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Original Article

ANTIMICROBIAL ACTIVITIES OF TWO NIGERIAN EDIBLE MACRO-FUNGI- *LYCOPERDON PUSILUM* (Bat. Ex) AND LYCOPERDON GIGANTEUM (Pers.)

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Two edible Nigerian macro-fungi – Lycoperdon pusilum and Lycoperdon giganteum were assayed in-vitro for their antimicrobial activities using water, methanol and ethanol as extractive solvents. Generally, the extracts were selectively active on few clinical pathogenic microorganisms. Ethanol was the best extractive solvent followed in order by methanol and water ($P \leq 0.05$). The best inhibitory zone (24.0mm) was recorded in ethanol extract of L. giganteum with inhibitory zone of 21.0mm. The least inhibitory zone, (4.0mm) was recorded with the aqueous extract of L. pusilum against Proteus vulgaris. The best antifungal activity (17.0mm), was recorded in L. giganteum extract against Microsporum boulardii. The minimum inhibitory concentration (MIC) for the ethanolic extract was between 0.75 and 4.0mg/ml for bacteria, and between 9.00 and 13.75mg/ml for fungi. The extracts were found to be stable at temperatures up to 50^{o}C. As the temperature was increased from 60 to 100^{o}C, there was a significant decrease in stability of the extract. The implications of these observations are discussed.

Key Words: Antimicrobial activities, macro-fungi, extractive solvents, Inhibitory zone

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INTRODUCTION

Lycoperdons otherwise known as 'Puffballs' are common edible mushrooms, which are well distributed in the tropics. (Zoberi, 1972). They belong to phyllum basidiomycota, class gasteromycetes, order Lycoperdales and family Lycoperdaceae (Miller and Miller, 1988). Puffballs grow on tree stumps, decaying logs or, on ground among the fallen leaves during the raining season (Zoberi, 1972).

Lycoperdon giganteum is an enormous puffball, which reaches 30-50cm or more in diameter. This fungus, which grows in the damp moist places, is readily noticed by its giant ball-like nature. (Smith *et al*, 1981). *Lycoperdon pusilum* is globose and about 1.5 to 2.0cm in diameter. It grows in open places, lawns and fields on moist soils. (Miller and Miller, 1988). The spherical mass of both *L. giganteum* and *L. pusilum* are attached to the ground by short rod like cord which is covered with a white or greyish envelop (Zoberi, 1972).

Mushrooms producing antibiotics are undoubtedly numerous, but so far, they have not been sufficiently studied especially in their natural habitat. Traditional doctors have successfully used puffballs to cure sores, abrasion or bruises, deep cut, h*emorrhage* as well as urinary tract infection (Buswell and Chang, 1993; Oso, 1977) but, these claims have not been proved scientifically. So much work has been carried out on the antimicrobial activities of lower fungi but higher fungi especially edible mushrooms have not been adequately explored. Therefore, the objective of this work was to provide information on the antimicrobial activities of *L. pusilum* and *L. giganteum* from Nigeria.

MATERIAL AND METHODS

Test microorganisms and Culture Condition: The bacteria used include Bacillus cereus, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris, Pseudomonas aeruginosa* and *Staphytococcus aureus*. The test fungi

include *Aspergillus niger, Aspergillus flavus, candida albicans, Microsporum boulardii and Trichophyton concentrum*.

The stock culture of these organisms had already been identified and typed. All bacteria were cultured aerobically at 37^{o}C for 24 hours on was blood agar and antimicrobial testing were carried out on the nutrient agar plates. All fungi were also grown aerobically at 30^{o}C on the saboraud agar plates.

Mushroom Samples

Lycoperdon pusilum (Bat. Ex) and *L. giganteum* (Pers.) were collected from Imini village in Afijio Local Government Area of Oyo State. They were identified by comparing their morphological, anatomical and physiological characteristics with the standard description of Zoberi (1972) and that of Alexopolous et al (1996).

Extract Preparation: The extraction of the mushroom sporophores was carried out using three solvents (water, methanol and ethanol). For water extraction, 2 litres of sterile distilled water were dispensed into a conical flask containing 200g of powdered mushroom sporophores. This was allowed to stand for 72hours with intermittent agitation. The mixture was filtered using Whatman's filter paper No. 1 and the filtrate was concentrated under a reduced pressure in a rotatory evaporator until a semi solid substance was obtained. This was dried inside the crucible under a controlled temperature (45^{o}C) to obtain solid extract.

For methanol and ethanol extraction, 200g of the pulverized sporophore was separately soaked in 3 litres of absolute methanol and ethanol inside 5 litre conical flasks. These were covered with aluminum foil and allowed to stand for 7 days for extraction.

Antimicrobial Activities of Mushroom Extracts: The antimicrobial activities of aqueous, methanolic and ethanolic extracts were determined by filter paper disc and agar well diffusion methods of Norrel and Messely (1997).

Sterile filter paper discs (7.0mm diameter) were soaked with the test extracts and dried at 40^{o}C for 1 hour. The disc was placed on a bacteria seeded plate and placed in the refrigerator for 12 hours to allow the diffusion of the extract into the growing medium. The plates were incubated for 24 hours at 37^{o}C after which the zone of inhibition was observed and measured. Each experiment was replicated 3 times. The concentration of the extract used was 20mg/ml and sterile distilled water was used in the dilution of extracts.

In the agar well diffusion method, pure isolate of each bacterium was cultured in peptone water for 18 hours. These were seeded on the sterile nutrient agar plates containing 7mm wells. Drops of the extract were introduced into the bore agar well and incubated as described in the previous experiment.

For the fungi, 20mg of the extract were mixed with 5g of ointment base and introduced into the bore wells on the saboraud agar plates.

Minimum Inhibitory Concentration (MIC): This test was carried out according to the method described by Hirasawa *et al* (1999). Different concentrations (1.0 – 2.0mg/ml) were prepared using sterile distilled water as the diluent. Agar well diffusion method was used and the test was carried out in triplicate and, their means recorded.

Effect of heat Treatment on the Antimicrobial Efficacy of the Extracts: Ethanol extract (which was observed as the most effective of all the extracts) was used for this study. Standard concentration (20.0mg/ml) was prepared for *L. pusilum* and *L. giganteum* ethanolic extracts and heated at 50, 60, 70, 80, 90 and 100^{o}C. These were tested against microorganisms using hole diffusion method. Incubation was carried out at 37^{o}C for 48hours. The size of the inhibitory zones were observed and recorded.

RESULTS AND DISCUSSION

From the results obtained, it could be observed that ethanol was the best solvent for extracting antimicrobial substances from these mushrooms (Tables 1,2 and 3). This suggestion was based on the number of organisms inhibited and the diameter of inhibitory zones produced. It could also be seen from Table 1 that, *L. pusilum* and *L. giganteum* extracts were different in their antimicrobial effectiveness depending on the extractive solvent used. This result agrees favourably with the suggestion of Oloke and Kolawole (1988), that bioactive components of any medicinal plant may differ in their solubility depending on the extractive solvents used. Takazawa *et. al.*, (1982), suggested that there is a need to employ broad range of extractive solvents in the extraction of possible phytochemicals from medicinal plants. Kawagishi *et. al.,* (1988), observed that some of the active polysaccharides and their haptenes are soluble in alcohol but insoluble water as in the case of *Agaricus blazei*.

Table 1 shows that water and methanol were not good solvents of extracting *L. pusilum* and *L. giganteum* fruit bodies because they could not be compared with the effectiveness of ethanol ($P \leq 0.05$). The widest inhibitory zone (19.0mm) in the filter paper disc method (Table 1) was demonstrated by *L. pusilum* against *E. coli* for the ethanolic extract. But, this value dropped to 15.0 and 12.0mm respectively when methanolic and aqueous extracts were tested against the same bacterium.

Table 1: Antibacterial Activities of *L. pusilum* and *L. giganteum* Extracts Using Filter Paper Disc Method.

Test Bacteria	Aqueous Extract Lyp	LyG	Methanol Extract LyP	LyG	Ethanol Extract LyP	LyG
	Zone of Inhibition (mm). Mean of three replicates					
B. cereus	9.0a	5.0a	10.0b	9.0a	13.0b	12.0b
E. coli	12.0a	8.0a	15.0a	11.0a	19.0a	17.0a
K. pneumoniae	I	7.0a	11.0b	9.0a	14.0b	11.0b
P. vulgaris	4.0b	9.0a	8.0b	11.0a	10.0bc	12.0b
P. aeruginosa	I	I	I	7.0a	13.0b	13.0b
S. aureus	5.0b	8.0a	10.0b	7.0a	14.0b	9.0b

Keys: *LyP = L. pusilum, LyG = L. giganteum, I = Inactive values followed by the same letter along each vertical column are not significantly different by Duncan's multiple range test (P=0.05).*

Table 2: Antibacterial activities of *L. pusilum* and *L. giganteum* extracts Using hole diffusion Method

Test Bacteria	Aqueous Extract Lyp	LyG	Methanol Extract LyP	LyG	Ethanol Extract LyP	LyG
	Zone of Inhibition (mm). Mean of three replicates					
B. cereus	13.0a	7.0a	15.0ab	12.0bc	18.0b	14.0bc
E. coli	15.0a	10.0a	20.0a	16.0a	24.0a	21.0a
K. pneumoniae	I	9.0a	16.0ab	12.0bc	19.0b	16.0b
P. vulgaris	7.0b	11.0a	12.0b	14.0ab	15.0b	17.0b
P. aeruginosa	I	I	I	10.0c	17.0b	15.0bc
S. aureus	10.0b	11.0a	14.0b	11.0c	17.0b	13.0c

Legend: *LyP = L. pusilum, LyG = L. giganteum, I = Inactive. Values followed by the same letter along each vertical column are not significantly different (P = 0.05).*

Also, 17.0mm was observed for *L. giganteum* inhibitory zone against *E. coli* for the ethanolic extract whereas, 11.0 and 8.0mm were recorded for methanolic and aqueous extracts respectively. Antibacterial properties exhibited by *L. pusilum* and *L. giganteum* confirms the report of Buswell and Thang (1993), that traditional doctors have successfully used Lycoperdons to cure sores, abrasions and wound infections.

When filter paper and hole diffusion methods were compared, the latter was found as the more sensitive method of evaluating antimicrobial activities of *L. pusilum* and *L. giganteum* extracts (Table 1 & 2). The zone of inhibition was 24.0mm for *L. pusilum* against *E. coli* when hole diffusion method was used whereas, it was 19.0mm for the filter paper disc method (Tables 1 and 2). Toda *et al* (1991), suggested that in hole diffusion method there is a better contact and diffusion of the extracts into the media and organisms, but, filter paper disc may act as barrier between the extract and the organisms. There may not be proper diffusion and total release of active components adsorbed by the discs into the media.

The best antifungal activity (17.0mm) was recorded in *L. giganteum* ethanolic extract against *M. boulardil* (Table 3). This suggests that this higher fungus is a promising antifungal agent. Generally, the observed values for all other extracts against pathogenic fungi were low. This result supports the suggestion of Takazawa *et al* (1982), that antifungal antibiotics are not common among basidiomycetes.

Table 3: Antifungal activities of *L. pusilum* and *L. giganteum* extracts

Test Fungi	Aqueous Extract LyG	Methanol Extract LyP	Methanol Extract LyG	Ethanol Extract LyP	Ethanol Extract LyG
	***Zone of Inhibition (mm). Mean of three replicates**				
A. niger	I	5.0b	6.0b	9.0a	10.0a	11.0b
A. flavus	7.0a	I	8.0ab	I	12.0a	I
C. albicans	I	8.0a	9.0a	10.0a	11.0a	11.0b
M. boulardii	I	I	I	5.0b	I	17.0a
T. concentrum	5.0a	I	6.0b	I	7.0b	I

Keys: LyP = *L. pusilum*, LyG = *L. giganteum*, I = Inactive *Values followed by the same letters along each vertical column are not significantly different (P = 0.05).

Table 4: Minimum Inhibitory Concentration (MIC) of *L. pusilum* and *L. giganteum* extracts against the test microorganisms

Test Microorganisms	**Aqueous Lyp**	**Extract LyG**	**Methanol Lyp**	**Extract LyG**	**Ethanol Lyp**	**Extract LyG**
	M I C (mg/ml). Mean of three replicates					
B. cereus	5.73c	6.25c	2.75b	3.00bc	1.50b	1.50b
E. coli	7.25c	5.00c	3.00b	1.75c	1.25b	0.75b
K. pneumoniae	N.D	N.D	3.75b	5.00b	2.00b	3.75b
P. vulgaris	10.50bc	9.25bc	5.25b	6.50b	3.25b	4.00b
S. aureus	11.25b	4.75c	4.00b	5.75b	2.75b	3.50b
A. niger	N.D	20.0a	13.00a	14.75a	11.75a	10.50a
A. flavus	18.25a	19.25a	15.00a	15.00a	13.25a	13.50a
C. albicans	N.D	N.D	15.25a	13.50a	12.75a	10.50a
M. boulardii	N.D	N.D	N.D	15.00a	N.D	13.50a

Legend: LyP = *L. pusilum*, LyG = *L. giganteum* ND = Not detectable
*Values followed by the same letters along each vertical column are not significantly different (P = 0.05).

Table 5: Effect of Heat Treatment on the Antimicrobial Efficacy of *L. pusilum* and *L. giganteum* extracts

Mushroom Extract/Temperature (^{o}C)	*Zone of Inhibition (mm)					
	B. cereus	*E. coli*	*K. pneumoniae*	*S. aureus*	*C. albicans*	*A. niger*
L. pusilum (50)	19.0a	27.0a	21.0a	18.0a	15.0a	11.0a
L. giganteum (50)	16.0ab	23.0a	17.0ab	15.0ab	12.0ab	13.0a
L. pusilum (60)	18.0a	18.0bc	17.0ab	13.0b	10.0ab	10.0ab
L. giganteum (60)	16.0ab	22.0ab	13.0bc	12.0bc	10.0ab	9.0ab
L. pusilum (70)	16.0ab	10.0c	15.0b	10.0c	8.0bc	10.0ab
L. giganteum (70)	14.0b	18.0bc	13.0bc	10.0c	10.0ab	8.0b
L. pusilum (80)	10.0bc	5.0d	10.0c	8.0c	5.0c	8.0b
L. giganteum (70)	9.0c	12.0c	13.0bc	7.0cd	7.0bc	7.0b
L. pusilum (80)	7.0cd	I	I	5.0d	5.0c	7.0b
L. giganteum (80)	7.0cd	8.0cd	10.0c	4.0d	5.0c	6.0bc
L. pusilum (90)	4.0d	I	I	I	5.0c	5.0c
L. giganteum (90)	3.0d	6.0d	4.0d	3.0	3.0c	4.0c

Legend: *I = Inactive; *Values followed by the same letters along each vertical column are not significantly different (P = 0.05).*

The minimum inhibitory concentration (MIC) values of ethanol, methanol and aqueous extracts varied from 0.75 – 13.50, 1.75 – 15.0 and 4.75 – 20.0mg/ml respectively (Table 4). The MIC against *P. vulganis* in *L. giganteum* ethanolic extract was similar to that of methanolic extract of *L. pusilum* against *S. aureus*. This result indicates that *L. pusilum* and *L. giganteum* possess broad-spectrum antimicrobial properties. These 2 mushrooms offer a promising therapeutic agent that could be active at low concentrations against some medically important microorganisms.

There was an initial increase in antimicrobial effectiveness when the temperature was increased to 50^{o}C from that of room temperature (30^{o}C) (Table 5). These values dropped gradually as the temperature was increased from 50 to 100^{o}C. *L. pusilum* had activity of 27.0mm against *E. coli* at 50^{o}C but, the activity dropped sharply to 18.0mm at 60^{o}C and at 100^{o}C, it was 6.0mm. This indicates that high temperature treatment may lower the antimicrobial effectiveness of these mushrooms. This result disagrees with that of Diker *et al* (1991), who obtained no change in the antimicrobial activities of tea extracts. But, the result agrees with that of Hirasawa *et al* (1999), who obtained similar gradual decrease in the antimicrobial effectiveness of the extracts of *L. edodes* as the temperature, was elevated.

In conclusion, this study has shown that different extracts (aqueous, methanol and ethanol) have been used in-vitro to inhibit the growth of some disease causing bacteria and fungi. It can therefore be suggested that, they are promising antimicrobial agents. Work is already in progress in identifying bioactive phyto-chemicals in *L. pusilum* and *L. giganteum*. The next report will also be focused on comparing the properties of these extracts with that of the commonly used standard antibiotics.

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