Original Research Article

FUNGI ISOLATED FROM POULTRY DROPPINGS EXPRESSES ANTAGONISM AGAINST CLINICAL BACTERIA ISOLATES

ABSTRACT (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Abstract

Aim: This study was conducted to isolate antibiotic producing fungi from poultry droppings.

Place and Duration of Study: Poultry droppings were collected from poultry farms and markets within Ihiala Local Government Area, Anambra State between March 2017 and October 2017.

Methodology: One hundred and fifty poultry dropping samples were analyzed. One (1) g of fresh poultry droppings were weighed and serially diluted. The dilutions were cultured on Sabouraud Dextrose Agar and Potato Dextrose Agar for five days. Isolates were characterized morphologically and microscopically. Isolates showing antagonism were subjected to submerged fermentation. The Screening and determination of minimum inhibitory concentration (MIC) of the secondary metabolite extracts was done using agar well diffusion method. The isolates were screened for antagonism against four bacteria isolates namely, *Escherichia coli*, *Salmonella typhi*, *Bacillus subtilis* and *Staphylococcus aureus*.

Results: Five isolates namely, *Aspergillus niger, Aspergillus tubingensis, Rhizomucor variabilis, Aspergillus aculeatus* and *Candida rugosa* were identified. *Aspergillus tubingensis* and *Rhizomucor variabilis* showed antagonism against the test bacteria during preliminary screening. *Aspergillus tubingensis* and *Rhizomucor variabilis* showed antagonism against *Bacillus subtilis*. After fermentation, the secondary metabolite extracts from *Aspergillus tubingensis* and *Rhizomucor variabilis*, were active against *Bacillus subtilis* at different concentrations with MIC of 20.27mg/ml and 12.72mg/ml respectively.

Conclusion: The extracts from two fungal isolates namely; *Aspergillus tubingensis* and *Rhizomucor variabilis*exhibitedantagonism against *Bacillus subtilis*only. The extracts when purified, may serve as a new drug molecule produced from natural source.

Keywords: Antagonism, Antibacteria, Fungi, Minimum Inhibitory Concentration (MIC), Poultry droppings, Secondary metabolites,

1. INTRODUCTION

Antibiotics produced by microorganisms have been very useful for the cure of certain human diseases caused by bacteria, fungi, and protozoa(Rahman *et al.,* 2011).With regards to increasing number of drug-resistant pathogens, especially the acquired multidrug resistant strains, there is need for more antimicrobial discovery for better treatment of infections in the

community including hospitals where antibiotic resistance is becoming life-threatening(Kavitha *et al.,* 2017).Antibiotic-resistant strains of bacteria and fungal infections have emerged and this resistance is rapidly transmitted to other bacterial strains and species. In recent years, Center for Disease Control and Prevention (CDC), USA, estimated that each year, nearly 687,000 people in the United States have acquired an infection while in a hospital, resulting in 72,000 deaths in 2015 (CDC, 2016).More than 70 percent of the bacteria that cause these infections are resistant to at least one of the antibiotics commonly used to treat them (Mahami *et al*., 2011). This situation has called for the need of naturally occurring antibiotics in order to curb the problems of ineffectiveness of existing antibiotics for the control of newly emerging antibiotic-resistant microbial strains (Tawiah *et al.,* 2012).

Natural products are still one of the major sources of new drug molecules today. They are derived from prokaryotic bacteria, eukaryotic microorganisms, plants and various animals. Microbial and plant products occupy the major part of the antimicrobial compounds discovered until now (Berdy, 2005). Furthermore, the study of different environments throughout the world has yielded a lot of microbial isolates with antimicrobial potentials that are of great value for the treatment of many infectious diseases (Singh and Mishra, 2013). These environments among others may include soil, aquatic, human and animal excreta especially poultry droppings.

Poultry farms provide a good ecology for microbiological activities due to the interplay of biotic and abiotic activities (Okoli *et al*., 2006). The colonization of such poultry farms by microorganisms makes such environment a potential source of antibiotic- producing strains.

The present work was therefore undertaken to screen for fungi isolates from poultry droppings with antagonistic effects on clinical bacterial isolates and subsequent production and extraction of secondary antimicrobial metabolites.

2. MATERIAL AND METHODS

2.1 Sample Collection

Poultry dropping samples were collected from local chickens and commercial fowls (broilers, layers and turkeys) vendors in Nkwo Ogbe Market Ihiala town, Ihiala Local Government Area Anambra State Nigeria. Fresh droppings from chicken houses were scooped using sterile plastic spoons. Poultry droppings which could not be collected using a plastic spoon, were swabbed by passing a sterile swab over each sample until it turned dark as described in another study by Hostettmann (Hostettmann, 1999).

The samples were labelled serially and immediately transported to the laboratory in a ziplock bag for processing within one hour of collection.

2.2 Sample Processing

One (1)g of poultry droppings were weighed and homogenised in 10ml of sterile water. The mixtures were serially diluted (10 fold). Using aseptic technique, 10^{-5}, 10^{-6} 10^{-7} and 10^{-8} were plated on Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar containing chloramphenicol. The culture plates were incubated for five (5) days as described by Maghraby and his colleagues (Maghraby *et al*., 1991).

2.3 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 as suggested by Norhafizah (Norhafizah, 2012).

2.4 Identification and Classification of Fungal Isolates

The morphologies of the fungal isolates were identified through macroscopic and microscopic observations. The pure culture plates were observed for seven (7) days for physical and colony cultural characteristics such as top and reverse colour, parameter, growth behavior, mycelia mat, and changes in medium color (Norhafizah, 2012).

2.5 Microscopic examination

For each fungal isolate, a small sample of the cell and agar were cut out from the fungal culture and transferred onto microscope slide. The slides were stained using lactophenol cotton blue and covered appropriately with a cover slip. The slides were examined at a low power (X40) using a light microscope. Microscopic characteristics such as mycelial end, branching, structure of hyphae, and presence of spore were observed and recorded.
Identification of fungal isolates was made by comparing the result of their cultural and morphological characteristics with those of known taxonomy in fungal atlas for identification (Norhafizah, 2012; Adegunloye and Adejumo, 2014).

2.6 Bacterial organisms used for the screening

The test organisms (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli*and *Salmonella typhi*) were clinical isolates obtained from Nnamdi Azikiwe Teaching Hospital, Nnewi Anambra State. The organisms were subjected to confirmatory biochemical tests before use.

2.7 Preliminary screening of fungi isolates for antagonism

An agar culture of the isolated strains of interest were made in Potato dextrose agar by spreading on the plate surface and incubated for five days at $30^0 \pm 2^0$ 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 typhi* and *Staphylococcus aureus*). These were 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 of inhibition zones around the agar plug (Balouiri *et al*., 2016).

2.8 Antibiotic production and extraction of secondary metabolite

Based on the zone of inhibition in primary screening, fungal isolates with inhibition zones were selected for submerged fermentation and subsequent extraction of antimicrobial secondary metabolite. The selected antagonistic fungal isolates were inoculated into 100ml of potato dextrose broth in Erlenmeyer flask as described by Jose *et al*. (2013) with little modifications. At room temperature, each of the fermentation medium was inoculated with agar plug of pure culture of the fungal isolates. These were incubated at 30^0C for 14 days. Each of the culture medium was occasionally shaken throughout the incubation period (Jose *et al.,* 2013). After incubation, the mycelial cells were removed from fermentation medium through filtration using Whatman no 1 filter paper.
Equal volume of Ethyl acetate (100 ml) was added to the filtered fermentation medium and shaken for 2hrs in an incubator shaker at 130 rpm. 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 surface evaporated and concentrated using a rotary evaporator at 40^0C and 100rpm (Gebreyohannes *et al.,* 2013).

2.9 Determination of the antibiotic activity of crude extracts

Antibacterial activity of the extracellular crude extracts was determined by agar well diffusion method in Muller-Hinton Agar plates using amoxicillin as a control. McFarland standardized 24 h broth culture of the clinical bacteria isolates (*Escherichia coli, Salmonella typhi, Bacillus*

subtilis and *Staphylococcus aureus*) were swabbed with sterile cotton swab on the surface of already prepared Muller Hinton agar. Agar wells were prepared in the plate using sterile cork borer (6 mm in diameter). One hundred (100) µl of crude extracts (100 mg/ml concentration) and ciplofloxacin (10 µg/ml concentration)as control test drug were carefully dispensed into designated wells and allowed to diffuse for 2 h and incubated at 37 ℃ for 24 h.) was used as the control test drug. After incubation, the zones of inhibitions were measured and recorded (Thenmozhi and Kannabiran 2010).

2.10 Minimum inhibitory concentration (MIC) determination

MIC was determined using agar diffusion technique as described by Ikegbunam *et al*. (2018) Different concentrations (200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml) of the extract were 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 was determined according to the method explained by Bloomfield (1991) but with little modifications. The value of X^2 was plotted against the log concentrations of the double fold serial dilutions of the crude extracts.

$$X^2 = \left[\frac{mean\ IZD - well\ diameter}{2}\right]^2$$

Where Well diameter is the diameter of the cork borer.

2.11 Statistical Analysis

The data collected and generated in this study were organised and presented using SPSS version 20 and Microsoft Excel version 2007.

3. RESULTS

3.1 Identification of Fungal isolates from poultry droppings.

The outcome of macroscopic and microscopic observations made on the individual isolates is shown in table 1. With respect to cultural and microscopic characteristics, the majority of the isolates were observed to be *Aspergillus niger, Aspergillus tubingensis, Rhizomucor variabilis, Aspergillus aculeatus, Candida rugosa.*

Specie frequency of occurrence in poultry dropping samples as shown in Fig.1 revealed that *Aspergillus niger*, *Aspergillus tubingensis*, *Rhizomucor variabilis*, *Aspergillus aculeatus* and *Candida rugosa* had 48%, 20%, 18%, 2%, and 12% respectively.

Table 1: Cultural and Microscopic characteristics of Fungi isolates.

Isolate	Macroscopy	Microscopy

Aspergillus niger	The surface color of the colony was dark brown to black. The reverse side was without color. The elevation was umbonated and the growth was rapid.	It has branched septate hyphae. The conidiophores length was 200-400 micrometers, diameter was 7-10 micrometers and the vesicle to globose. The conidia head was blackish brown. The length of the conidia was 30-70 micrometers. The phialides were biseriate. The cleistothecia were present
Aspergillus tubingensis	The surface color of the colony was black. The colony diameter was 2-7cm.	It has branched septate hyphae. It has bunch of spores arrangement and the spore shape was round.
Rhizomucorvariabilis	The surface of the colony was brown to tan and were hairy, with reverse side that was buff to brown in color.	It has branched round sporangia arising from hyphae which possessed rhizoids between the stolons. It has ellipsoidal, smooth-walled sporangiospores.
Aspergillus aculeatus	The surface color of the colony was black to dark brown. The reverse color was pale to yellow with distinct radial furrows.	It was uniserate with very large and globose conidia head. The vesicle measured 45-73 micrometers in diameter, and globose in shape. Conidia sizes ranged between 4-5 micrometers.
Candida rugosa	The surface of the colony was white to cream colored smooth, glabrous, yeast like.	It has ellipsoidal to elongated budding blastoconidia. It has short pseudohyphae.

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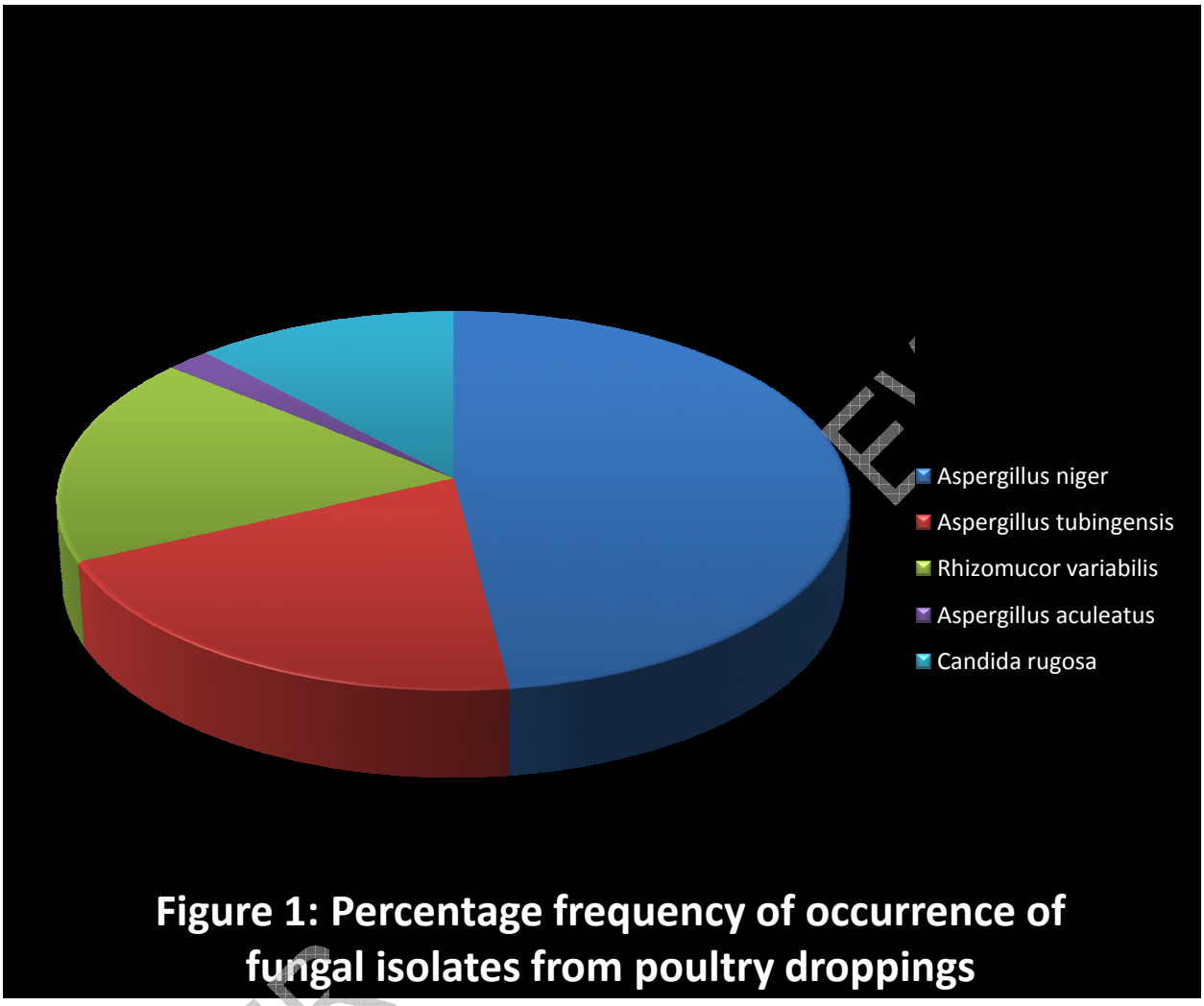

Figure 1: Percentage frequency of occurrence of fungal isolates from poultry droppings

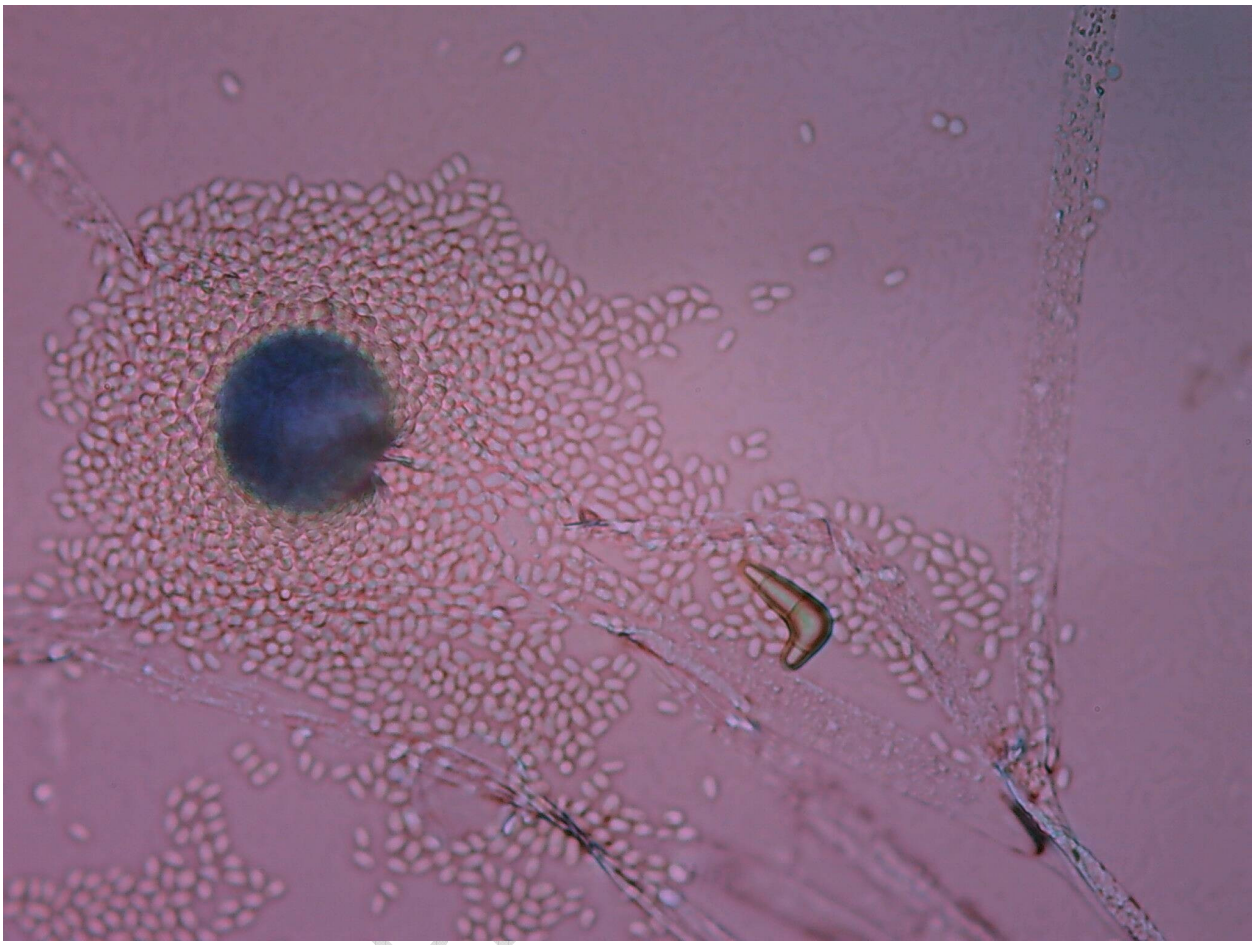

Figure 2: Micrograph of *Aspergillus niger*(Magnification x40)

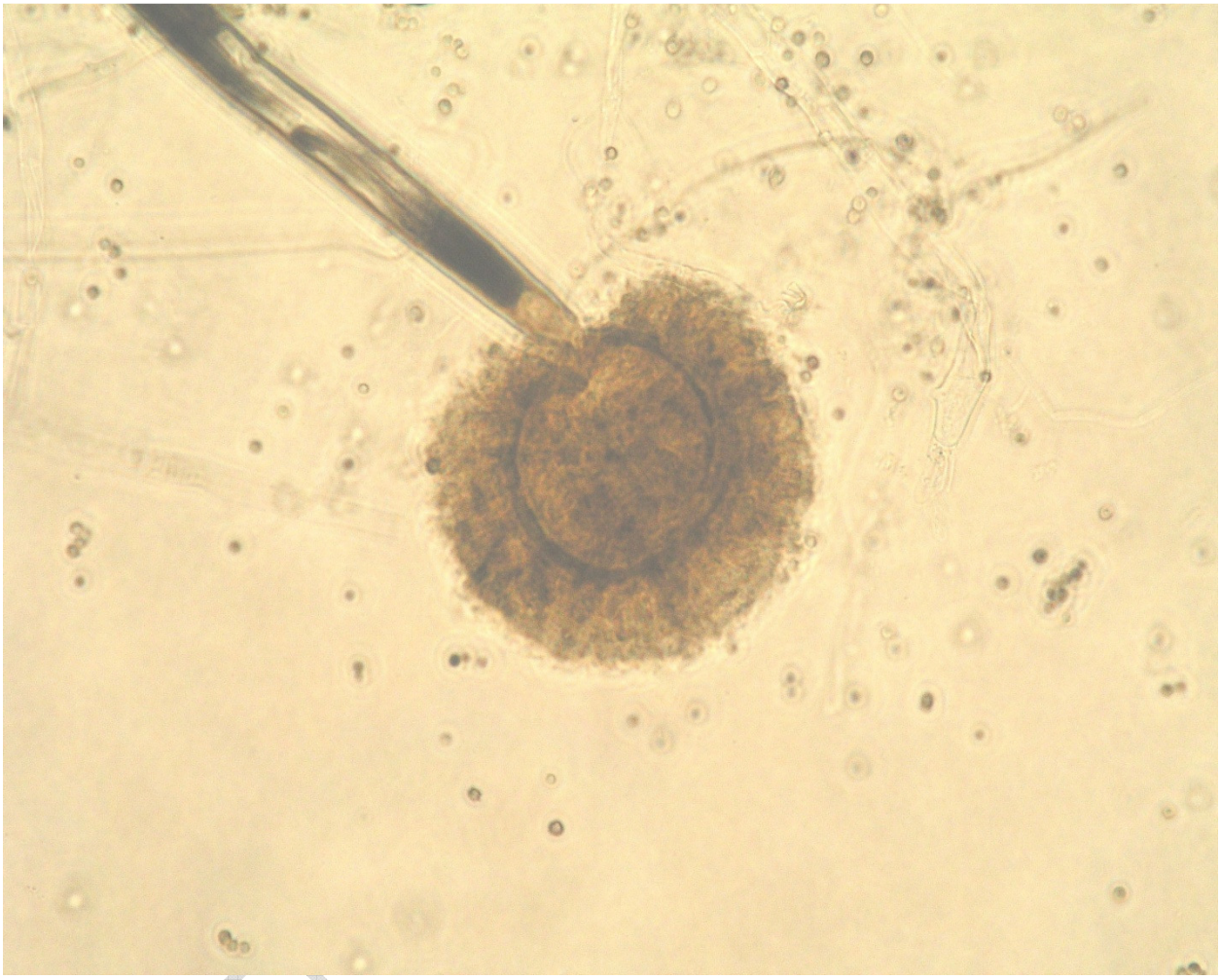

Figure 3: Micrograph of *Aspergillus tubingensis*(Magnification x40)

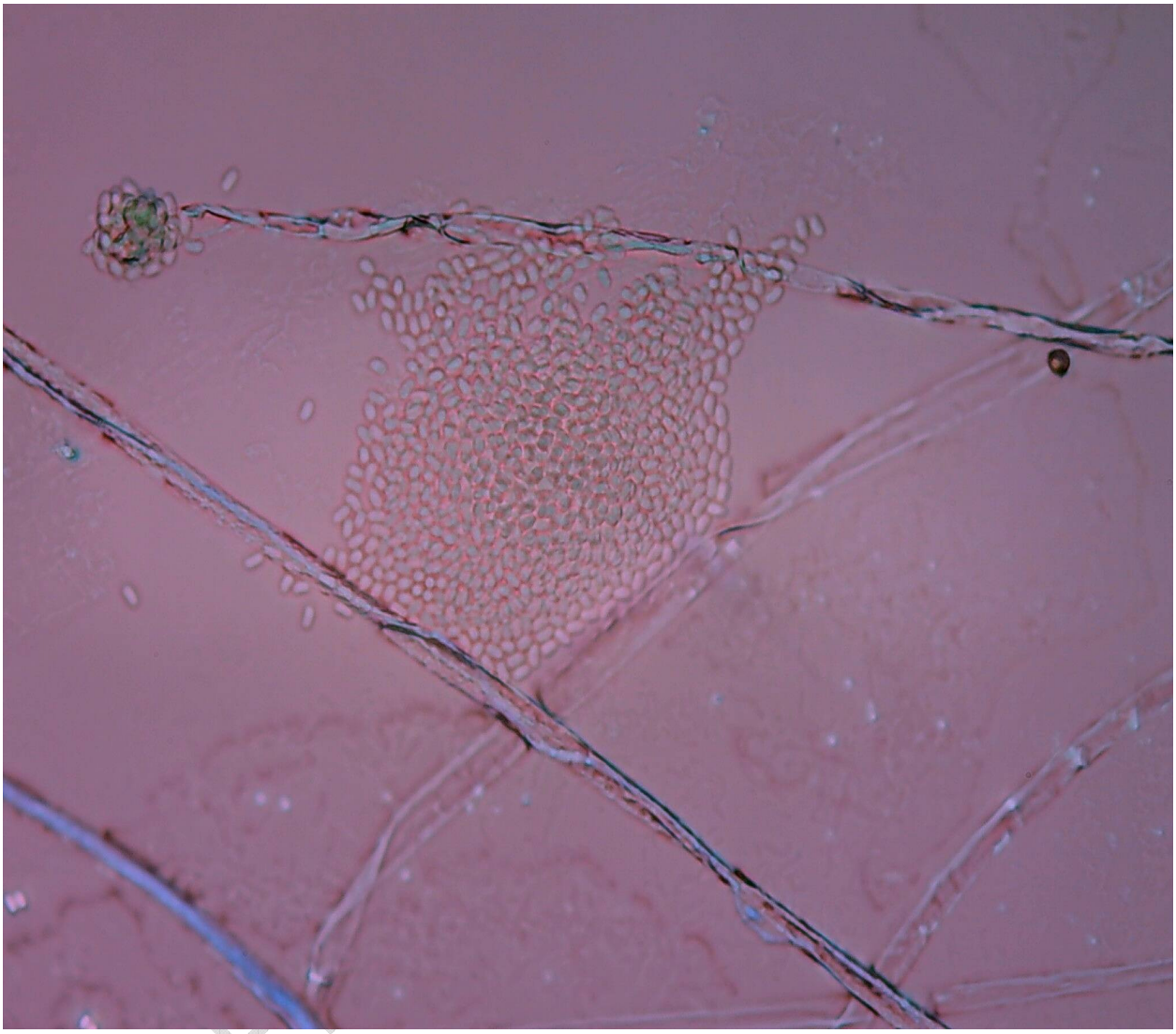

Figure 4: Micrograph of *Rhizomucorvariabilis*(Magnification x40)

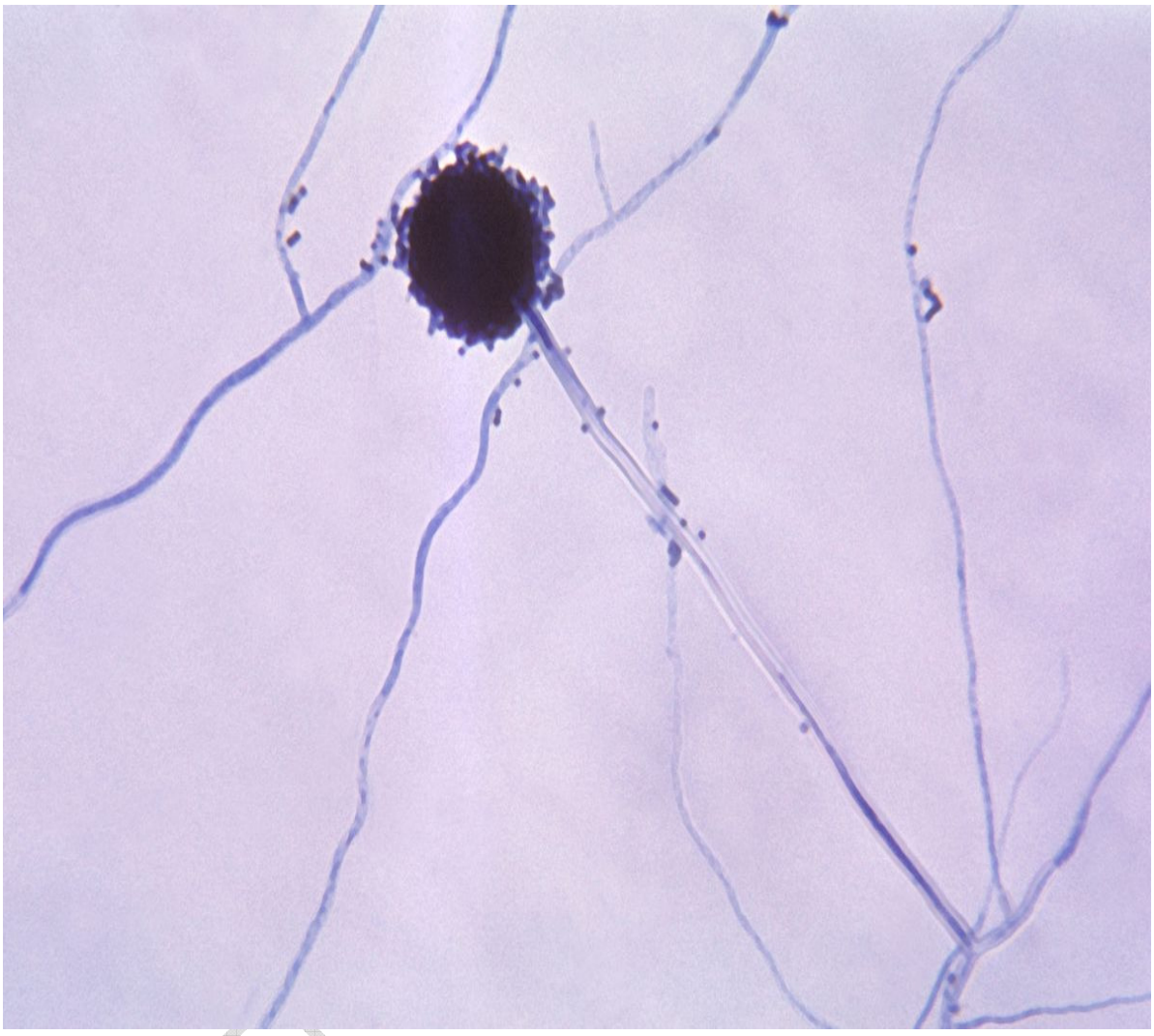

Figure 5: Micrograph of *Aspergillus aculeatus* (Magnification x40)

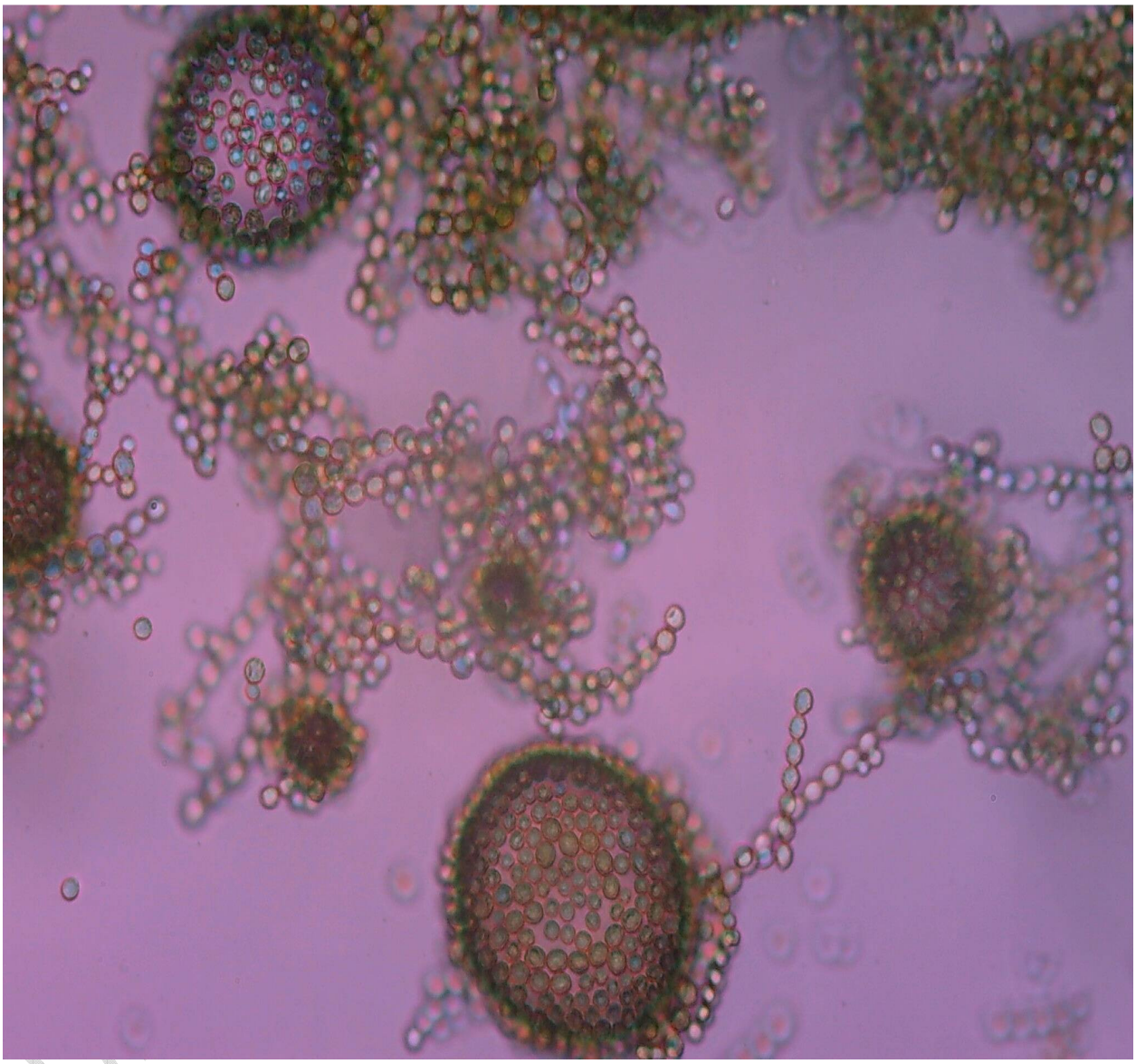

Figure 6: Micrograph of *Candida rugosa* (Magnification x40)

3.2 Antagonistic Effect of Fungal isolates on test organisms

Preliminary screening for antibiotic production of representative fungi isolates against selected clinical bacteria isolates revealed that only two isolates were able to exhibit inhibition zones to only *Bacillus subtilis* Figure 7.

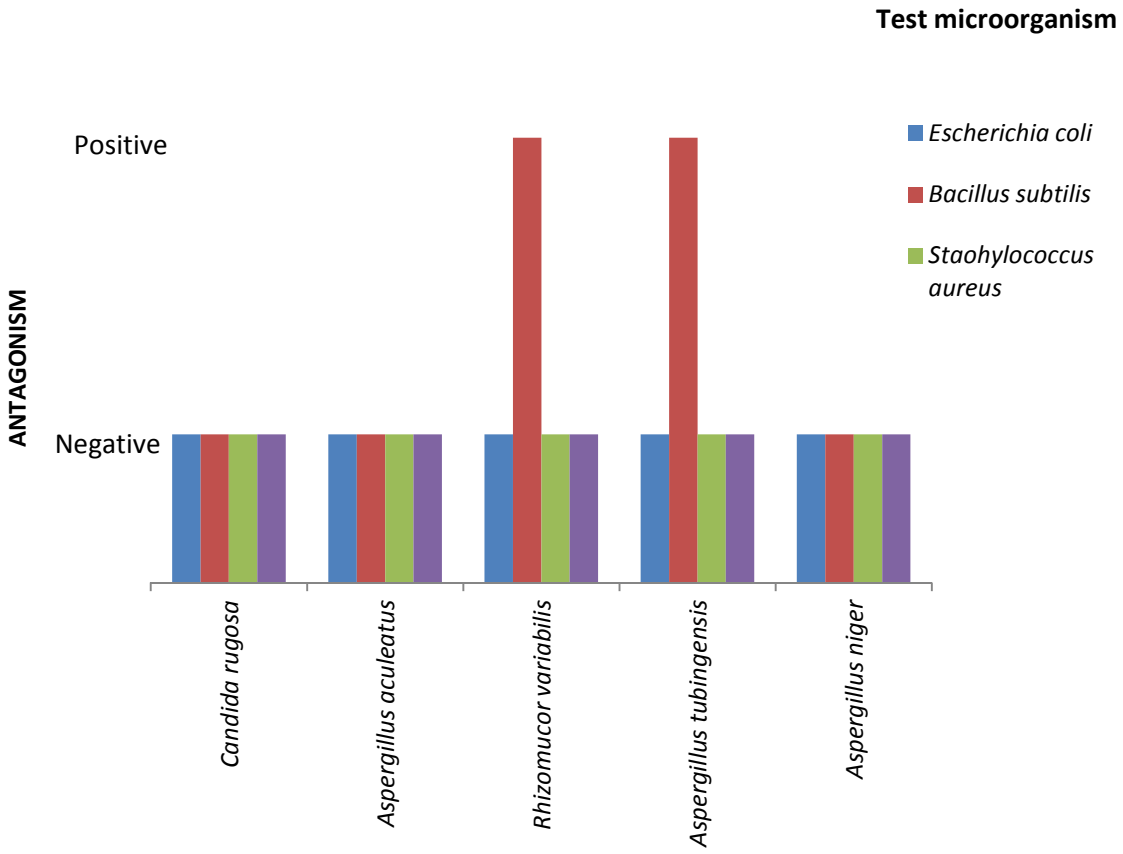

Figure 7. Isolates showing antagonism Effect on bacteria isolates

Antimicrobial evaluation of the extracted secondary metabolites showed that *Aspergillus tubingensis and Rhizomucor variabilis* extracts had activity against *Bacillus subtilis* with MIC of 20.27mg/ml and 12.72mg/ml respectively.

225 Table 2: Zone of inhibition (mm) in secondary screening of crude extracts of Fungi isolates
226 produced from submerged fermentation against *Bacillus subtilis* by using agar well diffusion
227 method

Concentration mg/ml (log conc)	Well diameter (mm)	Inhibition zone diameter (mm)	
		ATE	RVE
200 (2.3)	6	20	25
100 (2.0)	6	15	23
50 (1.7)	6	10	18
25 (1.4)	6	9	15
12.5 (1.1)	6	0	10
Amoxiliicilin (30µg/ml)			
Slope		1.308945613	1.104532
Mic (mg/ml)		20.27	12.72

228 ATE= *Aspergillus tubingensis* extract, RVE=*Rhizomucorvariabilis*extract
229

230 **3.3 Discussion**

231 Antibiotics are the most important bioactive compounds for the treatment of infectious
232 diseases. But now, because of the emergence of multi-drug resistant pathogens, there are
233 basic challenges for effective treatment of infectious diseases. Thus, due to the burden for
234 high frequency of multidrug resistant pathogens in the world, there has been increasing
235 interest for searching effective antibiotics.

236 In the present study, the randomly selected poultry dropping samples were taken from
237 different poultry farms within Ihiala for isolation of antibiotic/secondary metabolite producing
238 fungi. Previous studies showed that selection of different potential areas such as soil
239 rhizosphere and poultry droppings were an important activity for isolation of different types of
240 potent antibiotic/secondary metabolite producing fungi (Abo-Shadi *et al*., 2010).

241 Moreover, the results of primary screening using agar plug method indicated that, two (40%)
242 out of five isolates showed potential antimicrobial activity against one test bacteria (as shown
243 in figure 7). Observation of clear inhibition zones around the wells on the inoculated plates is
244 an indication of antimicrobial activities of antibiotic/secondary metabolite extracted from
245 isolated fungi (*Aspergillus tubingensis* and *Rhizomucor variabilis)* against test
246 microorganism.

In the secondary screening, crude extracts from *Aspergillus tubingensis* and *Rhizomucor variabilis* showed lower Inhibition zone (Table 2) diameter against *Bacillus subtilis* when compared with a standard antibiotic (amoxicillin 30µg/ml). Crude extract from *Aspergillus tubingensis* was active against *Bacillus subtilis* at different concentrations namely; 25mg/ml(10mm) and 12.5mg/ml(15mm) while *Rhizomucor variabilis* showed inhibition zone diameter across all the concentrations against *B. subtilis*.

In this research, the extracts from two of the fungal isolates showed antimicrobial activity against Bacillus *subtilis* only. The findings of this study, is in agreement with previous studies by Siqueira and colleagues who reported that, several extracts from fungi exhibited antimicrobial activity. According to them, sixteen (16) out of 203 isolates showed antimicrobial activity, although with a wider spectrum of activity, inhibiting Gram-positive and Gram-negative bacteria (Siqueira *et al.,* 2011). In contrast to the findings of this study that the fungi extracts could only exhibit antagonism against only one of the test bacteria isolates, other researchers had reported that 5 out of 21 isolates showed broad antagonistic activity against all the test microorganisms namely; *Bacillus subtilis, Staphylococcus aureus, Escherichia coli*and *Pseudomonas aeruginosa* (Kaaria *et al.,* 2012). Further investigation may yield novel compounds with practical applications in a variety of biotechnological areas, that will help in production of drugs useful as therapeutics options for innumerable disease.

4. CONCLUSION

This research work showed that the extracts from only two isolates namely, Aspergillus tubingensis and Rhizomucor variabilis have antibacterial activities against Bacillus subtilis. With increased reports of increased resistance to commonly used antibiotics and newly emerging antibiotic-resistant microbial strains there is need for naturally occurring antibiotics in order to curb the problems of resistance to existing antibiotics.

The findings of this study, suggests that natural products especially from fungi may still be considered as one of the major sources of new drug molecules. This may decrease medical as well as financial burden, thereby improving effectiveness of drug molecules produced from natural sources. These predictors, however, need further work to validate reliability.

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