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Screening for antibacterial producing fungi species isolated from plant rhizosphere in Keffi, Nasarawa, State, Nigeria

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Abstract

Antibiotics were considered to be organic compounds produced by microorganisms which were toxic to other microorganism. This study is aimed at isolation and screening for antimicrobial producing fungi isolated from soil in Keffi. Standard microbiological method was used in isolation and screening for antimicrobial of activity of the crude extracts. Fungi were identified using lactophenol cotton blue stain, and view under x10 and x100 objective microscope lens. highest fungi were isolated from groundnut rhizosphere (58.3 %) and the least was from beans rhizosphere (25.0 %). The screening for antimicrobial producing fungi, isolate fgm8 shows activity against the test organism with 11.02±0.12mm inhibited zone against *E. coli* (5.33±10mm) against *S. aureus* and (9.03±0.03 mm) against *C. albica*. The antimicrobial activity of the fungi grow in complex medium with nitrogen sources showed different ranges of activity against the test organism were *Rhizopus* sp. only showed inhibition zone of 12.11±1.34 mm against *C. albicans*. These fungi species should be used to develop starter culture that will be used in local food production, as they show high antimicrobial activity against some pathogenic bacteria that cause infection and food spoilage.

Keywords: Antimicrobial activity; Test organism; Rhizosphere; Fungi; Screening

1. Introduction

Infections have been the major cause of disease throughout the history of human population especially bacterial infections, which is a threat to human health, some acute while others are fatal. Many people, especially from developing countries who are exposed to unhygienic conditions in their daily activities, are the worst sufferers of infectious diseases [1]. Their sufferings have been increased many-fold due to prolonged illness caused by widespread drug resistant pathogens and cost of treatment. It has been estimated that more than 70% of pathogenic bacteria are resistant to at least one existing antibiotic as a result of a steady increase in drug-resistant pathogens every year, there have been demands for the development of new and effective antimicrobial drugs.

Antibiotics have revolutionized medicine in many respects, and countless lives have been saved; their discovery was a turning point in human history [2]. Antibiotic is defined as a substance, produced by one microorganism [3], or of biological origin which at low concentrations can inhibit the growth of, or are lethal to other microorganisms [4]. Penicillin was the first antibiotic discovered in September 1928 by an English Bacteriologist [5], late Sir Alexander Fleming who accidentally obtained the antibiotic from a soil inhabiting fungus *Penicillium notatum* but its discovery was first reported in 1929 [2,4], and clinical trials first conducted on humans in 1940 [2]. It is well known that fungi remain one of the most important resources for the discovery of new bioactive compounds [6, 7]. It is thought that fungi rank as the second biggest kingdom of organisms in nature [5], and that as many as 1.5–5.1 million fungal species exist. From

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the history of drug discovery from microorganisms, fungal secondary metabolites have provided a number of important drugs, such as the antibiotic penicillin, the immunosuppressant cyclosporine and the anti hypercholesterolemic agents lovastatin and compactin [6], also play a vital role in ecosystems and as one of the most important tool in biotechnology [1]. In Keffi, a few investigators showed the existence of antibiotic producing microorganisms from different ecosystem. This research work focused on isolation and screening for antimicrobial producing fungal from plant rhizosphere in Keffi, Nigeria.

2. Material and methods

2.1. Sample collection

The soil samples were collected from rhizosphere of rice, groundnut and beans in some farm land within Nasarawa State University main metropolis as described as Makut, and Owolewa.[8].. The soil sample were collected by using spoon from depth of 5cm from the rhizosphere the plants by removing the debrides around the plant then spoon was used to scup the soil and it was transfer to sterile polytene bag and transported to Microbiology Laboratory, Nasarawa State University, Keffi, for analysis.

2.2. Isolation of fungi

The fungi species were isolated using a method described [8]. Ten grams (10 g) of the soil was suspended in 90ml of sterile distilled water and 10-fold dilutions was made. 1ml of the soil suspended (the stock) was pick using petite and transferred into first test tube containing 9ml of sterile water another 1ml of water was pick from the first test tube using petite and transferred into second test tube containing 9ml of sterile water this step was performed till 10 times and 0.3 ml of the aliquot was pick from 5^{th} tube and spread on potato dextrose agar incubated at 26 ℃ for 4 days. Growth colonies were sub-cultured again on potato dextrose agar and later stored in slants for further use.

2.3. Identification of fungi species

Identification of fungi was carried out by the method adopted by Makut and Ekeleme [9]. The cultural characteristics of fungi were determined by their growth appearance on culture plates and the morphological features were determined microscopically using lactophenol cotton blue staining technique, where lactophenol cotton blue strain was dropped on a clean grease free microscope slide, a small portion of mycelium or colony from the fungi culture plate was dropped on the lactophenol cotton blue with aid of mounted needle, the mycelium was spread well with the two mounted needle and covered with cover slip. The slide was then viewed under the microscope at x40 and x100 lens. The images were identified with reference to the work of [9] and fungi standard chart atlas.

2.4. Screening for antimicrobial activities of crude cell-free extract of fungi isolates

The screening of antimicrobial activities of fungi isolates against clinical isolates was carried out using agar well diffusion by Ramesha, *et al.* [10]. Fungi spore were harvested from stored slants by adding 9ml of sterile water containing 3 % tween 80 and glass beads which was shake for 1mins to dislodge spores. 1ml of the spores were inoculated into 9ml ml of potato dextrose broth and incubated at 26 ℃ for 4days. The agar wells were prepared by punching wells on a Muller-Hinton agar plates using 8mm diameter using cork borer. The 4days broth were centrifuged at 10000 rpm for 20 mins and 0.1 ml of the cell free extract was introduced in to the agar well made on Muller-Hinton agar plate and the plates were incubated at 30 ℃ for 24 h and the diameter zone of inhibition was recorded and the experiment was carried out in duplicate.

2.5. Evaluation of Antimicrobial Activities of fungi Under Complex Carbon (maize starch) and Nitrogen Source (soya beans).

The antimicrobial activities of antimicrobial-producing fungi under complex carbon (maize starch) and nitrogen source (soya beans) against clinical isolates was carried by method earlier described [11]. 1ml of 24 h potato dextrose broth culture of antimicrobial-producing fungi isolates were adjusted to 10^7cfu/ml with sterile water and inoculated into 49 ml M1 (0.98 g/L of K_2HPO_4; 0.4 g/L of $MgSO_4$; 0.4 g/L of $CaCO_3$; maize starch 5.0g/l, organic nitrogen sources (soya bean cake) 2.0g/l and 1litter of water and M2 (0.98 g/L of K_2HPO_4; 0.4 g/L of $MgSO_4$; 0.4g/L of $CaCO_3$; 10g/l glucose; 2.0g/l, organic nitrogen sources and soya beans 2.0g/l adjusted to pH of 7.4 and were incubated at 30 ^oC for 48 h. After incubation, the culture was centrifuge at 10000 rpm for 20 mins and 0.1 ml of the cell free extract was introduced in to the agar well made on Muller-Hinton agar plate streak with adjusted inoculums of bacteria and yeast (10^5 cfu) using swab sticks and the plates was incubated at 30 ℃ for 24 h and the diameter zone of inhibition was recorded and the experiment was carried out in duplicate.

2.6. Fungal genomic DNA extraction

Extraction was done using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa. A heavy growth of the pure culture of the fungal isolates was suspended in 200 microliter of isotonic buffer into a ZR Bashing Bead Lysis tubes, 750 microliter of lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR bashing bead lysis tube were centrifuged at 10,000 x*g* for 1 minute.

Four hundred (400) microliters of supernatant was transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000 x*g* for 1 minute. One thousand two hundred (1200) microliters of fungal/bacterial DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1600 microliters, 800 microliters was then transferred to a Zymo-Spin IIC colum in a collection tube and centrifuged at 10,000x*g* for 1 minute, the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microliters of the DNA Pre-Was buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000 x*g* for 1 minute followed by the addition of 500 microliters of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000 x*g* for 1 minute.

The Zymo-spin IIC colum was transferred to a clean 1.5 microliter centrifuge tube, 100 microliters of DNA elution buffer was added to the colum matrix and centrifuged at 10,000 x*g* microliter for 30 seconds to elute the DNA. The ultra pure DNA was then stored at -20 degree for other downstream reaction.

2.7. Internal Transcribed Spacer (ITS) Amplification

The ITS region of the rRNA genes of the isolates were amplified using the ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3, primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microliters for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95 ℃ for 30 seconds; annealing, 53 ℃ for 30 seconds; extension, 72 ℃ for 30 seconds for 35 cycles and final extention, 72 ℃ for 5 minutes. The product was resolved on a 1% agarose gel at 120 V for 15 minutes and visualized on a UV transilluminator.

2.8. Sequencing

Sequencing was done using the BigDye Terminator kit on on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25 µl BigDye® terminator v1.1/v3.1, 2.25µl of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition were as follows 32 cycles of 96°C for 10s, 55 °C for 5s and 60°C for 4min.

2.9. Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method.

3. Results

3.1. Isolation of fungi

The occurrence of fungi from rhizosphere is as given in Table 1. The highest fungi were isolated from rhizosphere of groundnut 7(58.3%) followed by beans 6(50.0%) and the least was from maize 5(41.6%) respectively.

3.2. Identification of fungi isolates

The cultural, morphological characteristics of fungi isolates from rhizosphere of beans, maize and ground nut plants from Nasarawa State University Main Campus, Keffi is as shown in Table.2.

3.3. Percentage occurrence of fungi species isolated from rhizosphere of selected plants

Percentage occurrence of different fungi isolated from rhizosphere of selected plants is as shown in Table 3. The highest percentage occurrence of fungi from maize rhizosphere was *Trichoderma harzianum* (25.0%) followed by Mucor sp and *Verticillium dahliae* (8.3%). From ground nut rhizosphere highest fungi isolated was Rhizopus sp (25.0%) followed by *Aspergillus flavus* (16.6%), *Trichoderma harzianum* and *Verticillium dahliae* was the lowest (8.3%). *Verticillium dahliae* was the highest isolated from beans rhizosphere (25.0%) followed by *Aspergillus flavus* (16.6%) and the least was Mucor sp (8.3%) respectively as given in Table 3

Table 1 Occurrence of fungi isolate from rhizosphere

Plants	No. samples	No. (%) isolated
Rice	12	5(41.6)
ground nut	12	7(58.3)
Beans	12	6(50.0)
Total	36	18(50.0)

Table 2 Cultural, morphological characteristics of fungi isolated from plant rhizosphere

	Characteristics	
Fungal Isolate	**Cultural**	**Morphological**
Rhizopus sp	White cottony mycelia, with black dots and covers the entire plate	Sporangiospores are produced inside a spherical sporangium. Columella is present on the top of the sporagiophore. Root-like rhizoids are found
Mucor sp	Large white colonies which turn into black later. appears to be a bit granular with green conidia distributed throughout. An irregular yellow zone without conidia was present around the inoculum	Erect sporangiophores are formed. Sporangiophore swells ate the tip to form sporangia which are globular shaped. Columella is present. globose whereas there was no conidia formation.
Trichoderma harzianum	Colonies are granular, flat, often with radial grooves, yellow at first but quickly becoming bright to dark yellow-green with age. Conidial heads are typically radiate, later splitting to form loose columns biseriate but having some heads with phialides borne directly on the vesicle cleistothecia	Macroconidia are hyaline several-celled, fusiform to sickle-shaped Conidiophore stipes are hyaline and coarsely roughened, often more noticeable near the vesicle. Conidia are globose to subglobose
Aspergillus flavus	Colonies are olive-grey to olive or whitish due to the mycelia growing upwards, and seem velvety to tufted with olive-black or olive-brown edges.	Conidiophores loosely branched, conidia, ramoconidia and presence or absence of chlamydospores
Verticillium dahliae	Are fast growing, suede-like to downy, white with yellowish-green conidial heads. Colonies become greyish-pink to brown with age and produce a diffusible brownish-red to wine-red pigment	Are spherical to ellipsoidal, 2-6 µm in diameter, and divide by fission rather than budding. Numerous short hyphal elements are also present

Table 3 Occurrence of species fungi isolated from rhizosphere

Bacteria species	No. sample	Maize No. (%)	Ground nut No. (%)	Beans No. (%)
Rhizopus sp	12	0(0.0)	3(25.0)	0(0.0)
Mucor sp	12	1(8.3)	0(0.0)	1(8.3)
Aspergillus flavus	12	0(0.0)	2(16.6)	2(16.6)
Trichoderma harzianum	12	3(25.0)	1(8.3)	0(0.0)
Verticillium dahliae	12	1(8.3)	1(8.3)	3(25.0)

3.4. Antimicrobial activity of fungi isolates from plant rhizosphere

Antimicrobial activity of cell-free extract of fungi isolates from rhizosphere of plant against clinical isolates is as shown in Table 4. Fungi isolate Fgm8 showed activity against all the test organism with11.02±0.12mm inhibition zone against *E. coli,* 5.33±0.10mm against *S. aureus* and 9.03±0.03mm against *C. albicans.* Fga3 showed activity with inhibition zone of 3.76±0.02mm against *E. coli,* no activity against *S. aureus* but had activity against *C. albicans* with inhibition zone of 12.61±1.11mm. isolate Frt6 did not have activity against *E. coli* but had activity against *S. aureus* with 7.56±1.33mm inhibition zone and 6.34±0.50mm against *C. albicans.* Similarly, Isolate Frc5 had varying degree of activity against all the three test organism ranging from 4.45±0.01mm against *E. coli,* 3.12±0.00mm against *S. aureus* and 13.51±2.56mm against *C. albicans.* Isolate Fbc3 had activity only against *C. albicans* with inhibition zone of 8.53±1.16mm.

3.5. Antimicrobial activity of different fungi isolated from plant rhizosphere grown in complex media

The antimicrobial activity of antimicrobial producing fungi isolated from plant rhizosphere is as shown in Table 5. The different species of antimicrobial producing fungi grown in complex media showed different ranges of activity against the test organism were *Rhizopus* sp only showed inhibition zone of 12.11±1.34mm against *C. albicans, Aspergillus flavus* showed inhibition zone 15.34±2.17mm against *C. albicans* while *Trichoderma harzianum* showed activity of 5.10±1.03mm inhibition zone against *E. coli,* 3.12±0.01mm against *S. aureus* and high inhibition zone against *C. albicans* with 9.02±1.14mm. also, *Verticillium dahliae* 1 showed activity against *S. aureus* with 4.15±0.22mm inhibition zone and 15.15±2.01mm inhibition zone against *C. albicans,* lastly, *Verticillium dahliae* 2 had activity against *S. aureus* with 4.11±0.11mm inhibition zone and 8.02±0.23mm against *C. albicans* respectively.

Table 4 Antimicrobial activity of cell-free extract of fungi isolates from rhizosphere of rice, beans and ground nuts against clinical isolates

	Inhibition zone (Mean± S.D) in Millimetre (mm)		
Isolates	***E. coli***	***S. aureus***	***C. albica***
Fgm1	0.0	0.0	0.0
Fgm8	11.02±0.12	5.33±0.10	9.03±0.03
Fgm11	0.0	0.0	0.0
Fga5	0.0	0.0	0.0
Fga3	3.76±0.02	0.0	12.61±1.11
Fgc4	0.0	0.0	0.0
Fgc9	0.0	0.0	0.0
Frm4	0.0	0.0	0.0
Frt6	0.0	7.56±1.33	6.34±0.50
Frt9	0.0	0.0	0.0
Frt3	0.0	0.0	0.0

Frc5	4.45±0.01	3.12±0.00	13.51±2.56
Fbm5	0.0	0.0	0.0
Fba8	0.0	0.0	0.0
Fba2	0.0	0.0	0.0
Fbc3	0.0	0.0	8.53±1.16
Fbc10	0.0	0.0	0.0

Table 5 Antibacterial Activities of fungi isolated against clinical isolates

	Inhibition zone (Mean± S.D) in Millimetre (mm)		
Isolates	***E. coli***	***S. aureus***	***C. albicans***
Aspergillus flavus	00.0	00.0	0.0
Trichoderma harzianum	0.0	0.0	4.06±0.04
Verticillium dahlia	0.0	00.0	7.12±0.11
Verticillium dahlia	0.00	0.0	5.12±0.03

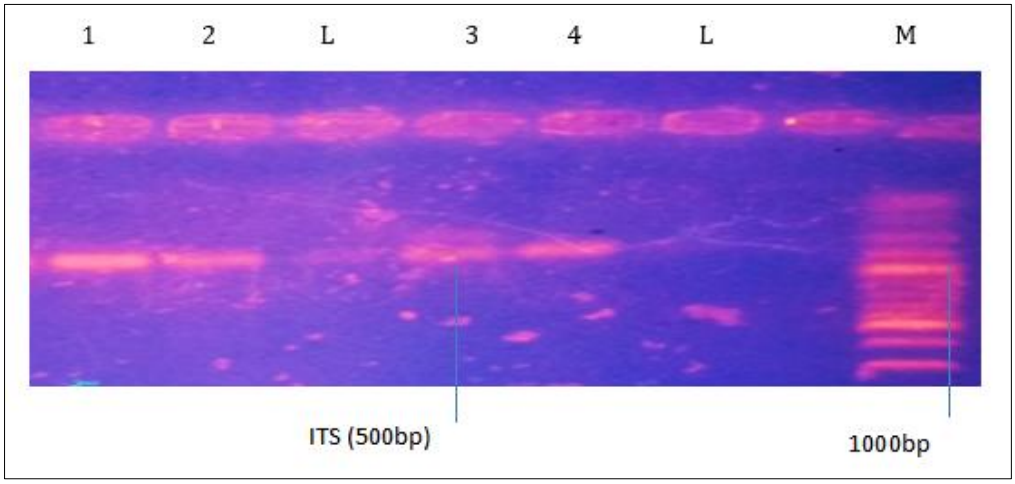

Figure 1 Agarose gel electrophoresis of the amplified internal transcribed spacer (ITS) fragment of the fungal isolates of some selected fungi isolates. Lanes 1= *Aspergillus flavus*, Lane2 = *Verticillium dahlia* lane 3= *Trichoderma harzianum* and lane 4= *Verticillium dahlia* represent the ITS bands (500bp), lane L represents= negative controls, Lane M represents the 1000bp molecular ladder

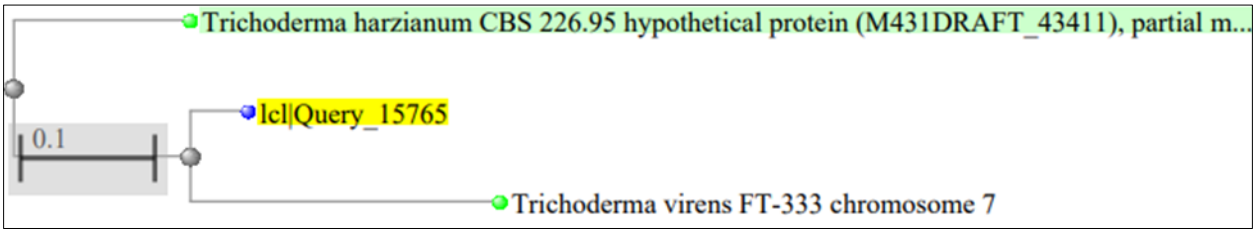

Figure 2 Phylogenic tree showing evolution relatedness of *Trichoderma harzianum*

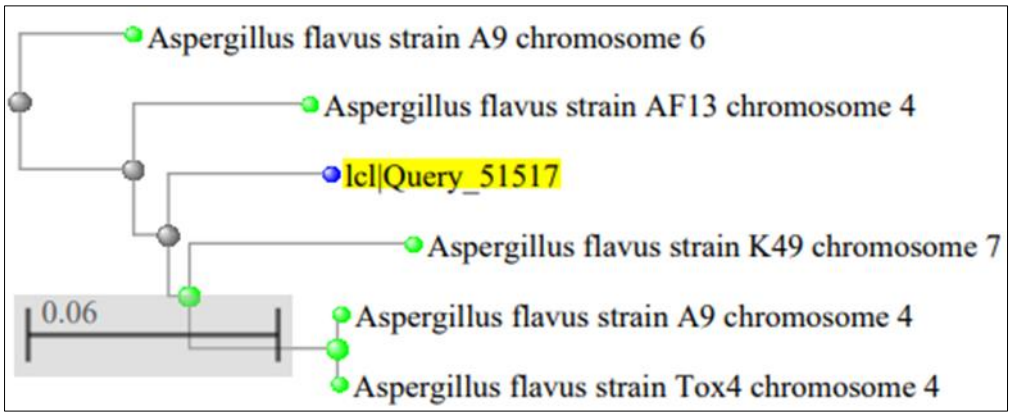

Figure 3 Phylogenic tree showing evolution relatedness of Aspergillus flavus

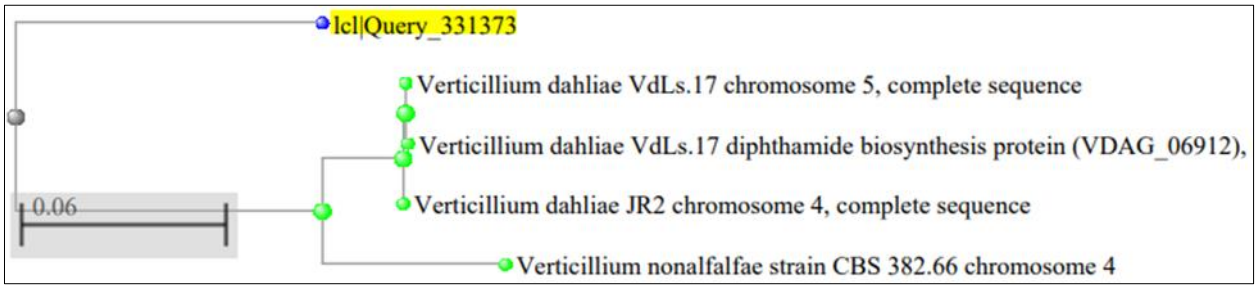

Figure 4 Phylogenic tree showing evolution relatedness of *Verticillium dahlia*

4. Discussions

Antimicrobial resistant has recently become a great consign in treatment of infection so there for there is need to develop or identify new microorganism that has the ability to produce substance that will be used in production of antimicrobial agents. Screening for antimicrobial producing fungi isolated from soil rhizosphere, in this study different fungi species were isolated from different rhizosphere of plants where the highest was observed from the rhizosphere of ground nut this is not surprising do the rhizosphere of ground nut harbor different microorganism which helps the plant in chemical cycling of nutrient at the node this similar to studies reported by Widawati *et al*., [12].

In this study different antimicrobial producing fungi species isolated and molecularly identified were *Trichoderma* harzianum, *Verticillium dahliae* and *Aspergillus flavus* as shown in the phylogenic tree reviewing their evolution relatedness also *Rhizopus* sp and *Mucor* sp these fungi are known to play an important role in increasing plant growth by various mechanisms that are carried out such as increasing nutrient absorption, as a biological control of pathogenic attacks to plants and produce growth hormones which are important to plant, this similar to study reported by [13].

The total occurrence of different fungi was eighteen (18) which was used to screen for antibiotic producing fungi, it was observed that at of the 18 only five were able to show antagonizing effective against the test organisms. The fungi that showed antagonizing effects are namely Mucor sp (Fgm8), *Aspergillus flavus* (Fga3), *Trichoderma harzianum* (Frt6), and *Verticillium dahliae* (Frc5), this is in agreement with studies reported by [14]. This confirms ubiquitous nature of fungi they are performing different activity in different environment where they are found. Their antagonistic effect clears that they are they're on the roots of those plants stopping pathogenic organism from attacking the plant.

Screening for antimicrobial activity of the fungi isolate using complex carbon and nitrogen source showed that the fungi isolates were able to inhibit the test organism with different degree of inhibition zone where the fungi isolates had more activity on yeast (*C. albicans)* than the bacteria namely *E. coli* and *S. aureus* to compare to the fungi grown in the synthetic carbon and nitrogen source which showed that the fungi isolates were able to have activity or antagonizing effect only on the yeast (*C. albicans*) and no effect on the bacteria. This finding suggest that source of carbon and nitrogen can play an important role on production bioactive compound or agent that will inhibit pathogenic organism. It was observed

that rapid utilizable carbon source did not support the antibiotic production as antibiotic synthesis does not totally depend on the growth of the fungi; rather, it depends upon the adverse conditions imposed by the medium or surroundings. This is similar to study reported by Jain and Jain [15] who reported the effect of media composition in production of bioactive agent.

5. Conclusion

From findings of this study the following are the conclusion different fungi species were isolated from different rhizosphere of plants. The isolation rate was high from rhizosphere of ground nuts and fungi such as *Trichoderma harzianum*, *Verticillium dahliae*, *Aspergillus flavus, Rhizopus* sp and *Mucor* sp were isolated. The fungi species were screened for antimicrobial activity against clinical isolates and the fungi species showed the ability to produce bioactive agents that inhibits the clinical isolates. Also, the fungi species that were grown in complex carbon and nitrogen source were able to show more activity against both the yeast and bacteria test organism.

Compliance with ethical standards

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Disclosure of conflict of interest

There was no conflict of interest all through the period of this research work or during the time of drafting of this paper

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