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Study on the Antibacterial Activity of Exopolysaccharides of *Lentinus subnudus* Using Swiss Albino Rats as Animal Model

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Abstract: The antibacterial properties of exopolysaccharides of *Lentinus subnudus* were investigated. The exopolysaccharides of *L. subnudus* was obtained by submerge fermentation using minimal salt media composition. The crude exopolysaccharides was harvested and injected intraperitoneally in aqueous solution (20mg ml^{-1}) into groups of Swiss albino rats infected with pathogenic strains of *Escherichia coli* and *Pseudomonas aeruginosa*. The prophylactic activities of the exopolysaccharides were monitored when administered 7 and 2 days before bacteria induction while therapeutic activities were monitored by administering the exopolysaccharides 1 hour after the bacterial induction. The exopolysaccharides injected into albino rats were seen to be active and showing improved haematological parameters, high sperm motility and reduction in the bacterial load per gram liver weight. Animals infected with pathogenic *E. coli* and *P. aeruginosa* without the exopolysaccharides had a low sperm count of 6.4±1.44 x 10^6 and 8.53±2.05 x 10^6/ml respectively while the control group showed an improved count of 28.08±2.36 x 10^6/ml. In summary, the exopolysaccharides of *L. subnudus* possesses antibacterial properties and is a, non-toxic substance of medicinal value.

Key words: *L. subnudus* · Exopolysaccharides · Prophylactic · *E. coli* · *P. aeruginosa*

INTRODUCTION

Mushrooms are ubiquitous group of fungi with many uses. They are used extensively, as food item and for medicinal purposes [1- 5] and due to their high economic value and the enzymes they produce they are also very useful for research purposes and in industrial applications [6-8]. Several exopolysaccharides possessing various physiological activities originate from fungi, especially edible and medicinal mushrooms [9-13]. Previous studies on mushrooms have demonstrated many interesting biological functions such as antitumor, hypoglycemic and immune stimulating activities [14-17]. A number of reports on the microbial polysaccharides obtained from higher fungi, such *as Ganoderma lucidum*, *Cordyceps* sp., *Lentinus edodes* and *Paecilomyces sp*., have been published [11, 17-22]. *Lentinus subnudus* is a highly-prized Nigerian mushroom, which can be picked in the wild during the rainy season. Research studies on the mushroom have shown its cultivability on uncomposted and composted substrates [23]. This paper reported the antibacterial potentials of exopolysaccharides of *L. subnudus* produced by submerge fermentation.

MATERIALS AND METHODS

Strain Collection and Maintenance: L. subnudus was collected from green vegetation environment specifically from a decaying mango log of wood of about six month old and then sub-cultured on potato dextrose agar (PDA). It was maintained on PDA slants at 4°C by sub-culturing every one month interval. The ambient temperature for culture of the fungus was 25-28°C and it takes a minimum of 72 hours for optimum mycelia elongation.

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Test Organisms: Pathogenic strains of *Escherichia coli* and *Pseudomonas aeruginosa* used were collected from Department of Medical Microbiology, College of Health Sciences, University of Ilorin Teaching Hospital, Ilorin Kwara State, Nigeria.

Extraction of Exopolysaccharides: The basal medium used was composed of glucose (20g/l), peptone (2.5g/l), yeast extract (2.5g/l), KH_2PO_4 (2.0g/l), $MgSO_4.7H_2O$ (1g/l) and $CaCl_2.2H_2O$ (1g/l). The medium inoculated with the fungal mycelia was maintained at 25°C for 10 days in a rotary shaker incubator. The exopolysaccharides were isolated from the shake flask culture. The filtrate was added to four volumes of 95% ethanol to precipitate the crude polysaccharide. The filtrate was maintained at 4°C over night. The precipitated polysaccharide was collected by centrigugation at 1350 x g for 15 min at 4°C and the supernatant was discarded [24] (En-Shyh Lin and Shu-Chiao Sung, 2006). The precipitate was then re-suspended in an equal volume of 75% ethanol and centrifuge again as earlier mentioned. The precipitated exopolysaccharides were dried at 60°C to remove residual ethanol.

Preparation of Aqueous Solution of the Exopolysaccharides: 20mg of the exopolysaccharides was dissolved in 1ml of de-ionised water and prepared according to Akanni *et al*. [25].

Experiment for Determining the Antimicrobial Effect of Exopolysaccharides

Animals and Rearing Conditions: Ninety Swiss albino rats of two and half months old with an average initial body weight of (77.00±12.75 g), were fed with food and water and allowed to acclimatize for three weeks prior to experiment.

Grouping of Experimental Animals: The rats were divided into following nine groups as follows: Group 1 (ECC)-*E. coli* control; group 2 (PAC)- *P. aeruginosa* control; group 3 (CUR)-untreated rats; groups 4&5 (1PBT)-1hr Post bacteria treatment; groups 6&7 (2DPT)-2 Day prophylactic treatment and groups 8&9 (7DPT)-7 Day prophylactic treatment. ECC and PAC were groups of animals infected with *E. coli* and *P. aeruginosa* respectively without the exopolysaccharide.

Investigating the Effects of the Exopolysaccharides on Experimental Animals: Seven and two day prophylactic and one hour post bacterial infection effects of the exopolysaccharides on *E. coli* and *P. aeruginosa* induced rats were investigated by modifying the method already used by Bill *et al* [26] and Oke [27] as follows: An aliquot of 200µl (4mg) and 400µl (8mg) of 20mgml^{-1} aqueous exopolysaccharides was administered intraperitoneally into each of the animals in the seven and two day prophylactic groups while the CUR group didn't receive any. About 0.2 ml (4.0 x 10^7/ml) of each of the test organism was administered intraperitoneally into each of the animals in the 7 and 2 day post exopolysaccharides administration. Three animals from each category were killed on the 5th day while any dead animal was dissected immediately. 1g of the excised liver was weighed aseptically and homogenized in 10 ml sterile water and further diluted. The multiplication and colonization of the test organisms was assessed by inoculating 0.1 ml of each dilution on MacConkey and nutrient agar for *E. coli* and *P. aeruginosa* respectively. The bacterial load per gram liver weight was calculated.

0.2ml (4.0 x 10^7 /ml) of each of the test organisms was administered intraperitoneally into each of the animal in each group except the CUR group. After 1 hour, aliquot of 200µl (4mg) and 400µl (8mg) of 20mg/ml aqueous exopolysaccharides was also administered as earlier mentioned.

Determination of Viable Bacterial Count: 20µl of the inoculum was dropped onto a nutrient agar medium where it was spread over an area of 1.5-2.0 cm diameter. Six plates were used for each inoculum which was incubated at 37°C for 48 hours for *E. coli* and *P. aeruginosa*. The number of colonies in each drop area was counted and recorded. The mean of the six counts which gave the viable counts per ml of the dilution was calculated. The drop area showed the largest number of colonies, without confluence (≥20).

Seminal Fluid Analysis: 1:20 dilution of the spermatozoa was made in sperm fluid and allowed to stand until mucous dissolves. The mixture was shaken vigorously and filled in a blood counting chamber. 1mm^3 area of one large square was counted and multiplied by 200,000 to obtain the number of spermatozoa per ml.

Determination of Packed cell Volume (PCV): Blood samples of the experimental animals were obtained from their tails at every five day interval and collected into a heparinized capillary tube. The tubes with the sealed end outwards were centrifuged and the PCV value was read on a haematocrit reader.

Statistics: Paired-sample t-test was used for assessing the test of significance at 5% level of probability, df (n-1). Fisher's $LSD_{0.05}$ was also calculated.

RESULTS AND DISCUSSION

The bacterial counts of *E. coli* and *P. aeruginosa* test organisms were greatly reduced in the animals injected with the exopolysaccharides (eps) as compared to the control group without (eps) administration and the clean untreated rats' group respectively (Tables 1 and 2). Although, there was a least significance difference (LSD) at 5% level of probability (1.19 and 0.72) for *E. coli* and *P. aeruginosa* experiment when the means of the samples were compared at 4mg and 8mg eps concentration respectively. This indicates that at either concentration of the eps, the same antibacterial effects were noticed on the tested organisms. There was also no significant difference in the sperm motility result at 4mg and 8mg metabolite concentration ($LSD_{0.05}$ = 1.32 and 2.54) as shown in Tables 3 and 4 for *E. coli* and *P. aeruginosa* respectively. The same insignificant result was also observed in the percentages of packed cell volume of *E. coli* and *P. aeruginosa* infected rats ($LSD_{0.05}$ = 2.33 and 1.66) respectively at the same concentrations of the eps as mentioned above (Fig. 1 and 2).

In this research, we investigated the potential of eps of *L. subnudus* in the area of chemotherapy. The extracted eps showed antibacterial activity against *E. coli* and *P. aeruginosa* which are of pathogenic origin. Also, the tolerance of the experimental rats to the exopolysaccharides was an indication of its non-toxicity.

Table 1: Bacterial counts of *E. coli* infected rats treated with exopolysaccharides of *L. subnudus*

Group	4mg metabolite CFU/g liver wt x 10^4	8mg metabolite CFU/g liver wt x 10^4	LSD (P<0.05)	df (n-1)
ECC	23.00±4.24	23.00±4.24		
CUR	0.00±0.00	0.00±0.00		
1PBT	0.00±0.00	0.00±0.00	*1.19	4
2DPT	5.00±1.41	2.30±0.58		
7DPT	3.50±0.71	1.67±0.53		

ECC= E. coli control;CUR= untreated rats;1PBT= 1hr Post Bacterial Treatment; 2DPT= 2-Day Prophylactic Treatment; 7DPT= 7-Day Prophylactic Treatment.

Table 2: Bacterial counts of *P. aeruginosa* infected rats treated with exopolysaccharides of *L. subnudus*

Group	4mg metabolite CFU/g liver wt x 10^4	8mg metabolite CFU/g liver wt x 10^4	LSD (P<0.05)	df (n-1)
PAC	21.67±2.89	21.67±2.89		
CUR	0.00±0.00	0.00±0.00		
1PBT	6.00±5.65	7.00±4.24	*0.72	4
2DPT	2.30±1.15	4.30±0.58		
7DPT	4.00±4.0	7.00±6.24		

PAC= P. aeruginosa control; CUR= Clean untreated rats 1PBT= 1hr Post Bacterial Treatment; 2DPT= 2-Day Prophylactic Treatment; 7DPT= 7-Day Prophylactic Treatment.

Table 3: Percentage sperm motility of *E. coli* infected rats

Group	4mg metabolite % motility	8 mg metabolite % motility	LSD (P<0.05)	df(n-1)
ECC	10.00±4.14	10.00±4.14		
CUR	70.00±14.14	70.00±14.14		
1PBT	15.00±7.07	15.00±2.21	*1.32	4
2DPT	45.00±7.07	32.50±10.61		
7DPT	65.00±7.07	35.00±7.07		

ECC= E. coli control;CUR= untreated rats;1PBT= 1hr Post Bacterial Treatment; 2DPT= 2-Day Prophylactic Treatment; 7DPT= 7-Day Prophylactic Treatment.

Table 4: Percentage sperm motility of *P. aeruginosa* infected rats

Group	4 mg metabolite % motility	8 mg metabolite % motility	LSD (P<0.05)	df (n-1)
PAC	5.00±2.07	5.00±2.07		
CUR	70.00±14.14	70.00±14.14		
1PBT	25.00±6.23	40.00±8.28	*2.54	4
2DPT	47.50±10.61	25.00±7.07		
7DPT	25.00±7.07	25.00±9.21		

PAC= P. aeruginosa control; CUR= untreated rats 1PBT= 1hr Post Bacterial Treatment; 2DPT= 2-Day Prophylactic Treatment; 7DPT= 7-Day Prophylactic Treatment.

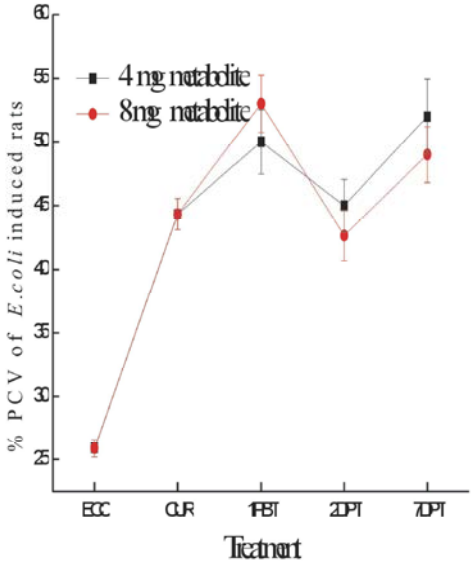

Fig. 1: Percentage of Packed Cell Volume of *E. coli* induced rats

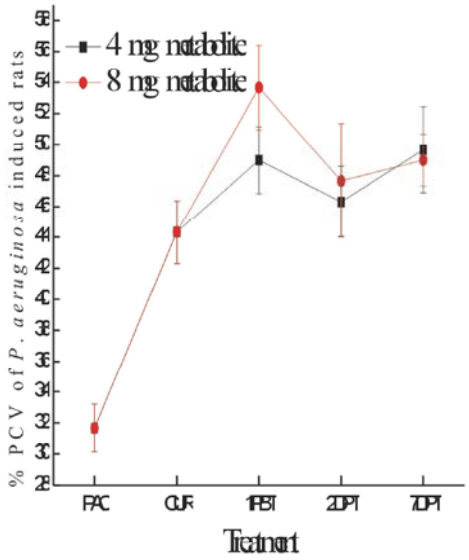

Fig. 2: Percentage of Packed Cell Volume of *P. aeruginosa* induced rats

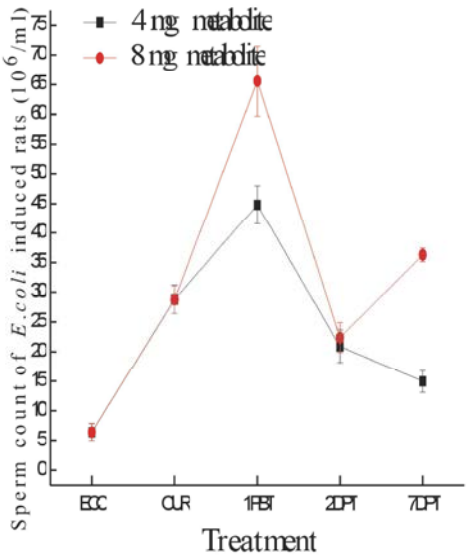

Fig. 3: Sperm count analysis of *E. coli* induced rats

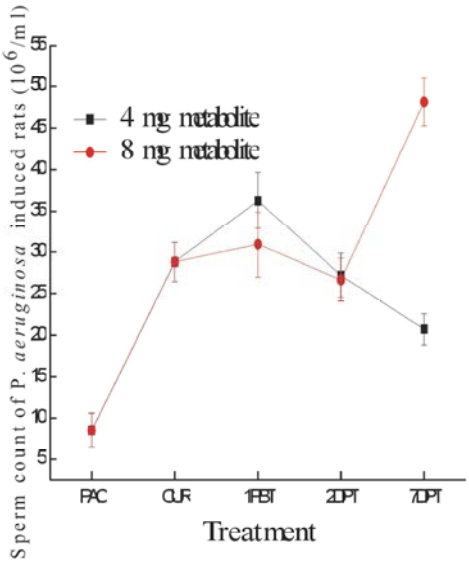

Fig. 4: Sperm count analysis of *P. aeruginosa* induced rats.

In early studies performed by Anchel, Hervey and Wilkins in 1941, diverse antibiotic activity was detected in basidiocarp or mycelia culture extracts of more than 2000 fungal species [28]. The prophylactic activity of the exopolysaccharides was ascertained when administered 7 days and 2 days before bacteria infection with no death of the animals recorded and reduction in the bacterial load per gram liver weight.

There was no bacteria in the group infected with *P. aeruginosa* and administered the exopolysaccharides 1 hour after injected bacteria. This illustrates the antibacterial potential of the exopolysaccharides. The result is in consonance with the report of Lindequist *et al*. [29] that antimicrobial compounds could be isolated from many mushroom species and some have proved to be of benefit for humans. The results of this study also revealed exopolysaccharides brought about improvement in the haematological parameters of the rats under investigation. A very low percentage of the packed cell volume (25.9 and 31.67%) was noticeable in the control rats (ECC and PAC) which did not administer the exopolysaccharides. An improvement of the percentages

of packed cell volume (PCV) of the exopolysaccharides treated rats was significantly observed as compared to the rats without exopolysaccharides and with the untreated rats control groups (Fig. 1 and 2). Our result is in line with that of Akanni *et al*. [24] who reported similar effects of the administration of metabolite of *Pleurotus ostreatus* and *P. pulmonarius* in the enhancement of the haematopoietic activity of tumour induced albino rats.

Acute and chronic infections can compromise spermatogenesis, resulting in their quantitative and qualitative reduction [30]. The animals infected with pathogenic *E. coli* (ECC) and *P. aeruginosa* (PAC) control without exopolysaccharides administration had a low sperm count of 6.4±1.44 x 10^6 and 8.53±2.05 x 10^6/ml respectively as compared with the untreated rats group (28.08±2.36 x 10^6/ml). There was significant improvement in sperm count of infected rats with 7 day prophylactic treatment as well as 1 hour Post bacterial treatment when compared with the control groups as shown in Fig. 3 and 4. This suggests that spermatozoa can subsequently be affected by infections at different points in their development and maturation. Direct interactions of bacteria and spermatozoa have been discovered for different bacterial species such as *E. coli* [31], Mycoplasmas, *U. urealyticum* [32] and Chlamydia species [33]. The direct inhibitory effect of *E. coli* on progressive motility of spermatozoa is found to depend upon the bacterial concentration. Our result also revealed low percentage motility in ECC and PAC without the eps to be 10±4.14% and 5±2.07% respectively as compared to the CUR (70±14.14%). The administration of the exopolysaccharides to the infected rats brought about improvement on the percentage of sperm motility but still lower than that of untreated rats (CUR) as shown in tables 3 and 4. *E. coli* probably represents the most frequently isolated micro-organisms in genito-urinary infections [34]. It rapidly adheres to the spermatozoa *in vitro*, resulting in agglutination of spermatozoa. A profound decline in motility of spermatozoa is evident over time caused by severe alterations in sperm morphology [35].

From this study, it can be concluded that the exopolysaccharides of *L. subnudus* is a bioactive secondary metabolite that possesses antibacterial properties which can be explored in the treatment of bacterial infections.

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