Special Conference Edition, November, 2017
http://dx.doi.org/10.4314/bajopas.v10i1.95S
Bayero Journal of Pure and Applied Sciences, 10(1): 498 - 502
ISSN 2006 – 6996

SCREENING AND IDENTIFICATION OF ANTIBACTERIAL AGENTS PRODUCED BY *Aspergillus* SPECIES FROM THE SOIL OF BAYERO UNIVERSITY KANO.

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ABSTRACT
This study was conducted to isolate antibiotic producing fungi in soil samples collected from Bayero University, Kano. Soil samples were suspended in deionised distilled water and inoculated on potato dextrose agar (PDA) by spread-plate method for the isolation of fungi. Primary screening of antibiotic producers was conducted using agar well diffusion method. All the isolates were identified to be in the genus Aspergillus. Out of the sixty (60) fungal isolates, three (3) species (A. flavus, A. fumigatus and A. niger) were selected for fermentation process and extraction of metabolites using ethyl acetate. The extracts were dried and subjected to disc diffusion assay against Streptococcus pneumoniae, Staphylococcus aureus, E. coli and Pseudomonas aeruginosa. The extracts showed inhibitory activity against all the test organisms at concentration of 1000µg and 800µg with little or no activity at 600µg against the tests organisms. Highest activity was observed from the metabolites of A. fumigatus on all the test organisms with MIC values of 250µg/ml. Thin layer chromatography of all the three extracts revealed four sports per chromatogram. The chromatogram study reveals many bioactive compounds such as Oleic acid, n-Hexadecanoic acid known to have antibacterial activity against range of bacteria. Similarly, the extracts showed some level of toxicity to brine shrimp. The extracts were found to have some pharmaceutical applications and therefore should be subjected for further bioassay such as anticancer and antioxidant.
Keywords: Antibiotic producing fungi, Soil, Aspergillus, Oleic acid.

INTRODUCTION

Antibiotics are substances produced by the natural metabolic processes of secondary metabolic processes of some microorganisms that can inhibit or even destroy microorganisms completely(Taylor *et al.*, 2003;Denyer *et al.*, 2 004). They are originally referred to as organic compounds produced by some species of actinomycetes, fungi, and bacteria, which are harmful to other microorganisms (Walsh, 2003; Denyer *et al.*, 2004).

There are so many different potential sources where antibiotics can be discovered, such as medicinal herbs (Sharma *et al.*,2013, Rajaperumal *et al*,.2013) and soil. However, soil, which is a naturally occurring loose mixture of mineral and organic particles (Nejad *et al* 2013), still remains the most important target for most researchers in their efforts to discover novel antibiotics which have pharmaceutical values.

Due to their pharmaceutical potentials secondary metabolites of fungi have been studied for more than 80 years. The search for new drugs from fungi started with the discovery of penicillin (Fleming, 1929; Dreyfuss *et al.*,1976). It was first discovered as an antifungal metabolite and later found to be immunosuppressive which made cyclosporine useful for the treatment following organ transplantation (Goodman *et al.*,1985). The antifungal agent griseofulvin being isolated from *Penicillium griseofulvum* (Rehm,1980) and the cholesterol biosynthesis inhibitor lovastatin isolated from *Aspergillus terreus* are two further examples supporting today's great interest in new secondary metabolites from fungi (Dreyfuss, 1986). *Aspergillus niger* is now widely used industrially for production of metabolites and as a source of enzymes α-amylase, cellulose and pectinases. The fungus is also known for production of various mycotoxins including ochratoxin A 5 and fumunisin B_1. This project aimed at screening and isolating fungal species with potential to produce antibiotics from the soil of Bayero University, Kano.

MATERIAL AND METHODS

Sample site

The sample was collected from Biological science garden and Sports complex environment of Bayero University Kano, which is in the Northern part of Nigeria. Kano lies on the geographical coordinates $13^{o}\,2^{1}$ N, $42^{o}\,4^{1}$ E.

Sample collection

Soil sample were collected as described by Tiwari *et al*. (2009). All the vegetation was removed from the surface of the soil. Spatula was used to collect the soil samples into 200ml sterile glass containers. Soil was sampled aseptically collected from a depth of 5cm and transported to Microbiology research laboratory of Bayero University, Kano

Isolation and identification of fungal isolates

Isolation of fungi was carried out using the pour plate technique after serial dilution using Potato Dextrose Agar (PDA) medium supplemented with 50µg/mL Streptomycin and incubated at 37°C for 4 to 7 days. Individual fungal colonies were removed and repeatedly sub-cultured until pure cultures were obtained (Ogbonna *et al*, 2013).

Direct Microscopic Mounts

A small portion of the isolated fungal colony was mounted on a clean microscope slide containing drop of Lactophenol Cotton Blue stain. This was covered with a cover slip and the preparation was squashed with the butt of the inoculation needle and the excess fluid was bloated off followed by observation under low power (David *et al*., 2007).

Test organisms

The test organisms that were used in this study were; *Candida albicans*, *Aspergillus niger*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus spp*. and *Staphylococcus aureus*. The organisms were obtained from Microbiology laboratory and were reconfirmed using standard biochemical tests.

Fermentation of fungal and crude extraction

Fermentation was in accordance with the procedure described by Shamim *et al*. (2011). Test fungi were grown in conical flasks (500 ml) containing 400 ml Potato Dextrose Broth, plugged with cotton wool and autoclaved at 121°C for 20 minutes. The flasks were inoculated with 5 mm disc, cut from the margin of growing culture of test fungi. The flasks were incubated for 15 days at room temperature (25°C) as stationary mat culture. Test fungi were filtered through Whatman No. 1 after 15 days of fermentation. This was then extracted using solvent extraction method (Chacko *et al.*, 2012). Ethyl acetate was added to the filtrate in the ratio of 1:1(v/v) and shaken vigorously for one 1 hour for complete extraction. The organic phase was finally evaporated in water bath at 40ºC.

Sensitivity test for the isolated bioactive compounds

Various test concentrations of 1000µg, 800µg, and 600µg, were prepared from dry fungal metabolites extract in accordance with the dilution method described by Baker *et al.*, (1993). The discs prepared were kept at distance apart to prevent any overlapping of zones formed. The plates were inverted and allowed to stand for 10mins to allow pre-diffusion of the extract into the agar and subsequently incubated at 37ºC for 18hours. Measurement of the diameter of zone (mm) of inhibition was conducted (Mukhar and Okafor, 2002).

RESULTS AND DISCUSSION

From the analysis temperatures of the sampling sites ranged between 25°C and 37°C. Soil textural properties from biological and ecological garden were identified to be loamy, while that from sports complex are sandy in their texture. The pH of the sampling sites was found to range between 8.3 - 6.8. (Table 1). The fungal colonies counted from Biological science garden and sports complex were observed to be 6.43±0.51CFU/mL and 5.10±0.53CFU/mL respectively (Table 2). There is no statistical difference between the mean fungal colony counts of the two sites ($p > 0.05$).

Table 1: Physical parameters and fungal counts of the two sampling locations.

Location	Soil type	pH	Temp (ºC)	Fungal Count (CFU/mL)
Biological Garden	Loamy	8.3	25	$6.43\times 10^{4}\pm 0.51^{a}$
Sports Complex	Sandy	6.9	26	$5.10\times 10^{4}\pm 0.53^{a}$

Values are mean ±S.D, values with similar letter within the same column were observed to be not significantly different ($p > 0.05$).

All the samples collected yield a total of sixty (32) fungal isolates. Twenty one (21) isolates from Biological science garden (65.63%), and eleven (11) from Sports complex field (34.37%), as shown in Table 2. The isolates were subjected to primary screening in which 21 isolates (found to be *A. flavus, A. fumigatus* and *A. niger*) were found to inhibit both Gram positive and Gram negative and are then selected for further analysis (Table 3).

Table 2: Percentage occurrence of fungi in the sampling sites

Location	Total fungal isolate	Percentage (%)
Biological garden	21	65.63
Sport complex	11	34.37

Table 3: Primary Screening of the isolated fungi against some bacteria

Test organisms	Sensitivity to isolate	Resistance to isolate	Percentage sensitive
E. coli	12	40	23.08
S. aureus	9	43	17.30

The residue obtained from the fermentation of four liters of each fungi in the potato dextrose broth showed that, strain A (*A flavus*) metabolite has the highest yield of 5.7g/L, followed by metabolite produce by strain B (*A. fumigatus*) with a total yield 5.0g/L, the least was obtained from metabolite of strain C (*A niger*) with 2.7g/L, as shown in (Table 4)

Table 4: Metabolite Recovered from the different fungal strains

ISOLATED CODE	ISOLATED STRAIN	YIELD OF METABOLITE(g/L)
A	*A. flavus*	5.7
B	*A. fumigatus*	5.0
C	*A. niger*	2.7

The sensitivity test results of isolate *A. flavus* showed that, the metabolites has activity on all test organisms in two concentration, and have no activity in 600µg. Table 5 showed the highest zone of inhibition against *S. aureus* at concentration of 1000µg, followed by *E. coli* and *S. pneumoniae* with zones of inhibition of 16.33mm and 15.66mm respectively.

Table 5: Antimicrobial activity of *A. flavus* metabolites against test microorganisms

Concentration	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. pneumoniae*
Amx 30µg	27.66 ±0.57[a]	22.66±1.15[a]	24.00±1.00 [a]	25.33±0.57 [a]
Disk	6.00 ±0.00[b]	6.00 ±0.00[b]	6.00±0,00 [b]	6.00 ±0.00 [b]
1000µg	15.66±1.15[c]	12.66±0.57[c]	16.66±1,15 [c]	15.66±0.57 [c]
600µg	12.33±0.57[d]	8.66 ±0.57[d]	10.33±0.57 [d]	12.66±1.15 [d]
600µg	6.33±0.57[e]	6.00 ±0.00[e]	7.33±1.15 [e]	6.00 ±0.00 [e]

Values are mean ±SD, values with different superscript within the same column are considered

The diameter inhibition zones from *A. fumigatus* was observed in all the concentrations except *P. aeruginosa* which is susceptible in concentration of 600µg. The highest zone of inhibition was recorded in the concentration of 1000µg in *S. aureus* with zone of inhibition of 18.66mm and the least zone was in concentration of 600µg with no activity in *P. aeruginosa* (Table 6).

Table 6: Antimicrobial activity of *A. fumigatus* metabolites against test microorganisms

Concentration	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. pneumonia*
Amx 30µg	31.33±0.57 [a]	22.00±1.73 [a]	22.00±0.00 [a]	23.00±1.00 [a]
Disk	6.00±0.00 [b]	6.00 ±0.00 [b]	6.00 ±0.00 [b]	6.00 ±0.00 [b]
1000µg	27.33±0.57 [c]	19.33±0.57 [c]	21.00±1.00 [c]	21.66±0.57 [c]
600µg	15.66±0.57 [d]	13.66±0.57 [d]	13.66±0.57 [d]	13.33±0.57 [d]
600µg	7.33 ±0.57 [e]	6.00 ±0.00 [e]	23.00±1.00 [e]	7.00 ±0.00 [e]

Values are mean ±SD, values with different superscript within the same column are considered

Table 7 showed the results from the metabolite extracted from *A. niger* with a zone of inhibition of 21.66mm against *S. aureus* at concentration of 1000µg, *E. coli* and *S. peumoniae* both have 7mm in the concentration of 600µg.

Table 7: Antimicrobial activity of *A. niger* metabolites against test microorganisms

Concentration	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. pneumonia*
Amx 30µg	30.33±0.57 [a]	19.66±0.57 [a]	24.00±1.00 [a]	25.66±1.53 [a]
Disk	6.00 ±0.00 [b]	6.00 ±0.00 [b]	6.00±0,00 [b]	6.00±0.00 [b]
1000µg	16.66±0.57 [c]	13.66±1.15 [c]	16.66±1,15 [c]	21.66±3.78 [c]
600µg	12.33±0.57 [d]	12.33±0.57 [d]	10.33±0.57 [d]	12.00±1.00 [d]
600µg	7.33 ±0.57 [e]	6.00±0.00 [e]	7.33±1.15 [e]	7.00±1.00 [e]

Values are mean ±SD, values with different superscript within the same column are considered significantly different (p<0.05)

The results from this study revealed the presence of microbes capable of producing antimicrobial metabolites in these habitats. A number of isolates (12) exhibited inhibitory action in the master plates and the high proportion of antibiotic producers isolated from the master plates may be associated with an ecological role, serving as a defensive mechanism to maintain their niches, or enabling the invasion of an established microbial community (Denyer, 2004). Although three (3) out of thirty two (32) isolates amounting to 9.38% showed antimicrobial activity in secondary screening of this study, However some inhibitory screening investigations have recorded values closed to what was obtained in this study while other recorded different values either higher or lower than this study. In a study, carried out by Adelaide (2011) reported that out of 119 isolate from soil source 23% of the isolates were active against test organisms. Ivanova *et al*. (1998) reported that out of the 491 bacteria isolated from different marine sources, 26% of the isolates shows activity on the test bacteria. Zheng *et al*. (2005) also reported that eight out of twenty-nine (29) strains, representing 28 % of the microbes considered in their study were able to inhibit the growth of at least one of the target microorganisms.

The results of this investigation have further confirmed that soil dwelling antibiotic producing fungi are mostly in the genus *Aspergillus* as reported by (Schlegel 2003,). Therefore the antibiotic producing isolate from the soil environment of Bayero University Kano can be harnessed for the production of novel antibiotics. The quantity of the metabolites recovers from the fermented broth varies among the fungi (Table 4), in which *A. flavus* has high yield here 5.7g/liter of the broth was recover, follows by *A. fumigatus* with 5g/liter, and the least was *A. niger* with 2.7g/l respectively. Ghada *et al*. (2011) have recovered 500mg and 400mg of crude extract from *A. niger* and *A. flavus*, this result is a little lower than this finding, probably the difference comes due to the difference in the media composition, they made use of malt extract broth and this research potato dextrose broth was employed. Also Zafar *et al*. (2015) in their research using *A. niger* recovered 300mg/liter, this also is slightly lower than this result, and it may be due to the difference of incubation period, where they incubate the broth for five days and the incubation period of this research was fourteen days.

CONCLUSION

The fungal isolates from Biological science garden and Sports complex of BUK were found to produce antibacterial metabolites. Out of the 32 isolated fungi, only three (*A. flavus, A. fumigatus* and *A. niger* were found to have potential for antibiotic production.

Recommendation

The present study showed that, the metabolites produced by *Aspergillus* species exhibited antibacterial activity however, further studies are recommended on the use of mutant strains for possible high yield, and possible identification of any new bioactive compounds. Also the metabolites should be subjected to other biological assays such as anticancer and antioxidant activities testing.

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