extract	Test organism	MIC (mg/ml)	MFC (mg/ml)
Ethanol	*C.albicans*	15.63	31.25
	C.glabata	125	125
Methanol	*C.albicans*	250	250
	C.glabata	ND	ND
Aqueous	*C.albicans*	7.81	15.25
	C.glabata	62.5	125

ND = NOT DETERMINED

Table 3: PHYTOCHEMICAL ANALYSIS OF *PLEUROTUS SQUARROSULUS* IN DIFFERENT SOLVENT

Solvents	Methanol	Ethanol	Aqueous
Saponin	++	+	+
Tannins	+	++	+

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Flavonoid	+	++	+
Alkaloid	+	+	-
Proteins	++	+++	+ +
Glycosides	++	+++	-
Carbohydrates	++	++	++

Legend: - = not present, + = present in low concentration, ++ = moderate, +++ = present in high concentration,

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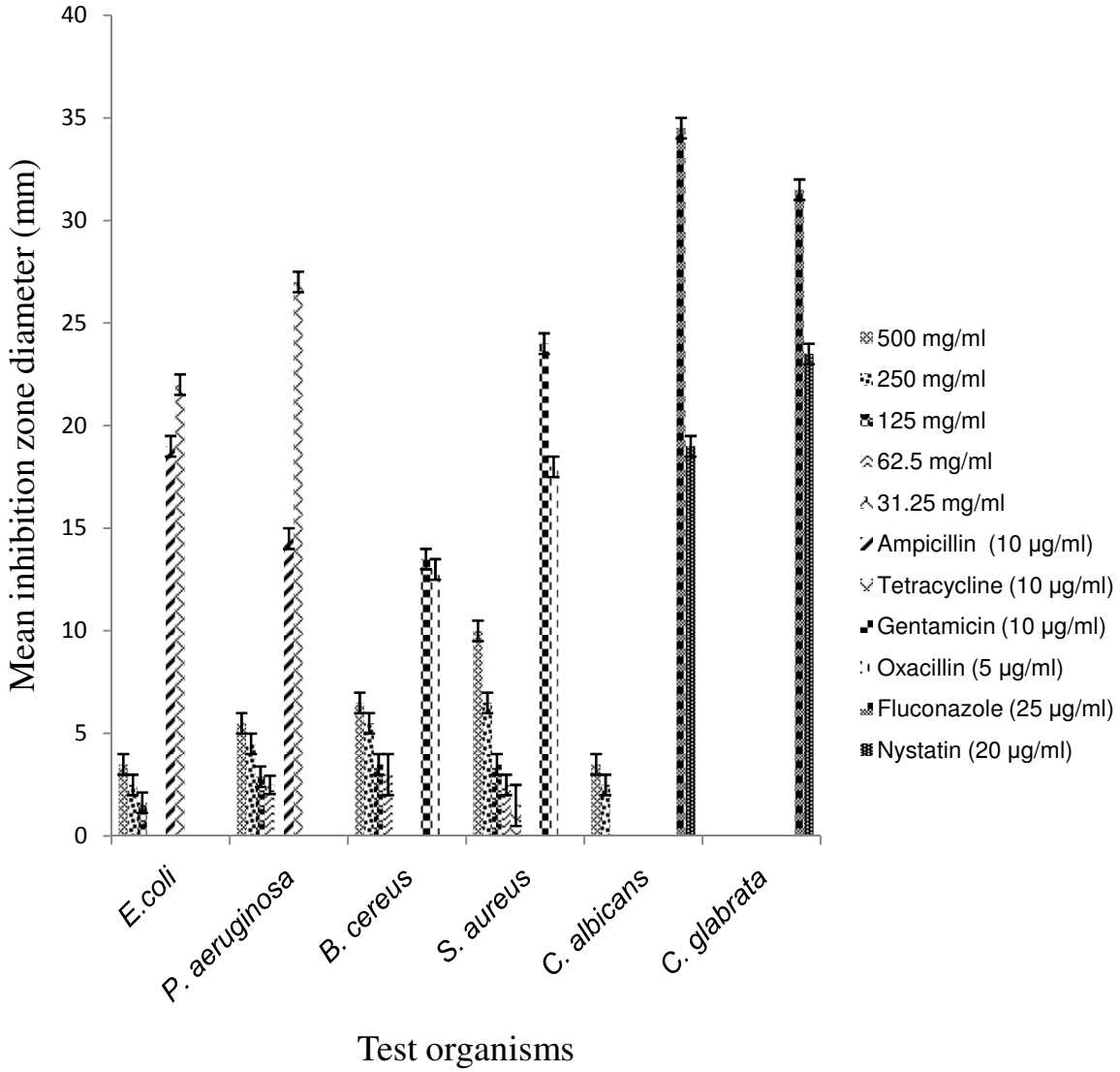

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219 Figure 1: The antimicrobial activity of *Pleurotus squarrosulus* methanol extract on the test

220 organisms

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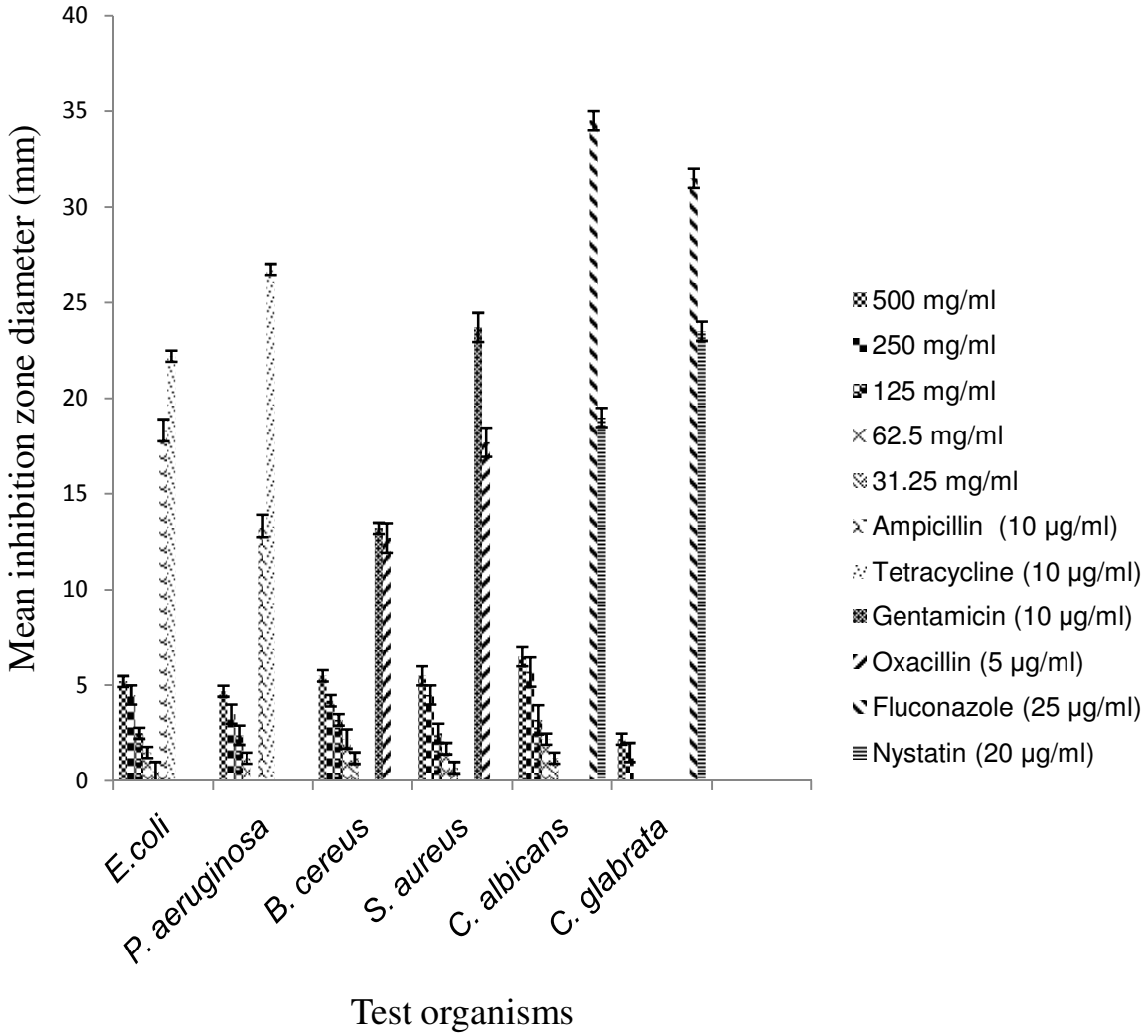

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223 Figure 2: The antimicrobial activity of *Pleurotus squarrosulus* ethanol extract on the test

224 organisms

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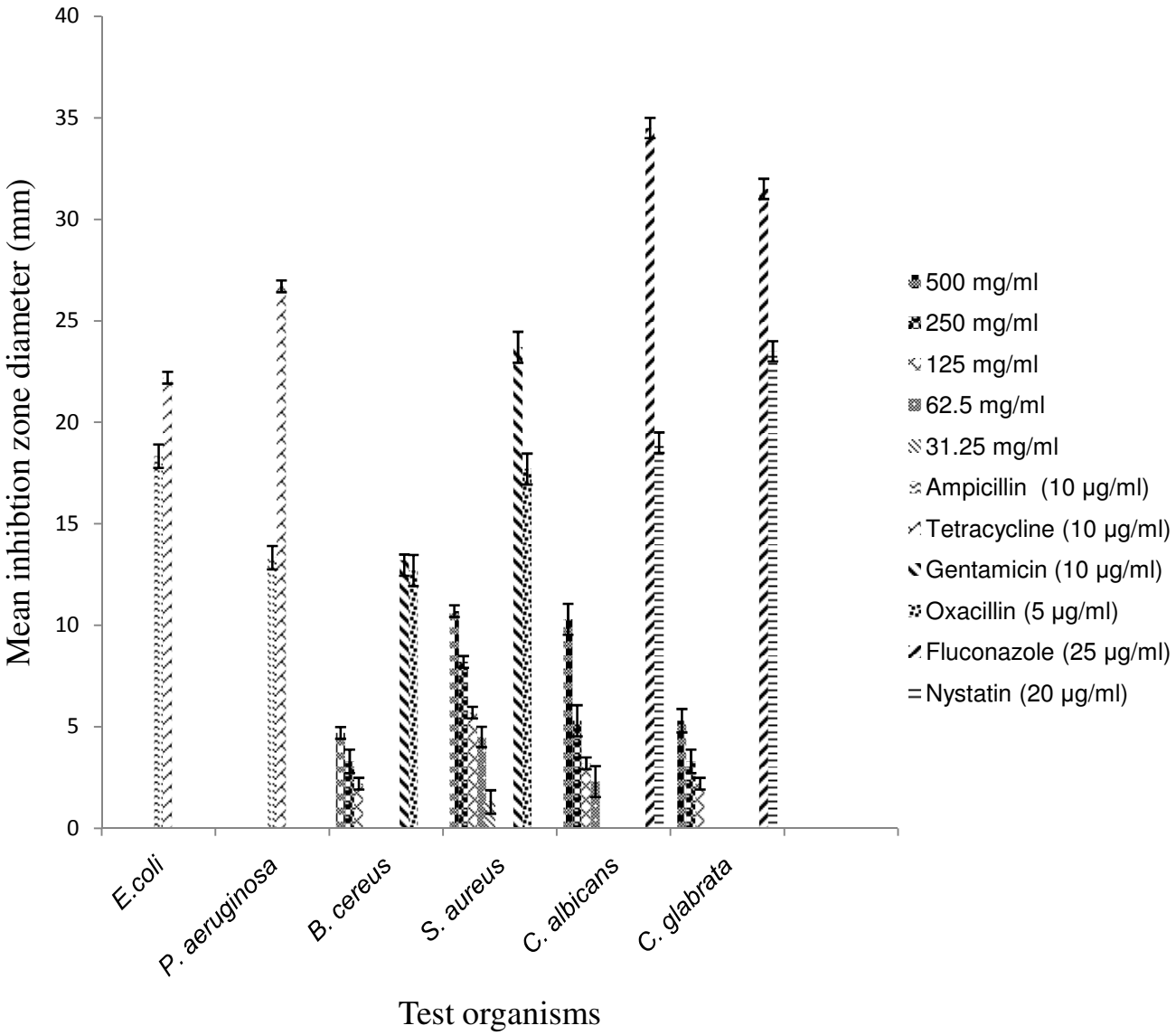

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227 Figure 3: The antimicrobial activity of *Pleurotus squarrosulus* aqueous extract on the test

228 organisms

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