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Antibacterial Activities of Fungi Isolated from Soil Samples in Refuse Dumping Sites at IFITE-AWKA in AWKA South Local Government Area of Anambra State

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Abstract: ***In the study of antibacterial activities of fungi isolated from soil samples in refuse dumping sites at Ifite-Awka in Awka south local government area of Anambra state, one sample each was collected from 5 different sites A-E and analyzed for fungi isolations using conventional methods. A total number of 9 fungal specie were isolated which includes Aspergillus niger, Mucor microsporus, Candida albicans, Aspergillus fumigatus, Rhizopus stolonifer, Rhizopus microsporus, Penicillium janthinalium, Mucor indicus and Alternaria alternata were isolated and screened for antibacterialactivities against 4 different test bacterial which includes; Bacillus subtilis, E. coli, Salmonellaenterica serovarTyphi and Staphylococcus aureus clinically isolated from urine, stool, mouth and sour swab samples of patients in the intensive care unit of Nnamdi Azikiwe universityteaching hospital Nnewi in Anambra State. The bacterial isolates were subjected to biochemical confirmatory test before use. Thecrude extracts of isolates with highest zone of inhibition in all the sampling sites showed that in site A, E. coli shown clear zone of inhibition of 18mm for Mucor indicus while in site B, Staphylococcus aureus, E. coli and Salmonella enterica serovar Typhi showed 5mm, 10mm and 13mm respectively for Aspergillus niger. In site C, Salmonella enterica serovar Typhi showed the lowest zone of inhibition of 4mm for Aspergillus niger while the highest zone of inhibition of 19mm was shown in site D by S. enterica serovar Typhi for Aspergillus niger.Site E showed 16mm clear zone of inhibition as the highest zone of inhibition respectively. The minimum inhibition concentration (MIC) and minimum bacteriocidic concentration (MBC) for S. enterica serovar Typhi showed negative growth while E. coli, Staphylococcus aureusand Bacillus subtilis showed positive growth against Aspergillus niger respectively at the lowest concentration of 1.6mg/ml. Salmonella enterica serovarTyphi had the highest clear zones of inhibition for Aspergillus niger in all the sampling sites indicating that it produced bioactive antibacterial compounds that inhibits bacterial growth followed by E. colifor Mucor indicus while Salmonella enterica serovar Typhi also showed the lowest zone of inhibition for Rhizopus microsporus.***

Keywords: Antibacterial, Pathogenic, Metabolites, Bioactive compounds, Minimum inhibition concentration, Minimum bactericidal concentration, Antagonism, Refuse dumping sites

1. Introduction

Antibacterial agents are a group of materials that fight against pathogenic bacterial. Thus, by killing or reducing the metabolic activities of bacteria and their pathogenic effects in the biological environment will also be minimized. Maryam P. and Hooshmand T. (2019). They are agents that selectively destroy bacteria by interfering with bacterial growth or survival.

Antibiotics are compounds that are produced by living organisms, derived from bacterial, fungal, mold, plants and animal sources and are used to treat bacterial infections. Maryam P. and Hooshmand T. (2019).

Soil is considered one of the most suitable environments for microbial growth. Cavalcanti et., al.(2006). Soil is a very rich source of bioactive compounds that can be explored as pharmaceuticals. Bahram et., al. (2018).The need for this research is based on the fact that there is a remarkable increase in antibiotics resistant bacterial species, Kina (2003), Motta et., al. (2004) leads to search for new sources of antibiotics through the isolation and identification of new types of microorganisms such as bacteria, fungi, and actinomycetes. The antibiotics produced by bacteria have been gaining importance by many researchers. Bacterial species producing antibiotics have been used as biocontrol agents against pathogenic fungi. Yilmaz et., al., (2005), Gebreel et., al., (2008). The genus Streptomycets which is antibiotics producer has been isolated from Yemen. Ahmed (2010). Also, one hundred bacterial were isolated from six different samples collected from Egypt, 20 of them could antagonized some *Aspergillus sp, Fusarium oxysporum, Penicillium digitatum* and *Alternaria solani*. Gebreel et., al., (2008). Moreover, 20 bacterial strains isolated from soil stressed ecological niches of eastern Utta Pradesh, India showed strong antimicrobial activities. Singh et. al., (2009).

Soil samples were collected from 5 different refuse dumping sites at IfiteAwka, in Awka south local government area of Anambra state. Using conventional method, 26 fungal isolates were isolated and identified.3 out of the 26 identified fungal isolates namely *Aspergillus niger, Rhizopus microsporus* and *Mucor indicus* showed antibacterial activities for clinical isolated bacterial test organisms namely *Salmonella enterica serovar Typhi* and *E. coli* respectively. This indicates that these fungal isolates produced bioactive compounds that had antibacterial activities to those tested clinical bacterial isolates.

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2. Materials and Methods

1) Study Area

This study was carried out at Ifite - Awka in Awka south local government area of Anambra state in the Eastern part of Nigeria. Ifite-Awka is a well-developed residential area in Awka- south, located very close to Nnamdi Azikiwe University Awka. The neighborhood is comprised of residential, student hostels and staff quarters. Residence dumps their trash at dumping sites at the center of a nearby bush in the area. The dumping sites were where the soil samples are collected for this research.

2) Sampling

Soil samples were collected from different soil sediments in 5 different sites using sterilized spatula 6-12 inches dept from the ground. The soil samples collected were inserted into sterile polythene bags, labeled, sealed tightly and transported immediately to the laboratory for isolation of fungi.

3) Preparation of soil samples.

The samples were air drive for 3hrs at 37ºC, crushed and sieved before use.

4) Sample Processing

One (1) g of soil sample was weighed and homogenized in 9ml of sterile water. The mixtures were serially diluted (10-fold). Using aseptic technique, 10 -5, 10 -6, 10 -7 and 10 -8 was plated on sabouraud Dextrose Agar (SDA) containing chloramphenicol. The culture plates were incubated for five (5) days as described by Maghraby et., al.(1991)

5) Isolation of Fungi

The mixed fungi culture was purified by sub-culturing into new SDA media to obtain the pure culture. Then, the plates were incubated for another five (5) days at room temperature Norhafizah (2012).

6) Identification and Classification of Fungi Isolates

The Morphology of the fungi isolates were identified by physical observations such as top and reverse color, parameter, hyphae and conidia structure, growth behavior, mycelia mat and changes in medium color were observed and recorded. Norhafizah (2012).

7) Microscopic examination

For each fungus isolate, a small sample of the cell and agar was cut out from the fungal culture and transferred into microscope slide. The slides were stained using lactophenol cotton blue and covered appropriately with a cover slip. The slides were examined using X40 lightmicroscope. Cultural characteristics such as mycelia end, branching, structure of hypha, and presence of spore were observed and recorded. Identification of fungal isolate was done by comparing the result of the cultural and morphological characteristics with those of known taxonomy in fungal atlas for identification Norhafizah, (2012) Adejumo and Adegunloye (2014).

8) Test bacterial Organisms used for antibacterial screening.

The test bacterial (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Salmonella enterica serovar Typhi*) clinical isolates were obtained from patients in the intensive care unit of Nnamdi Azikiwe university teaching hospital Nnewi. The organisms were subjected to confirmatory biochemical tests before use.

9) Preliminary Screening of Fungi Isolates for antagonism.

An agar culture of the isolated strains of interest was made in SDA by spreading on the plate surface and incubated for five daysat 30*C. After incubation, an agar plug was cut aseptically with flame-sterilized spatula and deposited on the agar surface of other plates previously inoculated with the test microorganisms (*Bacillus subtilis, Escherichia coli, Salmonella enterica serovarTyphi,* and *Staphylococcus aureus)* and was allowed to stand for 2hrs. for proper diffusion from the agar plug into the culture media as described by Balouiri et., al.(2016). Antagonism was described as the appearance clear zones of inhibition around the agar plug Balouiri et., al. (2016).

10) Submerged Fermentation and extraction of crude metabolic compound for antimicrobial activities from the screened isolates

Based on the zone of inhibition in primary screening, fungal isolates with the highest inhibition zones were selected for submerged fermentation and subsequent extraction of antimicrobial crude metabolite. The selected antagonistic fungal isolates were inoculated into 100ml of sabouraud dextrose broth in Erlenmeyer flask as described by Jose et., al. (2013)at room temperature, each of the fermentation medium was inoculated with agar plug of pure culture of the fungal isolates. This was incubated at 30^{o} C for 14 days. Each of the culture medium was occasionally shaken throughout the incubation period Jose et., al. (2013).

After incubation, the mycelia cells were removed from fermentation medium through filtration using what-man no 1 filter paper. Equal volume of Ethyl acetate (100ml) was added to the filtered fermentation medium and shaken for 2hrs in an incubator shaker at 130rpm. The mixtures were allowed to stand overnight. The solvent phase was separated from aqueous phase by using a separating funnel. To obtain the crude extract, the solvent phase was evaporated and concentrated using rotary evaporator at 40^{o}C and the residues were collected. Gebreyohannes et., al. (2013).

11) Secondary Screening of Crude Extracts for Determination of the Antimicrobial activities

Antibacterial activities of the extracellular crude extracts were determined by agar well diffusion method in Muller-Hinton Agar plates using ciprofloxacinas a control. Max-fahland Standardized twenty-four hours broth culture of the clinical isolate (*Escherichia coli, Salmonella enterica serovarTyphi, Bacillus Subtilis* and *Staphylococcus aureus*) was swabbed with sterile Cotton swap on the surface of already prepared Muller Hinton agar. Agar wells was prepared in the plate using sterile corkborer (6 mm in diameter). A volume of 1ml of10mg/ml of crude extracts was carefully dispensed into each well and allowed to diffuse for 2hrs and incubated at 37^{o}C for 24 hrs incubation period, the zones of inhibitions were measured and recorded. Thenmozhi and Kannabiran. (2010). Ciprofloxacin (10mg/ml) was used as the control textdrug.

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a) Minimum Inhibitory Concentration (MIC) Determination

Minimum inhibition concentration (MIC) was determined using agar diffusion technique as described by Ikegbunam et., al. (2018). Different concentration (50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml and 1.6mg/ml) of the extract was prepared and introduced into agar wells (6mm diameter) created on culture plates of test organisms and incubated at 37^{o}C for 18hrs. The zone of inhibition was measured and recorded. The minimum inhibitory concentration of the extract at which there was no visible growth for each organism was determined according to the method explained by Bloomfield (1991).

b) Minimum Bactericidal concentration (MBC) Determination.

From each negative result in the (MIC) assay, 1ml was transferred onto the surface of freshly nutrient agar (without antibiotics of the extracts) and the plates were incubated at 35^{o}C for 72 hrs. The lowest concentration showing no visible growth on nutrient agar was observed and recorded as minimum bactericidal concentration (MBC) for each test bacterial.

3. Results

Table 1 shows the characteristics of the fungal isolates identified and a total number of 9 isolates were observed in all the sampling sites.

Table 1: Characteristics of fungal isolates observed in this study

Size	Color	Nature of Hyphae	Conidia shape	Isolated Fungi
Medium	Black	Non-Septate	Rough Irregular	*Aspergillus niger*
Medium	White	Broad Non-Septate	Ellipsoidal	*Mucor microspores*
Medium	Cream	Short pseudo hyphae	Ellipsoidal	*Candida albicans*
Medium	Black	Non-Septate	Irregullar	*Aspergillus fumigatus*
Medium	Brown	Non-Septate	Globose	*Rhizopus stolonifer*
Large	Black	Non-Septate	Oval	*Rhizopus microspores*
Medium	Blue Green	Non-Septate	Oval	*Penicillium janthinalum*
Medium	White	Broad Non-Septate	Ellipsoidal	*Mucor indicus*
Medium	Black	Non-Septate	Macro Conidia	*Alternaria alternata*

Table 2 shows the occurrence of each isolate according to sampling sites (- or +)

Table 2: Occurrence of each isolates according to sampling sites

Sampling sites	Fungal isolates								
	A. niger	*M. microspores*	*A. fumifatus*	*C. albicans*	*R. stolonifer*	*R. microspores*	*P. janthinalum*	*M. indicus*	*A. alternata*
A	++++	++	++++	-	-	++	+	++++	++
B	++	+++	+	++	+	-	++	-	-
C	-	-	+++++	+++	-	++	-	++	-
D	++	-	+++	++	+	-	-	-	-
E	+++++	-	-	-	++	-	+	+++	+

KEY: A-E = Sampling sites, (-) = Negative, (+) = Positive.

Table3 shows the Primary screening of fungi isolates according to the sampling sites (A-E).

Table 3: Primary screening of the isolates according to sampling sites

Site A	Test Bacterial and their zones of inhibition in (mm)			
Fungal Isolates	Staph. aureus	E.Coli	Bacillus subtills	Salmonella enteric typhi
Aspergillus niger	-	-	-	3
Mucor microspores	-	-	-	-
Aspergillus fumigatus	-	5	-	12
Rhizopus microspores	-	-	-	10
Penicillium janthinalum	-	9	-	-
Mucor indicus	-	13	-	16
Alternaria alternata	5	-	-	3

Site B	Test Bacterial and their zones of inhibition in (mm)			
Fungal Isolates	Staph. aureus	E.Coli	Bacillus subtills	Salmonella enteric typhi
Aspergillus niger	-	6	-	18
Mucor microspores	3	-	-	-
Candida albicans	8	-	-	13
Aspergillus fumigatus	10	5	-	11
Rhizopus microspores	-	-	-	-
Penicillium janthinalum	-	9	-	-

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Site C	Test Bacterial and their zones of inhibition in (mm)			
Fungal Isolates	Staph. aureus	E.Coli	Bacillus subtills	Salmonella enteric typhi
Candida albicans	15	-	-	3
Aspergillus fumigatus	-	2	-	10
Rhizopus microspores	-	7	-	14
Penicillium janthinalum	-	6	-	8

Site D	Test Bacterial and their zones of inhibition in (mm)			
Fungal Isolates	Staph. aureus	E.Coli	Bacillus subtills	Salmonella enteric typhi
Aspergillus niger	-	13	-	20
Candida albicans	-	-	-	-
Aspergillus fumigatus	-	5	-	12
Rhizopus stolonifer	-	-	-	-

Site E	Test Bacterial and their zones of inhibition in (mm)			
Fungal Isolates	Staph. aureus	E.Coli	Bacillus subtills	Salmonella enteric typhi
Aspergillus niger	2	-	-	18
Rhizopus stolonifer	-	-	-	-
Penicillium janthinalum	-	13	-	-
Mucor indicus	-	14	-	16
Alternaria alternata	-	-	-	-
CPX	22	15	25	30

Key; CPX= Ciprofloxacin, (-)= no inhibition_____

Table 4 shows the secondary screening of crude extracts from isolates that has the highest inhibition concentration. *Salmonella enterica typhi* was highly susceptible against *Aspergillus niger.*

Table 4: Secondary screening of crude extracts that had the highest inhibition concentration in all the sites

Sampling Site (A-E)		Test Bacterial and their zones of inhibition in (mm)			
Site	*Isolates*	Staph. aureus	E.Coli	Bacillus subtills	Salmonella enteric typhi
A	*Mucor indicus*	-	18	-	-
B	*Aspergillus niger*	5	10	-	13
C	*Rhizopus microspores*	-	-	-	4
D	*Aspergillus niger*	-	-	-	19
E	*Aspergillus niger*	-	-	-	16
CPX		26	32	21	30

CPX= Ciprofloxacin, (-)= no growth

Table5 shows the Minimum inhibitory concentrations (MIC) of *Aspergillus niger* in site E. At a very low concentration of 1.6mg/ml there was no growth for *Salmonella enterica typhi* against *Aspergillus niger*.

Table 5: Minimum Inhibition concentration (MIC) of *Aspergillus niger* in site E.

Bacterial Isolates	*Aspergillus niger* inhibition concentration					
	50mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.125 mg/ml	1.6 mg/ml
Staph. aureus	-	-	+	+	+	+
E.Coli	+	+	-	-	+	+
Bacillus subtills	-	+	+	-	+	+
Salmonella typhi	-	+	+	-	-	-

(-)= no growth, (+)= growth

Table 6 shows the minimum bactericidal concentrations (MBC) of *Aspergillus niger* against the bacterial test organisms. At the lowest concentration of 1.6mg/ml the growth of Salmonella typhi was significantly negative indicating no growth.

Table 6: Minimum Bacteriocidic concentration (MBC)

Test Organisms	*Aspergillus niger* inhibition concentration					
	50mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.125 mg/ml	1.6 mg/ml
Staph. aureus	+	+	+	+	+	+
E.Coli	-	-	+	+	+	+
Bacillus subtills	+	+	+	+	+	+
Salmonella typhi	+	+	-	-	-	-

(-)= no growth, (+)= growth

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4. Discussion

The results of antibacterial activities of fungi isolated from samples in refuse dumping sites at Ifite-Awka in Awka south Local government area of Anambra state shows that out of 5 samples collected from different sites A-E, a total number of 26 fungal isolates were identified based on their cultural characteristics on Sabouraud dextrose agar. InsiteA, *Aspergillus niger, Mucor indicus, Aspergillus fumigatus, Rhizopus microsporus, Penicillium janthinalum, Mucor indicus* and *Alternaria alternate* were identified. In site B, *Aspergillus niger, Mucor microsporus, Aspergillus fumigatus, Candida albican, Rhizopus stolonifer*and *Penicillium janthinalum* were identified. In site C, *Aspergillus fumigatus, Candida albicans, Rhizopus stolonifer,* and *Mucor indicus* were identified. In site D, *Aspergillus niger, Aspergillus fumigatus, Candida albicans and Rhizopus stolonifer*were identified. While the isolates identified in siteE includes Aspergillus niger, Rhizopus stolonifer, Penicillium janthinalum Mucor indicus and *Alternaria alternata.*

The inhibitory effect of *Aspergillus niger* and *Alternaria alternate* for *Salmonella enterica serovarTyphi* in site A show 3mm each as the lowest zone of inhibition. *E. coli* show clear zones of inhibition of 5mm, 9mm and 13mm for *Aspergillus fumigatus, Penicillium janthinalum* and *Mucor indicus* respectively. *Salmonella enterica serovar Typhi* shows clear zones of inhibition of 12mm and 10mm for *Aspergillus fumigatus* and *Rhizopus microsoprus* respectively while the highest zone of inhibition of 16mm was shown by *Salmonella enterica serovar Typhi Mucor indicus.*In site B, *Staphylococcus aureus* shows clear zones of inhibition of 3mm, 8mm and 10mm for *Mucor microsporus, Candida albicans,* and *Aspergillus fumigatus* respectively.*E. coli* shows zones of inhibition of 6mm, 5mm and 9mm for *Aspergillus niger, Aspergillus fumigatus* and *Penicillium janthinalum* respectively while *Salmonella enterica serovar Typhi* shows clear zones of inhibition of 13mm and 11mm for *Candida albicans* and *Aspergillus fumigatus* respectively and the highest zone of inhibition of 18mm was shown by *Salmonella enterica serovar Typhi* for *Aspergillus niger.* In site C, *Staphylococcus aureus* shows the highest zone of inhibition of 15mm for Candida albicans. *E. coli* shows the lowest zone of inhibition of 2mm for *Aspergillus fumigatus,* 7mm and 5mm for *Rhizopus microsporus* and *Mucor indicus* while *Salmonella enterica serovar Typhi* shows 3mm, 10mm, 14mm and 8mm for *Candida albicans, Aspergillus fumigatus, Rhizopus microsporus* and Mucor indicus respectively. In site D, *Salmonella enterica serovar Typhi* shows the highest clear zone of inhibition of 20mm for *Aspergillus niger* while 12mm was shown for *Aspergillus fumigatus.E. coli* shows 13mm and 5mm as the lowest zone of inhibition for *Aspergillus niger* and *Aspergillus fumigatus* respectively. In site E, *Salmonella enterica serovar Typhi*shows the highest clear zone of inhibition of 18mm for *Aspergillus niger,* 16mm for *Mucor microsporus* while the lowest zone of inhibition of 2mm was shown by *Staphylococcus aureus* for *Aspergillus niger* and *E. coli* shows clear zones of inhibition of 13mm and 14mm for *Penicillium janthinalun* and *Mucor indicus* respectively.

The crude extracts of isolates with the highest zone of inhibition in all the sites shows that in site A, *E. coli* shows 18mm for *Mucor indicus* while in site B, *Staphylococcus aureus, E. coli*, and *Salmonella enterica serovar Typhi* show 5mm, 10mm, and 13mm respectively for *Aspergillus niger* in site C. *Salmonella enterica serovar Typhi* shows the lowest zone of inhibition of 4mm for *Aspergillus niger* in site D and also with highest zone of inhibition of 19mm in site D. Site E shows 16mm clear zone of inhibition in *Salmonella enterica serovar Typhi* for *Aspergillus niger.*

The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) for *Salmonella enterica serovar Typhi* shows negative growth while *E. coli, Staphylococcus aureus* and *Bacillus subtilis* shows positive growth for *Aspergillus niger* respectively at the lowest concentration of 1.6mg/ml.

5. Conclusion

The analysis of the antibacterial activities of fungi isolated from soil samples in refuse dumping sites at Ifite-Awka, in Awka south local government area of Anambra state shows that *Aspergillus niger* had the highest clear zone of inhibition for *Salmonella enterica serovar Typhi* in all the sampling sites indicating that it produces antibacterial substances that can inhibits bacterial growth. *Mucor indicus* also produces antibacterial substances that can inhibit the growth of *E. coli* while *Rhizopus microsporus* shows the lowest zone of inhibition for *Salmonella enterica serovar Typhi* in all the sampling sites indicating that it also contains antibacterial substances that can inhibit bacterial growth.

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