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THE PRODUCTION OF MEDICALLY IMPORTANT BIOACTIVE COMPOUNDS BY A NEWLY DISCOVERED FUNGI SPECIES (*PURPUREOCILLIUM* SPECIES) - PP537396

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ABSTRACT: *The clog in the wheel of progress in management of infectious diseases has necessitated a continuous search for suitable bioactive agents for desired relief. Thus this work was to specifically screen previously isolated, newly discovered fungi species for bioactive agents. The fungi species – Purpureocillium sp was used to produce a crude antibiotic substance, extracted with acetone and tested on clinical isolates – Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Candida albicans. The result of zones of inhibition was 20mm, 15mm, 16mm, 0mm and 20mm in order of listing of the organisms. The result of GC-MS test pin-points these compounds – t-9-octadecenoic acid, oleic acid, hexadecanoic acid methyl ester, z-9-octadecenamide, hexadecanoic acid and methylene chloride as been responsible for observed inhibition. The fungus was also used to produce a crude amylase and tested it on clinical organisms as above with no antibiotic activity. The enzyme activity of the crude enzyme was 70U/L. The antibiotic product holds a promise for a desired drug for mixed microbial infection just like the enzyme which can be coupled to a nano silver particle for drug delivery.*

KEYWORDS: Amylase, Antibiotic, Bioactive, Nano silver, Oleic acid, t-9-octadecenoicc acid.

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INTRODUCTION

A bioactive compound can be described as a substance with an inherent cell inhibition property obtained either from microorganisms, plants or animals. But its definition varies according to author. For instance, Segneanu *et al.* (2017) defined it as those secondary metabolites of plant origin that can exhibit therapeutic, preventing, toxicological and immune-stimulating activity. A bioactive compound can also be defined as any compound of microbial origin or other living thing that can exhibit antimicrobial and/or anti-tumour properties on other organisms and/or tumour cells (Shukla, 2015). One thing is common to all these definitions – a bioactive agent has the ability to alter the proliferation of unwanted cells in living systems among other desirable effects. Some of these bioactive agents are in the form antibiotics, enzymes etc.

The word antibiotic was introduced for the first time in 1942 by Selman Waksman (Rafiq *et al.,* 2018). He described it as any small molecule of microbial origin that has an antagonising effect on the growth of other microorganisms (Rafiq *et al.,* 2018; Clardy *et al.,* 2009). It is usually a secondary metabolite of microorganisms (bacteria and fungi) via the process of antibiosis (Uwalaka *et al.,* 2019; Rafiq *et al.,* 2018). Other authors defined antibiotics as biological substances (from microorganisms) that have deleterious effect to the metabolic activities of other microorganisms when delivered in small quantities (Muleta and Assefa, 2018).

Enzymes can be defined as biological substances that act as catalysts in a biological system and as such can be called bio-catalysts (Rupali, 2015). They can be produced from amino acids via digesting food proteins and are highly specific in nature (Raza *et al.,* 2016). Some enzymes have shown importance in medical field where they are used to diagnose and treat certain diseases. Majority of enzymes used today in industries (textile, food and feed) such as cellulases, pectinases and xylanases and in medicine (lipases, tyrosinases, collagenases etc) are of microbial origin (Ilesanmi *et al.,* 2020; Boroujeni *et al.,* 2012). One of the most important features of microbial enzymes making them being sort after is that they are more active and stable than that of plant, animal or chemical versions (Raza *et al.,* 2016). Enzymes such as amylases are used in the diagnosis of pancreatitis while other such as L-Asperaginase and Ribonuclease serve as anti-tumor and antiviral (Reshma, 2019; Sabu, 2003; Cheesbrough, 2000).

This work sets out primarily to establish the production of medically important bioactive compounds using a newly discovered fungus with the hope of finding a drug to usher in a desired relief in the management of infections caused by drug resistant infectious agents. Traditional microbiology methods were employed in this exercise.

MATERIALS AND METHODS

Sample collection

Sample was collected from a stock culture of the fungus (originally isolated from a sewage contaminated soil) and kept by the researchers. The organism was stored in Sabouraud Dextrose Agar (SDA) for 2 weeks prior to this research.

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Culture

The organism was sub-cultured on a SDA to establish purity and incubated for 7 days at room temperature with daily observation for growth.

Cultural and microscopic identification of isolate

Colonial and microscopic identification of the fungus was done using the method of Rafiq *et al.* (2018).

Primary screening for antibiotic

The method of Muleta and Assefa (2018) was adopted for primary screening of fungi isolates for antibiotic production. Pure isolates were streaked as a straight line across a Petri dish of nutrient agar and incubated for six days. After that, test organisms – *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Candida albicans* were streaked perpendicular to each isolate and incubated for 24 hours at 37^{o}C. Zones of inhibition were measured with meter rule.

Extraction of antibiotic substance

A simple nutrient broth containing peptone, yeast extract and sodium chloride was used for the production of antibiotic. A 100ml of water was measured into 500ml of Erlenmeyer flask. This was followed by the addition of 3.0 grams of peptone (Molychem, India) and yeast extract (RPI, Illin, USA) each. After that 0.5g of sodium chloride was added and the medium was autoclaved at 121^{o}C for 15 minutes. When the medium cooled, a 1.0 cm block of the organism was inoculated into the medium. It was incubated at room temperature for 6 days (Bhardwaj *et al.,* 2018).

The medium was filtered using Whatman no 1 filter paper and centrifuged at 4000rpm for 15 minutes. Extraction of the antibiotic substance was done with acetone by adding 3 parts of acetone to 1 part of extract. It dried in a water bath at 56^{o}C for 8 hours. The dried extract was then suspended in a 1.0ml of autoclaved water and used for drug sensitivity test (Felczykowska *et al.,* 2017).

Secondary microbial inhibition test

The dried extract was used for secondary microbial inhibition test by adding 1.0ml of autoclaved water to 50mg (50mg/ml) of extract produced. Mc Farland 0.5 turbidity standard was prepared (Aryal, 2021) as a guide to prepare saline dilution of pure clinical isolates. Nutrient medium was prepared in Petri dishes and wells made in the center using cock borer (7mm in diameter). A sterile swab was dipped into each test tube of the clinical organisms and spread evenly on the medium. A 100µl of each of the fraction was used to fill the well, left on the bench for diffusion to occur and later incubated at 37^{o}C over night. Zones of inhibition were recorded using a meter rule.

Screening isolate for amylase

The method of Elemary and Salem (2020) was adopted for this test. The organism was screened for amylase with pure colony. It was point inoculated on starch agar, incubated at room temperature for 7days. After the incubation period, the Petri dishes were flooded with

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Gram's iodine and were observed for halo around the colony. The diameter of the halo that formed was measured together with the diameter of the colony of organism. These values were then used to calculate starch hydrolysis rate (SHR) using the formula: SHR = diameter of clear zone (mm) / diameter of the colony growth.

Extraction of crude amylase

A starch-mineral medium of Luang-In *et al.* (2019) was used. The medium was autoclaved at 121^{o}C for 15 minutes; cooled to 50^{o}C and a culture block of 1cm of the isolate was inoculated into the medium and incubated at room temperature on shaker of 120 rpm for 7 days. At the end of the incubation period, the medium was filtered twice using Whatman no 1 filter paper before been centrifuged at 4,000rpm to obtain the crude enzyme. The crude enzyme was then used for further assay.

Crude Amylase activity (Somogyi method)

The method of Cheesbrough (2000) was adopted for this test. The test was then performed by labeling two clean test tubes as T and B (for test and blank). This is followed by addition of starch solution, water bath incubation at 37^{o}C for 3 minutes. Thereafter 20µl of the crude enzyme (substituted for serum) was added to the tube T. This was followed by water bath incubation for 71/2 minutes at 37^{o}C. Then 1mL of iodine reagent was added followed by 8mL of water. Absorbance was then taken in spectrophotometer set at 660nm. Amylase activity was calculated thus:

Amylase U/L = AB – AT ⁄ AB X 1480

Where amylase U/L = unit of amylase in international units per litre; AB = absorbance of blank; AT = absorbance of test and 1480 = a factor for converting amylase activity to international units per litre - U/L (Cheesbrough, 2000).

Microbial inhibition test with crude amylase

The crude extract of amylase was tested for inhibitory capacity using agar diffusion method. Nutrient agar was prepared and holes were made at the centre of the Petri dishes using a sterile cock borer. Dilutions of the test organisms (clinical isolates) were made equivalent to Mc Farland 0.5 standard. A sterile swab dipped in the tubes of the organisms was used to inoculate the medium spreading evenly. A 100µl of the crude enzyme was added into the wells accordingly and left to stand for 2 hours for diffusion of the extracts into the medium. Thereafter the Petri dish of *Candida albicans* was incubated at room temperature while others were incubated at 37^{o}C for 24 hours. The plates were then checked for zones of inhibition at the end of the incubation period.

Identification of constituents of antibiotic extract

The dried extract that showed inhibition was sent to Central Research Laboratory Federal University of Technology, Akure Ondo State for GC-MS analysis.

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Molecular identification of organism

The organism that showed inhibition identified via traditional method to be *Purpureocillium* species was sent to Lahor Research Laboratory Benin City, Edo State for sequencing and BLASTn.

RESULTS

The result of the presumptive identification of the fungus is shown in Table 1. The organism was identified microscopically as *Purpureocillium* species. Also plates 1 and 2 show the organism growing on SDA and its photomicrograph.

Next is the result of primary microbial inhibition test which is shown in Table 2. Also plate 3 shows the pictorial representation of the antibiotic activity.

Again the result of secondary microbial inhibition test with dried extract is shown Table 3. Also plates 4…8 show the pictorial representation of same result. The result of the screening for amylase is also on shown plate 9. Not only that the organism also produced a starch hydrolysis rate of 3.5 and an amylase activity of 70U/L but the crude amylase produced showed no antibiotic activity against any of the clinical organisms tested.

The result of the identification of the constituents of the extract showed that 6 compound were responsible for the observed microbial inhibition. They include – mehtylene chloride, n-hexadecanoic acid, z-9-octadecenamide, hexadecanoic acid methyl ester, oleic acid and t-octadecenoic acid. Figures 1….7 show the gas chromatogram of the extract and mass spectra of the compounds.

The result of the gel electrophoresis test is shown in plate 10. The BLASTn test indicated that the organism is related to fungi species strain F1 XL-2011 with accession number JF703665. Again in terms of percentage relatedness, the isolate has 99.6% relatedness with the closest fungus stated above with an E- value of 0. The result also indicated that the organism is a bit complex in that it shares genetic features between *Paraisaria heteropoda* strain FSFC64P3-4-OR405018 and *Purpureocillium lilacium* clone SF_833-MT530109. Since the organism shares microscopic features of *Purpureocillium* (figure 2) hence it bears the name of the genus. Phylogenetic tree (figure 8) of the organism shows no root – indicating no common ancestry according to the BLASTn result.

Table 1: Presumptive identification of fungi species

Macros.	Sur colour	Elevation	Margin	Rev colour	Gr on SDA	Organism
	Cream	Raised	Uneven/fold	Rev colour	None	
Micros.	Phialides Present	Vesicle Present	Metulae Absent	Conidia Oval/black		Purpureoc. species

Key: macros. – macroscopy, micros. – microscopy, rev – reverse, Gr – growth, SDA – sabouraud dextrose agar, purpureoc. – *Purpureocillium*

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Table 2: Zones of inhibition (mm) of the fungi isolate against organisms after primary test

Organisms	*Candida albicans*	*Staphylococcus aureus*	*Escherichia coli*	*Klebsiella pneumonia*	*Pseudomonas aeruginosa*
ZOI diamet	25mm	13mm	21mm	35mm	0.0mm

Key: ZOI diamet – zone of inhibition diameter

Table 3: Zones of inhibition (mm) of the dried extract against clinical organisms

Organisms	*Canndida albicans*	*Escherichia coli*	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
ZOI diameter	25mm	15mm	20mm	16mm	NA

Key: ZOI – zones of inhibition, NA – no activity

Plate 1: The newly discovered fungus growing on SDA

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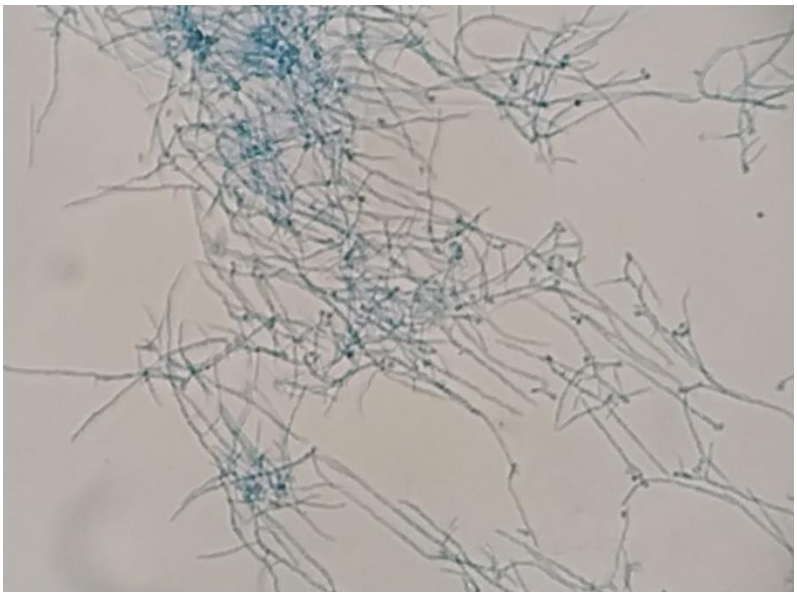

Plate 2: the organism's photomicrogragh

Plate 3: Pictorial presentation of antibiotic activity of the organism following primary test

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Plate 4: Antibiotic activity of the dried extract on *C. albicans*

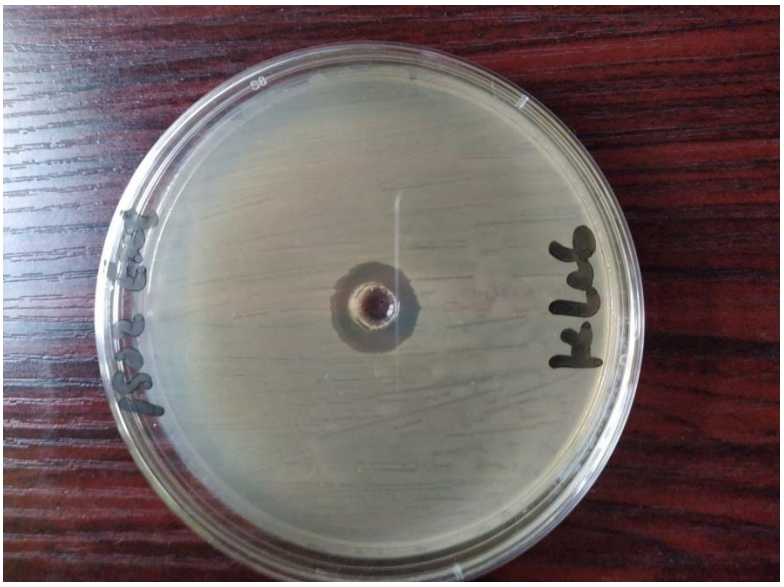

Plate 5: Antibiotic activity of the dried extract on *K. pneumoniae*

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Plate 6: Antibiotic activity of the dried extract on *E. coli*

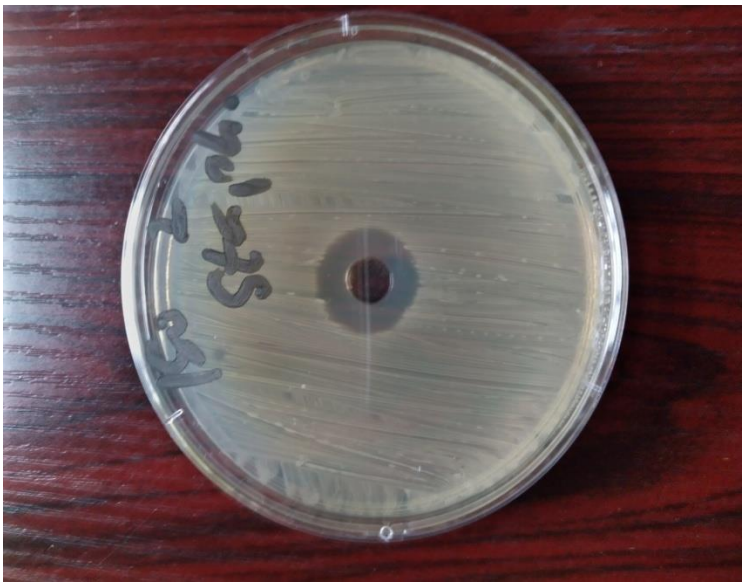

Plate 7: Antibiotic activity of the dried extract on *S. aureus*

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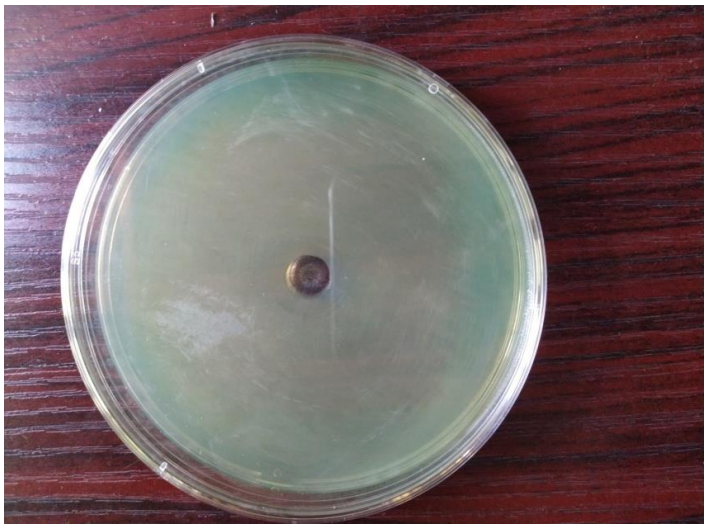

Plate 8: No antibiotic activity on *P. aeruginosa*

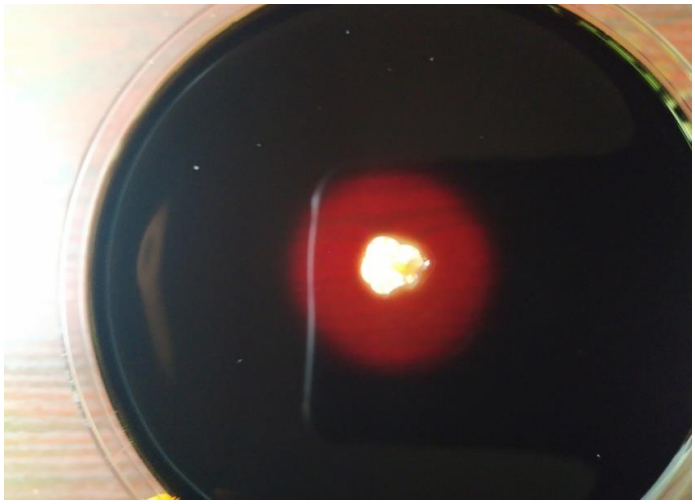

Plate 9: Amylase activity of the isolate (*Purpureocillium* sp) on starch agar

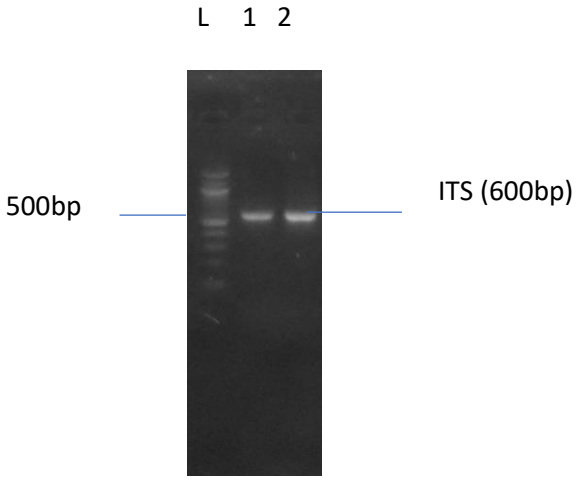

Plate 10: Agarose gel electrophoresis showing the amplified ITS of the fungi isolate. Lane 2 represent the ITS bands of 600bp of the isolate (*Purpureocillium* sp).

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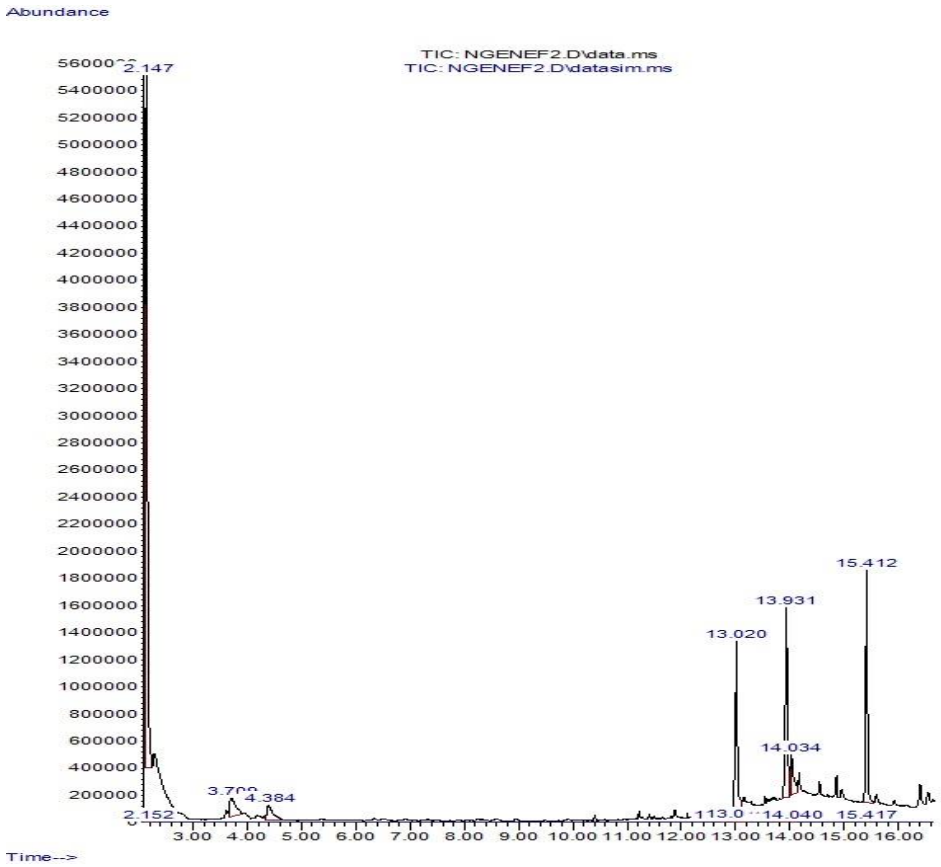

Figure 1: Gas chromatogram of extract of the fungus

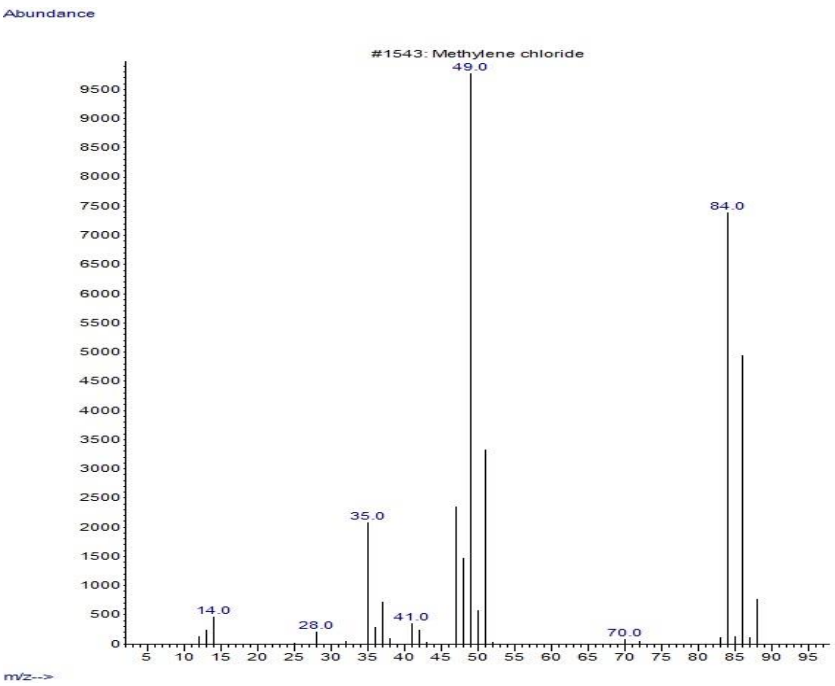

Figure 2: Mass spectrum of methylene chloride, the first compound to be identified

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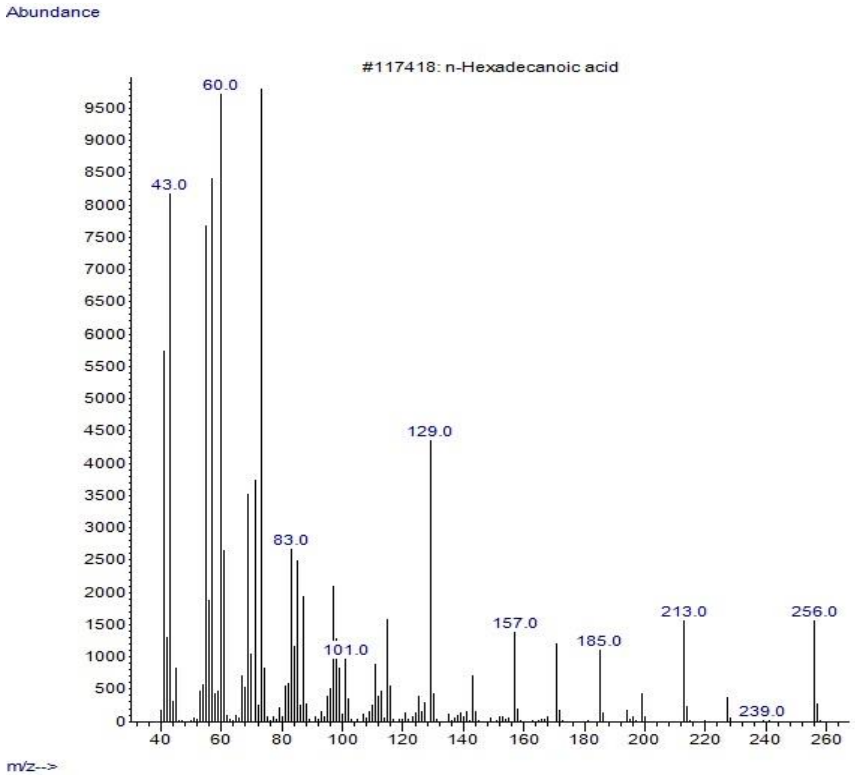

Figure 3: Mass spectrum of n-hexadecanoic acid, the second compound identified

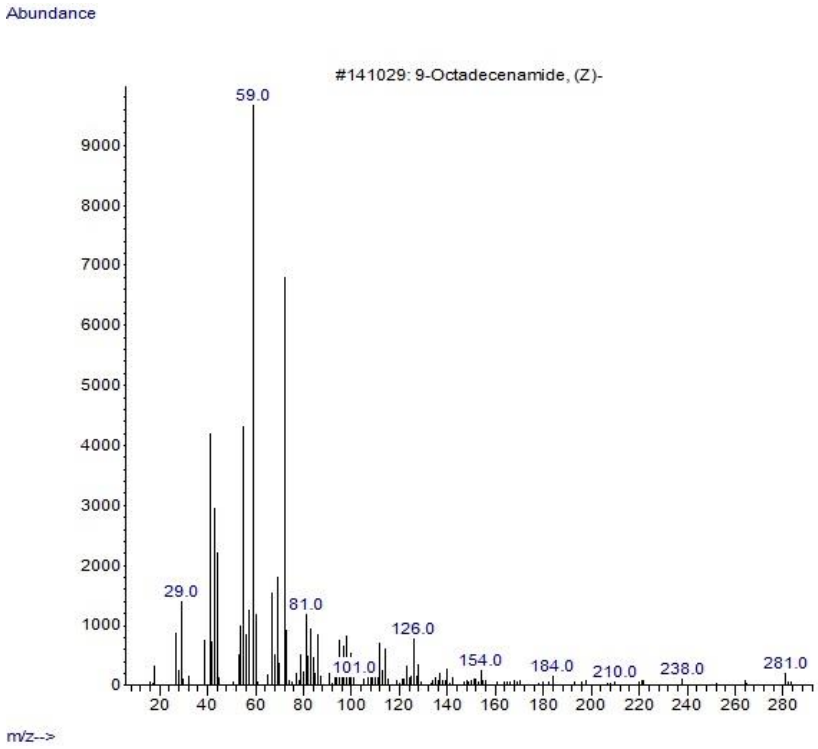

Figure 4: mass spectrum of z-9-octadecenoic acid, the third compound identified

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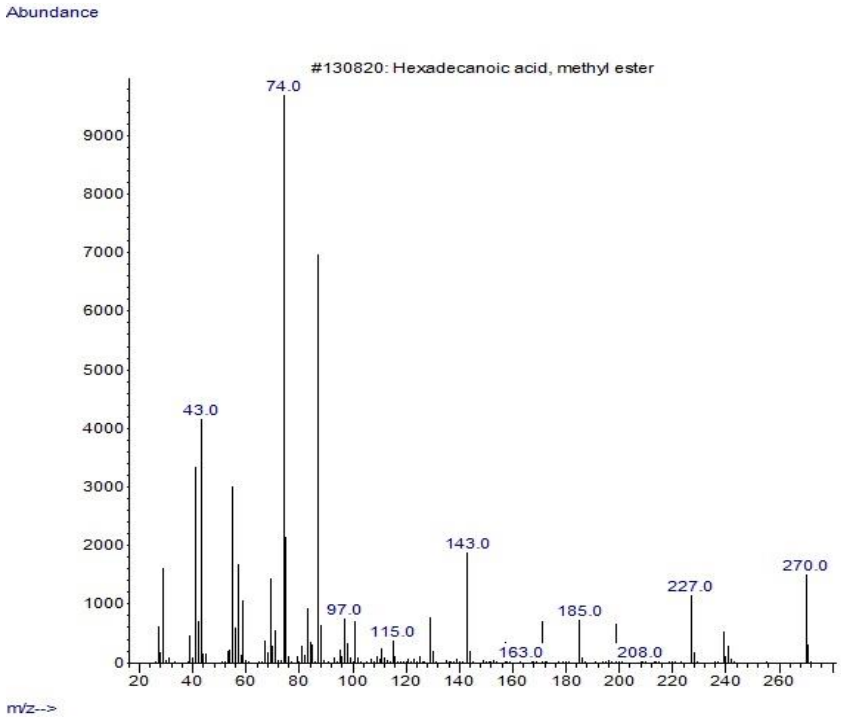

Figure 5: Mass spectrum of hexadecanoic acid methyl ester, the fourth compound identified

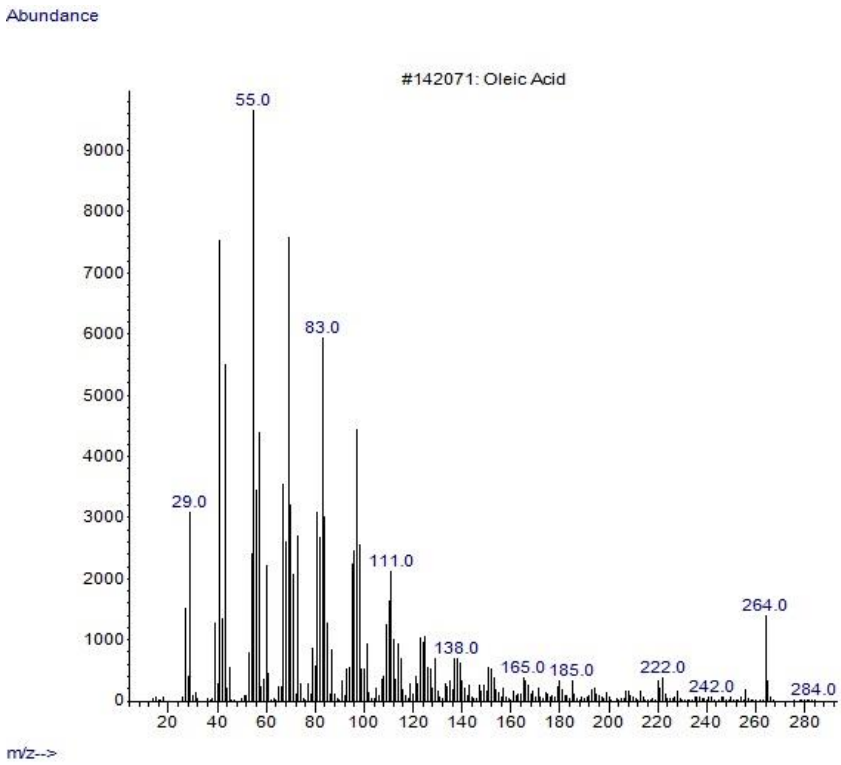

Figure 6: Mass spectrum of oleic acid, the fifth compound identified

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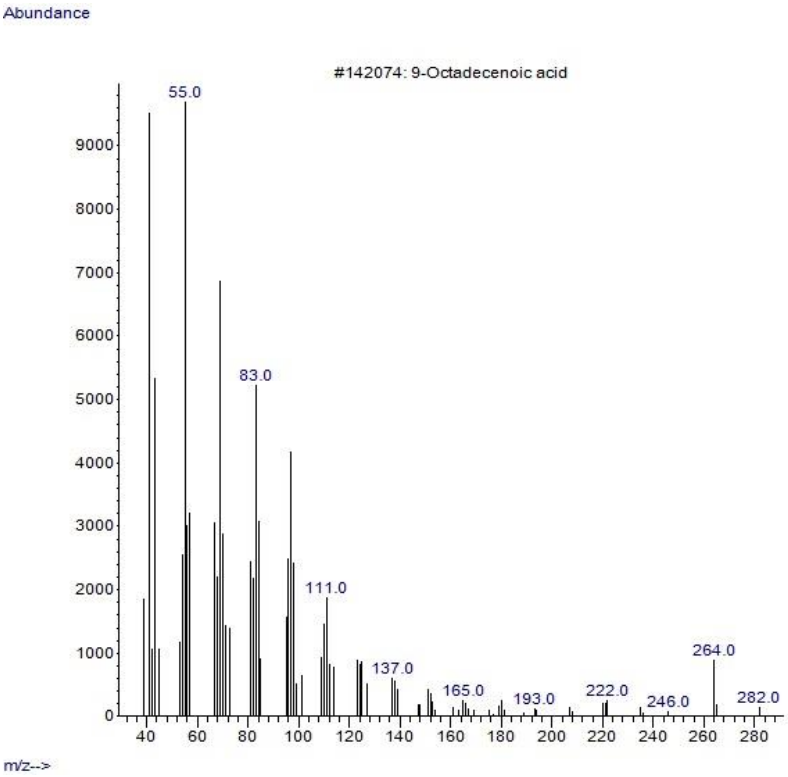

Figure 7: Mass spectrum, of t-9-octadecenoic acid (elaidic acid). The last compound identified.

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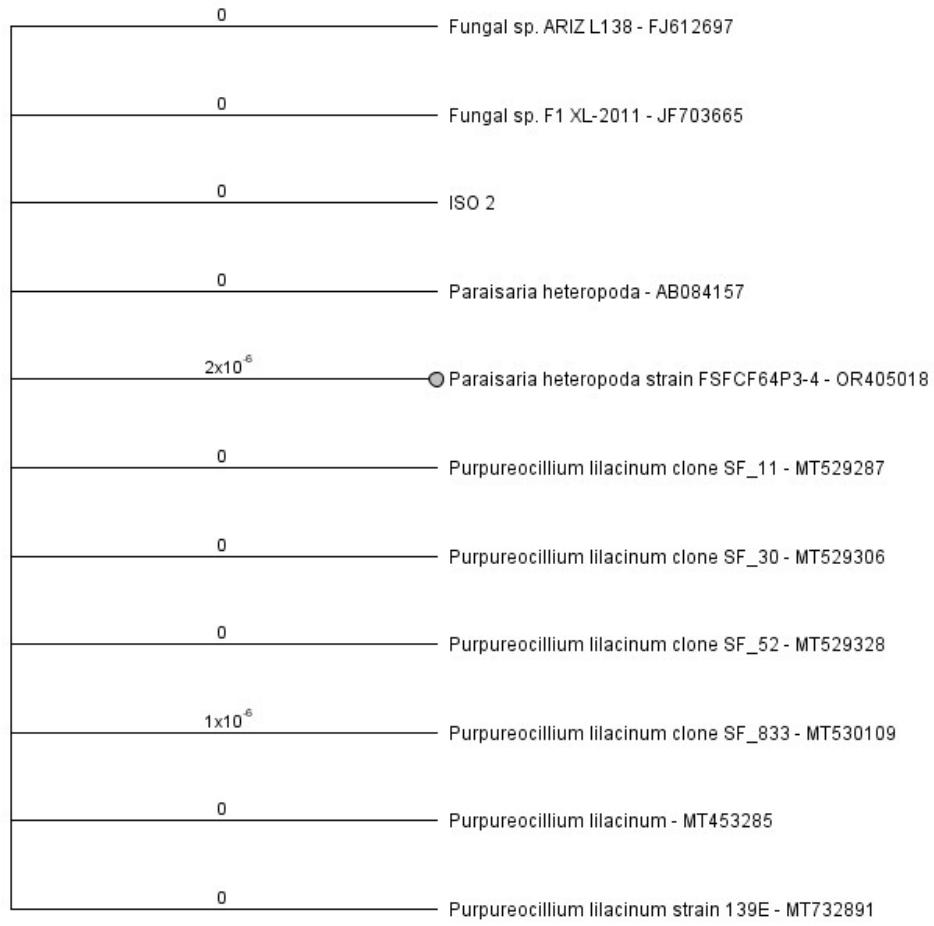

Figure 8: Phylogenetic tree of the isolate (*Purpureocillium* sp)

DISCUSSION

The results of this work revealed a newly discovered fungus (*Purpureocillium* sp) originally isolated from a sewage dump site with capacity to produce bioactive agents for man's use. This organism is considered one with great potential because its products are capable of helping man combat drug resistant infectious agents.

The result of the microbial inhibition test with the extract of the organism showed that both fungi (*C. albicans*) and bacteria commonly encountered in clinical practice were inhibited with zones of inhibition in the range of 15mm – 25mm (Table 3). This may be particularly useful in the management of mix microbial infections.

GC-MS analysis indicates that the extract of the organism is made up of organic fatty acids and amides. Their antibiotic capacity is had been documented. Methylene chloride which is the first compound detected has not been linked to any antibiotic ability other than an organic solvent for industrial extraction (Onyegbule *et al.,* 2019).

On the other hand, n-hexadecanoic acid had been linked to antibiosis before now (El-Shoubaky *et al.,* 2014; Kairmi *et al.,* 2015). Not only have that Tung *et al* (2021) detected

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hexadecanoic acid methyl ester and z-9-octadecenamide as possessing antibiotic capacity. It is not surprising to have identified these compounds as having antibiotic capacity in this work.

Again oleic acid has also been linked to antibiosis in the past against *E. coli*, *Salmonella typhimurium*, *P. aeruginosa* and *Bacillus cereus* using cocktail fatty acids according to the works of Sultan *et al* (2009) and Rasha (2019). Also Fung *et al* (2017) established that a combination of oleic, t-9-octadecenoic acid each with erythromycin had a good inhibition on methicilin-resistant *S. aureus*.

Amylase an enzyme important in the diagnosis of pancreatitis is also a product of this organism. The result of the amylase activity showed a 70U/L using Shomogy method (Cheesbrough, 2000). Also the microbial inhibition test done with this enzyme produced no activity unlike the results of some researches in the past – Pradeep *et al* (2022) and that of Anand *et al* (2019). Even though the primary aim of this enzyme was not met, its activity sets it on the road map to other means of antibiotic production and delivery such as amylase-nano silver particle couple especially against *S. aureus* (Pandey *et al.*, 2018).

CONCLUSION

This work has once again proved the indispensable nature of microorganisms in the fight against infectious agents via its products (fatty acids and amides – hexadecanoic acid, oleic acid, 9-octadecenamide, t-9-octadecenoic acid etc). Also new area of drug development and delivery via amylase-nano silver particle holds a holds a great hope for future antibiotic formulations.

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