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ANTIBIOTIC ACTIVITY OF A NEWLY DISCOVERED *ASPERGILLUS* SPECIES ISOLATED FROM SEWAGE DUMP SITE

Ngene M. Okechukwu[1*], Anyamene C. Okwudili[2], Ezebialu C. Uzoamaka[3], Iheukwumere Ikechukwu[4], Ozo N. Chinwendu[5], and Okorie Benson[6]

[1]Medical Laboratory Department, Comprehensive Health Center, Nnamdi Azikiwe University Teaching Hospital, Umunya Anambra, State.

[2,3,5]Department of Applied Microbiology, Nnamdi Azikiwe University Awka, Anambra State.

[4]Department of Microbiology, Chukwuemeka Odumegwu Ojukwu University Uli, Anambra State.

[6]Lahor Research Laboratory Benin City, Edo State.

[*]Corresponding Author's Email: okechukwu_michael@yahoo.com; Tel.: +2347031604508

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ABSTRACT: *Infectious agents causing diseases are becoming resistant to drugs produced to manage them. This has continued to spur scientific investigations for newer and better antibiotics to aid and/or replace existing ones. Soil samples were collected from sewage dump sites with the sole aim of isolating and screening fungi species for antibacterial substances using the cultural method. A newly discovered fungi species of Aspergillus was used to produce crude antibiotic, fractionated by column chromatography and tested on clinical isolates – Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Candida albicans and Pseudomonas aeruginosa. The result of zones of inhibition was 45 mm, 47 mm, 48 mm, 49 mm and 47 mm in order of listing of organisms. Gas chromatography mass spectrometry analysis of the fractionated extract revealed the following compounds as being responsible for the observed inhibition – methylene chloride, heptanoic acid, octanoic acid, methyl hydrogen phthalate, 2,4-di-tert-butylphenol, dodecanoic acid, z-10-tetradecen-1-ol acetate, tetradecanoic acid, 2-tetradecyloxy-ethanol, n-hexadecanoic acid, hexadecanamide, octadecenamide and octadecanamide. A cocktail of organic compounds – fatty acids and amides that displayed strong antimicrobial ability, if well tapped, hold a future in the development of new antibiotics.*

KEYWORDS: Fatty acids, Antimicrobial, Sewage, Heptanoic acid, Octanoic acid, Octadecanamide.

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INTRODUCTION

Microorganisms have continued to play a vital role in the existence of man as can be seen in industrial products useful to man or in the healthcare delivery service where they cause a lot of infectious diseases. The increasing resistant infectious agents seen in medical centers nowadays have elicited continuous search for better antibiotic agents from diverse ecosystems (Borreby *et al.,* 2023; Ghareeb *et al.,* 2022; Fung *et al.,* 2017). As a result of this scenario, many organic compounds – polyphenols, organic ethanolic compounds, fatty acids, amides, etc. – which are all products of microorganisms isolated from different ecosystems are now receiving focus for new potent antibiotic formulations to halt the menace of infectious agents (Tanvir *et al.,* 2018; Karimi *et al.,* 2015).

An antibiotic, as defined by Selma Waksman in 1942,is any small molecule of microbial origin that has an antagonizing effect on the growth of other microorganisms (Rafiq *et al.,* 2018; Clardy *et al.,* 2009). Thus, it is indispensable in the management of infectious agents especially now that drug resistant forms of these agents are on the increase. Since man's hope for success in the fight against infectious agents is a continuous process, microorganisms being known to produce numerous secondary useful metabolites will continue to be investigated in different habitats including sewage contaminated soil for new antibiotics (Muleta & Assefa, 2018; Rafiq *et al.,* 2018; Ladan, 2014).

The goal of this work was to establish the antibiotic activity of a newly discovered *Aspergillus* species against common infective microbial agents encountered in healthcare delivery service and whose resistances to available drugs are on the increase. This was achieved using traditional microbiological methods.

MATERIALS AND METHODS

Study Area

This work was carried out with soil samples taken from a sewage dump site (colloquially called oil rig by locals) in Nkwelle-Ezunaka, one of the communities in Oyi L.G.A of Anambra State with coordinates 6.2094^{o}N, 6.8405^{o}E.

Sample Collection

Soil samples were collected from ten different spots in the area of study. Samples were collected from 10 cm down the soil surface with the aid of sterile spatula in a sterile universal container and taken to the laboratory for culture (Muleta & Assefa, 2018).

Culture

The method of Rafiq *et al.* (2018) was adopted for the isolation of fungi from soil samples. 0.1 ml of serially diluted (10^4) soil samples were transferred to Potato Dextrose Agar (PDA) in Petri dishes and were spread evenly with a sterile glass spreader. The plates were then incubated at room temperature for seven days with daily examination.

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Cultural and Microscopic Identification of Isolates

Isolates were purified using Sabouraud Dextrose Agar (SDA) after which colonial features and microscopic examination were 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 perpendicularly to each isolate and incubated for 24 hours at 37^{o}C. Zones of inhibition were measured with meter rule.

Extraction of Antibiotic Substance

The *Aspergillus* species that showed inhibition in the primary test was used in solid state fermentation for the production of crude antibiotics using the method of Bhardwaj *et al.* (2017). 100 ml of water and 100 g of rice grain were weighed into a 500 ml Erlenmeyer flask and boiled to touch before being autoclaved at 121^{o}C for 15 minutes. When the medium cooled, a 1.0 cm block of the organism was inoculated into it and incubated at room temperature for fifteen days. Extraction of the antibiotic substance was done by adding 100 ml of autoclaved water into the flask. A sterile glass rod was used to break up the culture lump. Filtration using Whatman no 1 filter paper was done followed by centrifugation at 4000 rpm for 15 minutes. Fractionation of the extract was done using column chromatography (Kumar *et al.*, 2016).

Secondary Microbial Inhibition Test

The column fractions were used to carry out a secondary microbial inhibition test to know the fraction containing the inhibition agent. McFarland 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 cork borer (7 mm in diameter). A sterile swab was dipped into each test tube of the clinical organisms and spread evenly on the medium. 100 µl of each fraction was used to fill the well, left on the bench for diffusion to occur and later incubated at 37^{o}C overnight. Zones of inhibition were recorded using a meter rule.

Identification of Constituents of Extract

Fraction 4 of column chromatography done showed inhibition and was sent to Central Research Laboratory Federal University of Technology, Akure Ondo State for GC-MS analysis.

Molecular Identification of Organism

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

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RESULTS

The results of the presumptive identification of fungi isolates are given in Table 1. The genera of fungi species isolated include *Aspergillus*, *Mucor*, *Absidia*, *Penicillium* and *Purpureocillium*. The result of primary screening of *Aspergillus* species that showed inhibition (Isolate 1) is shown in Table 2 while its pictorial representation is shown by Plate 1. Also, Plates 2 and 3 show the organism grown on SDA and its photomicrograph.

The results of the secondary microbial inhibition test done using the fractionated extract is shown in Table 3 with Fraction 4 containing the inhibitory substance(s). The organism's extract recorded more than 45 mm zones of inhibition against all the test organisms – *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *C. albicans*. Plates 4–8 show the pictorial representation of the inhibition activity.

The result of the gas chromatography of the extract is shown in Figure 1. It showed that the extract consists of 13 compounds. The x-axis bears the retention time of the compounds while the y-axis bears the abundance of the constituent compounds. The mass spectra of the 13 identified compounds are shown in Figures 2, 3, 4, …, 14. The following are the names of the identified compounds: methylene chloride, heptanoic acid, octanoic acid, methyl hydrogen phthalate, 2,4-di-tert-butylphenol, dodecanoic acid, z-10-tetradecen-1-ol acetate, tetradecanoic acid, 2-tetradecyloxy-ethanol, n-hexanoic acid, hexadecanamide, octadecanamide and octadecenamide.

The result of the sequencing and BLASTn tests confirmed Isolate 1 to be an *Aspergillus* species. The gel electrophoresis result is shown in Plate 9 while the phylogenetic tree is shown in Figure 15.

Table 1: Presumptive Identification of Fungi Isolates

Macroscopy	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus terreus*	*Mucor* sp	*Absidia* species	Penic species	Iso 1 Asperg. sp	*Purpureocillium* sp
Surface colour	Greenish with white margin	Dark brown to black	Brown with white margin	White-gray	White	Bluish green with white edge	White to gray-white with age	white /cream coloured
Elevation	Slightly raised at centre	Slightly raised at centre	Flat with slightly raised centre	Fluffy/ thread like	Fluffy /slightly raised	Raised	Raised	Raised
Margin	Entire	Entire	Entire	uneven	uneven	Entire	Entire	Uneven, folded
Reverse colour	Cream coloured	No colour	Dull yellow	White	White	white	cream colour	None
Growth on SDA	Moderately	Rapid	Rapid	Rapid	moderate	Rapid	Moderate	Too slow
Microscopy								

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Sporangium/phialides	Phialides present	Phialides present	Phialides present	Round sporangium	Sporangium with swelling below it	Phialide bears conidia	Phialides bear conidia	Phialide present bear conidia
Hyphal vesicle/Columnella	Vesicle biseriate	Vesicle biseriate	Vesicle biseriate	Columella round	Columella has projection	Vesicle not pres	Vesicle monoseriate	Hyphae bear phialides
Metulae/sporangiophore	Metula present	Metula present	Metula present	Sporangiophore short and erect	Sporangiophore branched	Metulae present –brush-like appearance	Metula absent	Metula absent
Rhizoids	absent	absent	Absent	absent	Present	abs	abs	Abs
Conidia/sporangiospores	Round and light brown	Round and black	Round and light brown	Round	Sporangiospores oval / round/ blue	Conidia present in long chains	Conidia round/ sphericle	Oval, black conidia

Key: abs – absent, penic sp – *Penicillium* species, Asper. sp – *Aspergillus* species

Table 2: Zones of Inhibition (mm) of Isolate 1 (*Aspergillus* sp) on Test Organisms at Primary Test

Organisms	*Candida albicans*	*Klebsiella pneumoniae*	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
DZI	10 mm	15 mm	0.0 mm	0.0 mm	0.0 mm

Key: DZI – diameter of zones of inhibition

Table 3: Zones of Inhibition (mm) of Fractionated Extract (F4) of Isolate 1 against Test Organisms

Organisms	*Staphylococcus aureus*	*Candida albicans*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Escherichia coli*
Zoi frt extr1	45 mm	49 mm	48 mm	47 mm	47 mm

Key: Zoi – Zones of inhibition diameter, frt – fractionated, extr – extract, 1 – isolate 1 (*Aspergillus* species)

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Plate 1: Inhibition of test organisms by Isolate 1 (*Aspergillus* sp) at primary level testing of inhibitory capacity

Plate 2: The newly discovered *Aspergillus* sp on SDA

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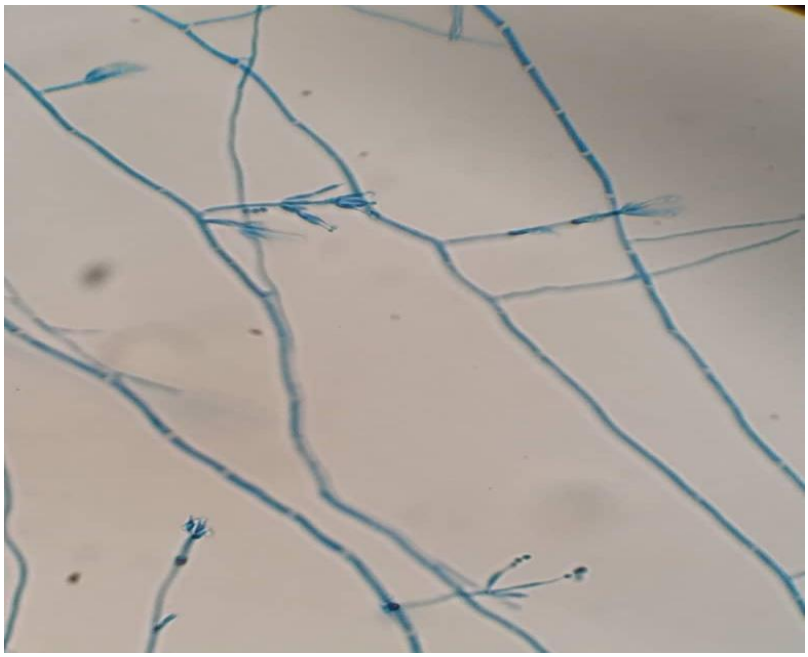

Plate 3: Photomicrograph of the newly discovered *Aspergillus* sp

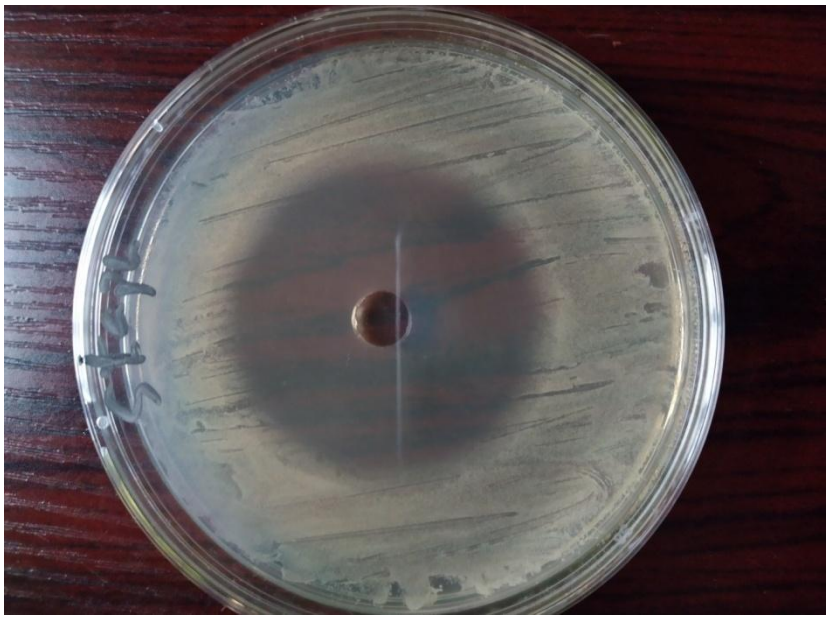

Plate 4: Inhibitory action of fractionated extract of Isolate 1 against *S. aureus*

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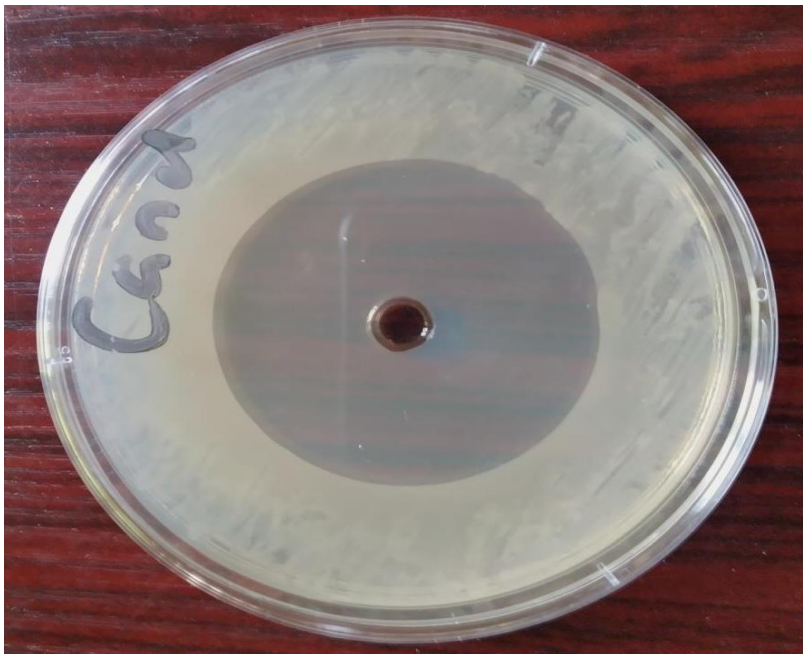

Plate 5: Inhibitory action of fractionated extract of Isolate 1 against *Candida albicans*

Plate 6: Inhibitory action of fractionated extract of Isolate 1 against *K. Pneumoniae*

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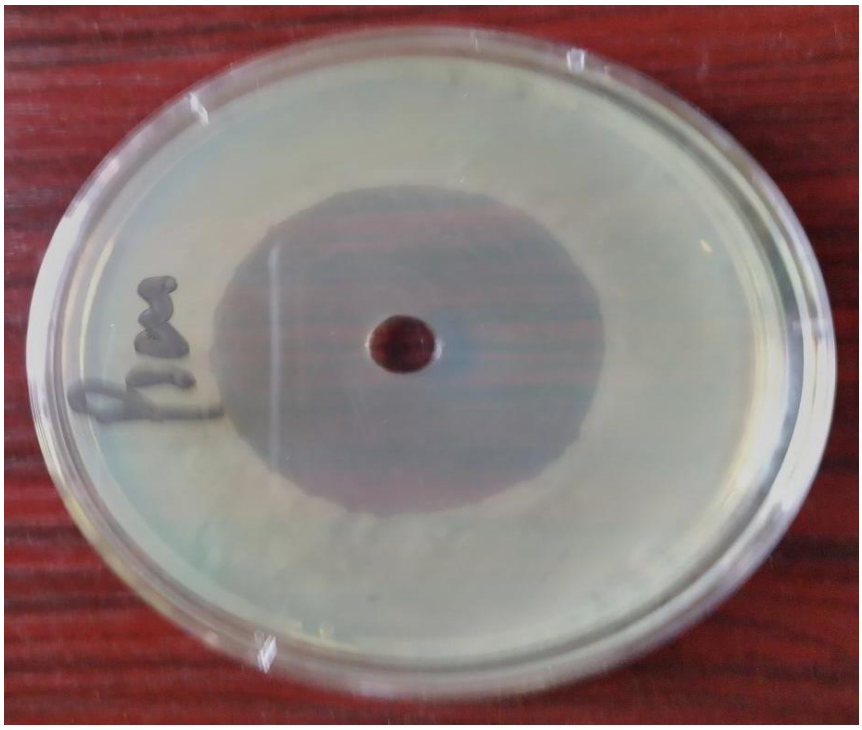

Plate 7: Inhibitory action of the extract of Isolate 1 against *P. aeruginosa*

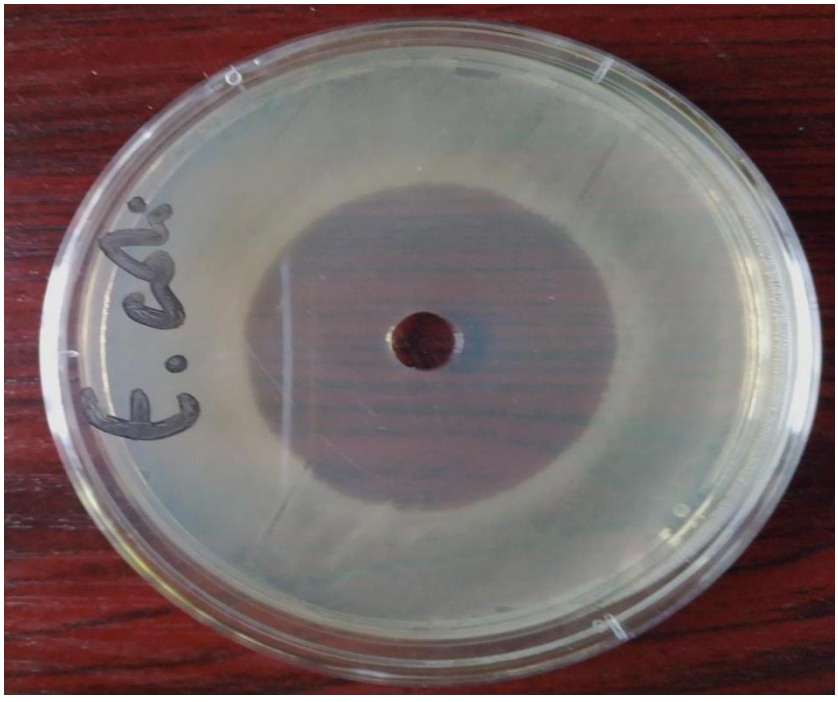

Plate 8: Inhibitory action of fractionated extract of Isolate 1 against *E. coli*

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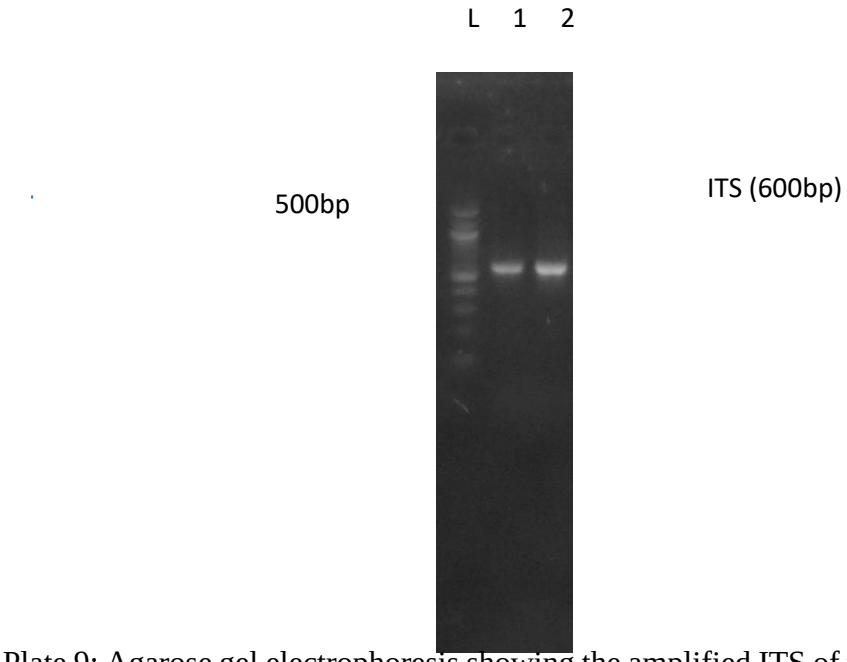

Plate 9: Agarose gel electrophoresis showing the amplified ITS of the fungal isolates. Lane 1 and 2 represent the ITS bands at 600bp of isolate 1 (*Aspergillus* species and *Purpureocillium* species while lane L represents the 100bp molecular ladder.

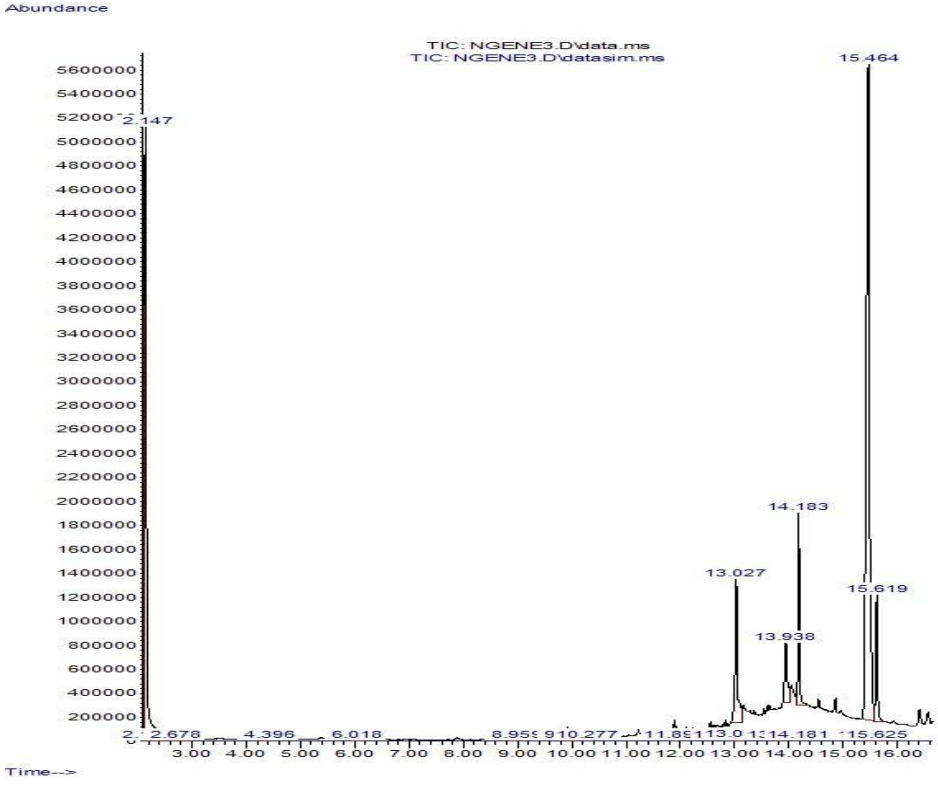

Figure 1: Gas chromatography of the extract of Isolate 1 (*Aspergillus* species)

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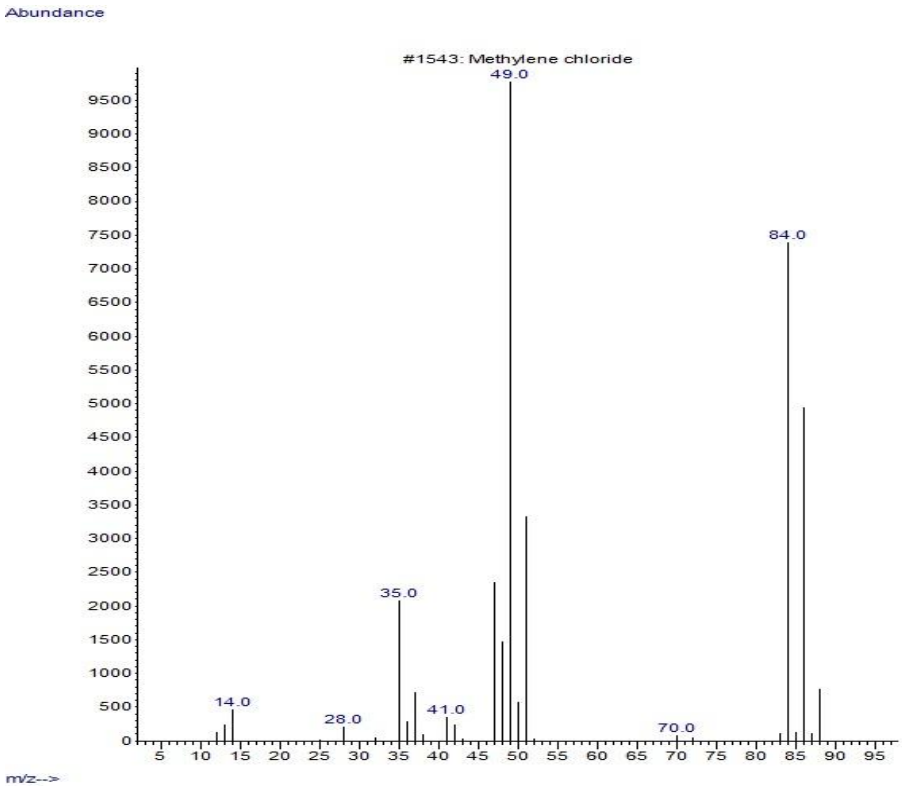

Figure 2: Mass spectrum of methylene chloride

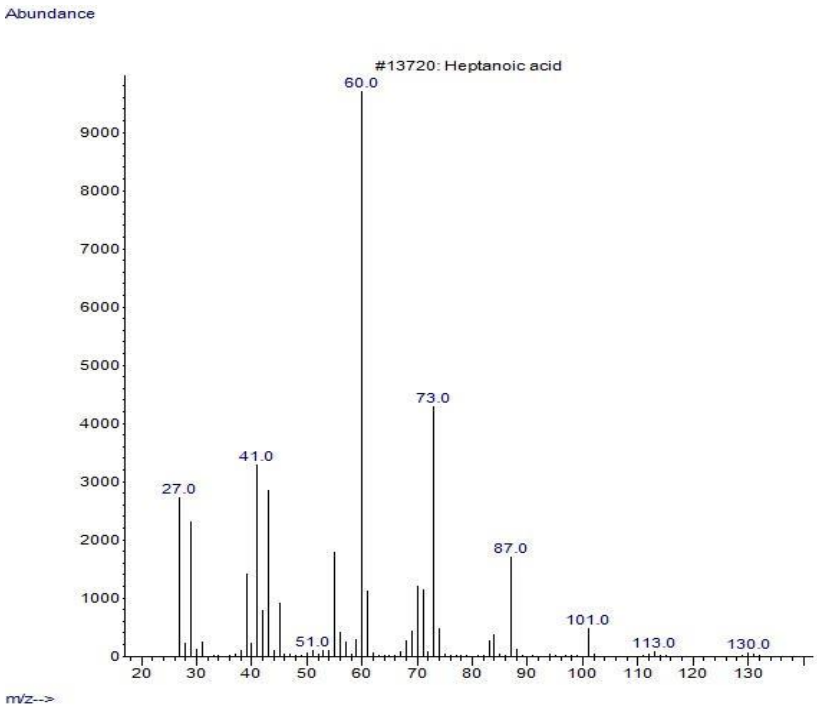

Figure 3: Mass spectrum of heptanoic acid

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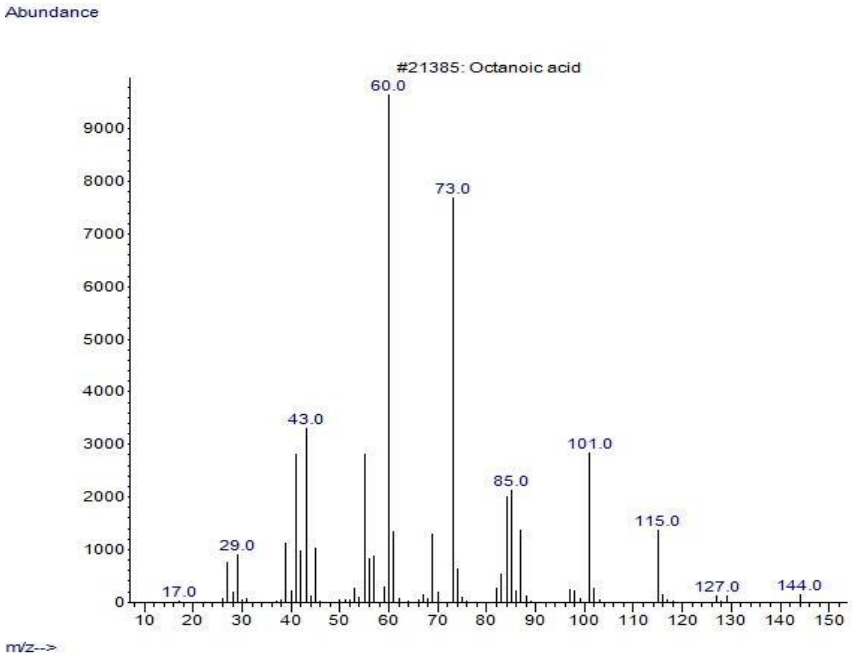

Figure 4: Mass spectrum of octanoic acid

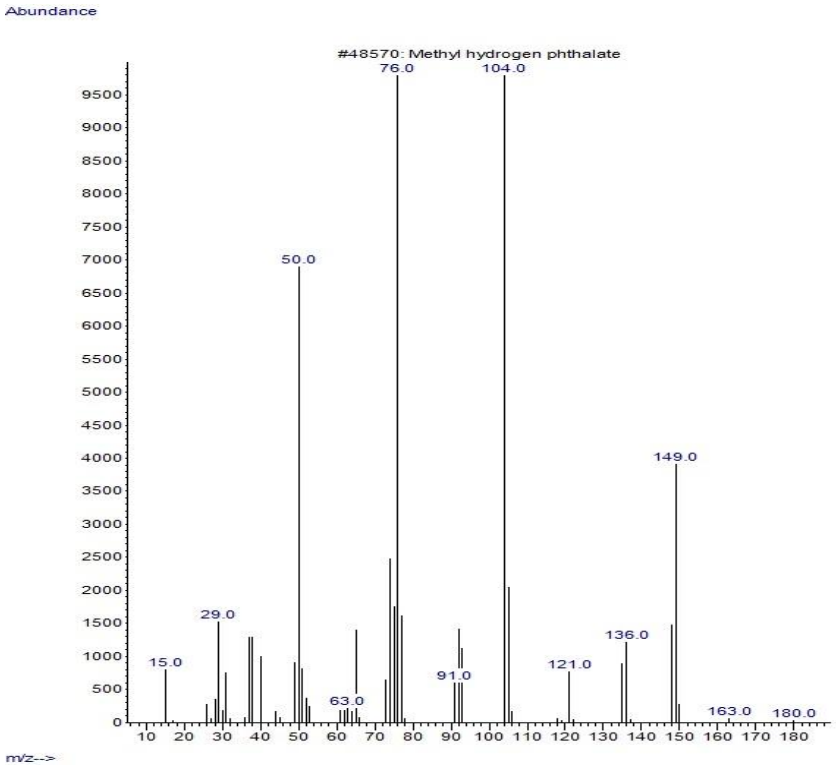

Figure 5: Mass spectrum of methyl hydrogen phthalate

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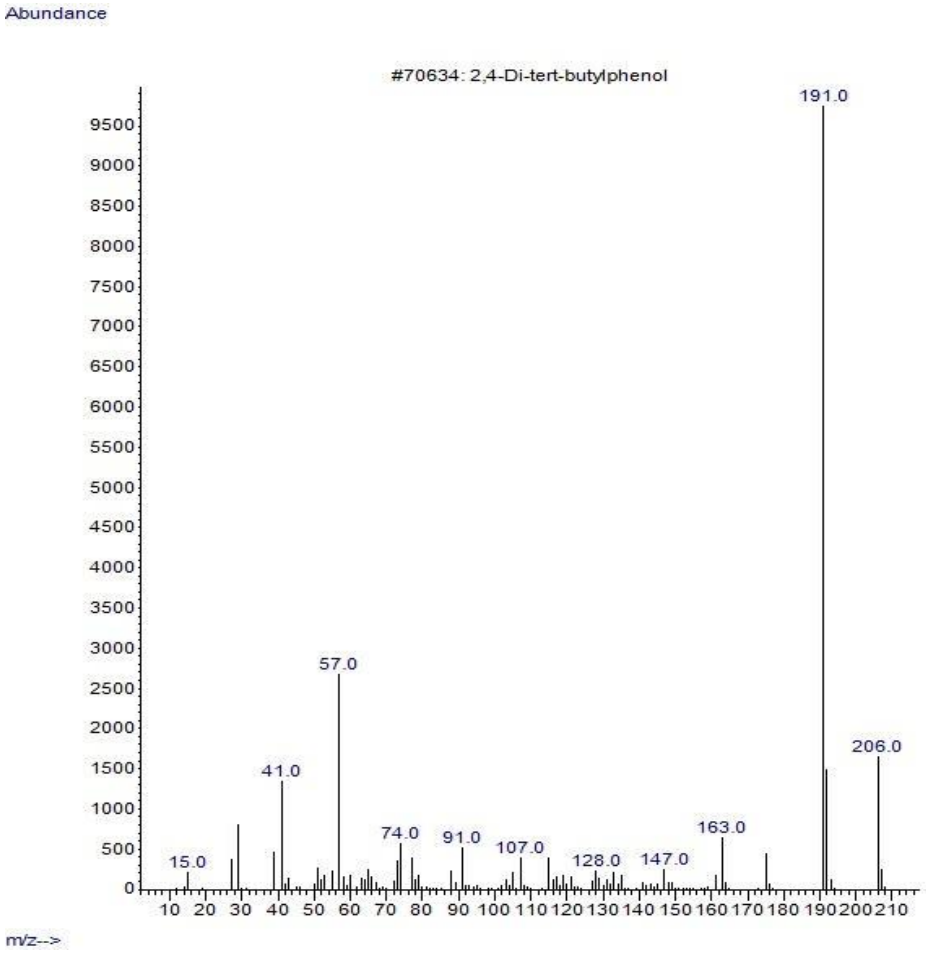

Figure 6: Mass spectrum of 2,4-di-tert-butylphenol

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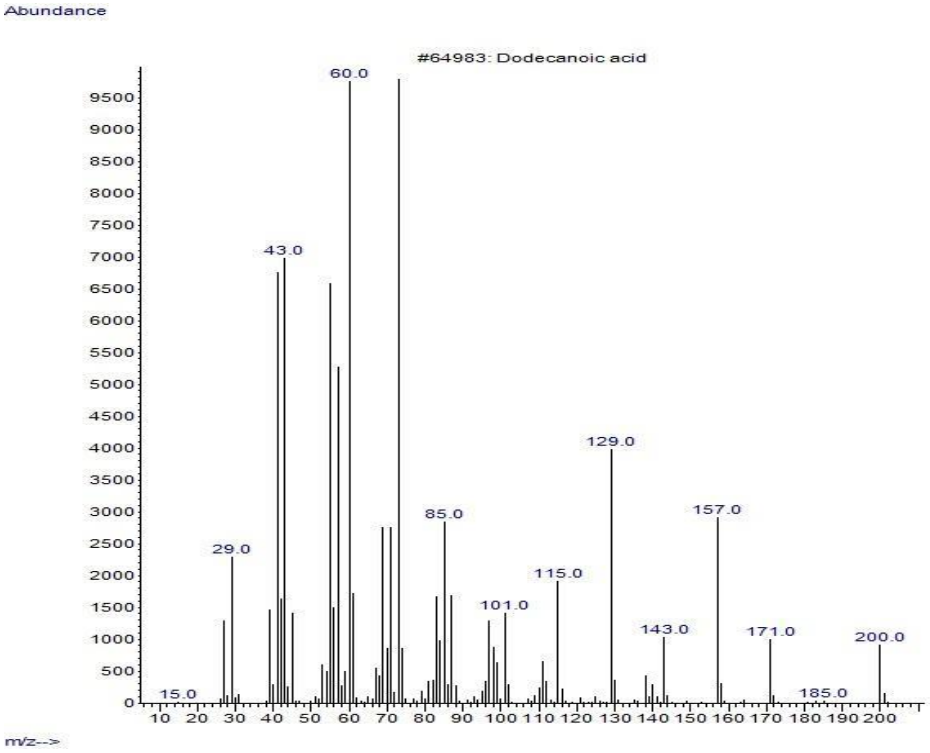

Figure 7: Mass spectrum of dodecanoic acid

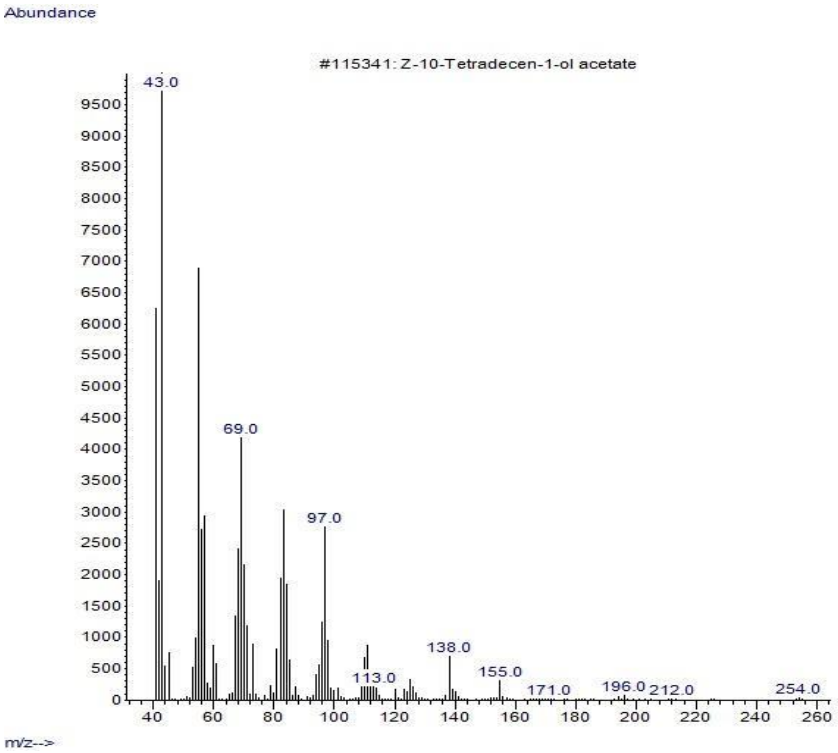

Figure 8: Mass spectrum of z-10-tetradecen-1-ol acetate

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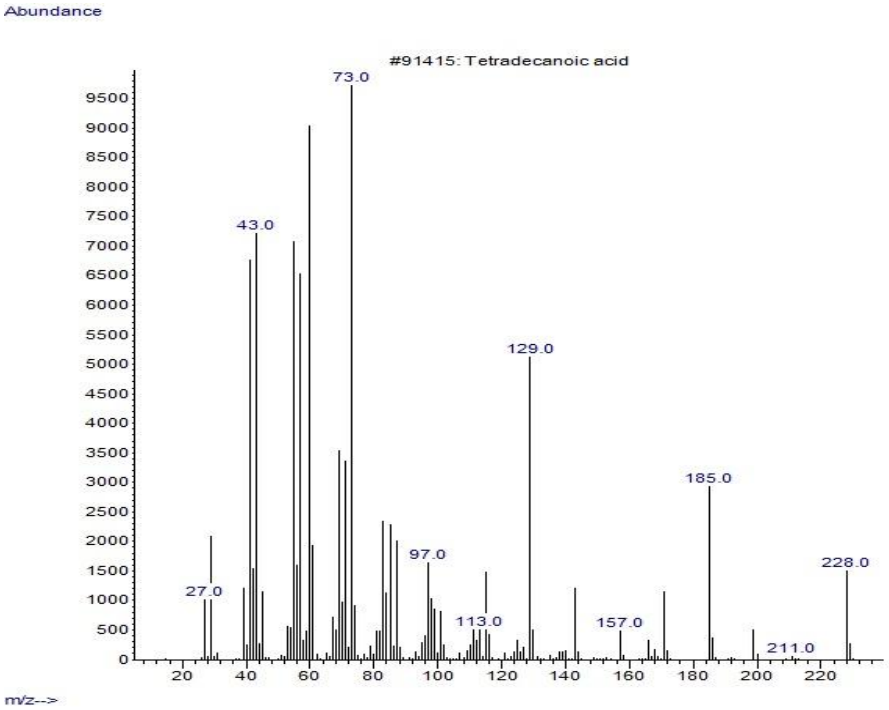

Figure 9: Mass spectrum of tetradecanoic acid

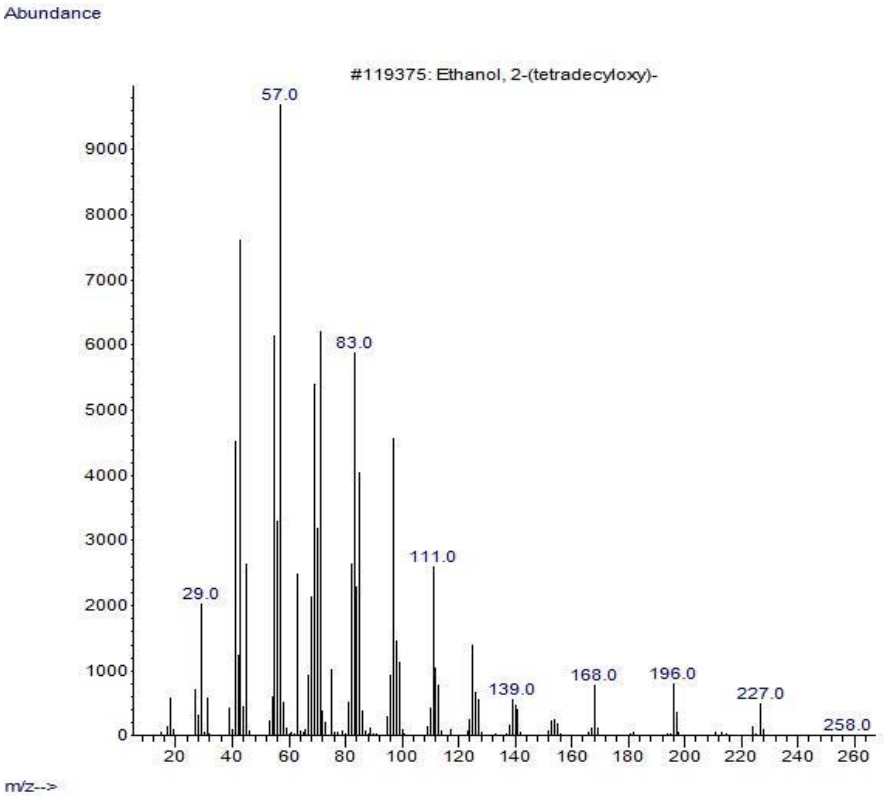

Figure 10: Mass spectrum of 2-tetradecyloxy ethanol

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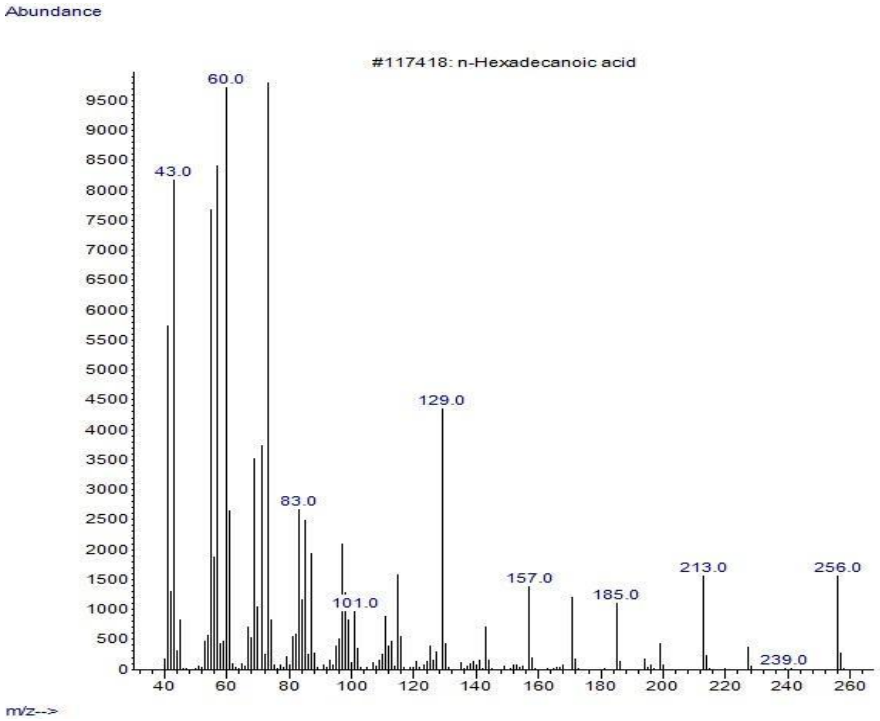

Figure 11: Mass spectrum of n-hexadecanoic acid

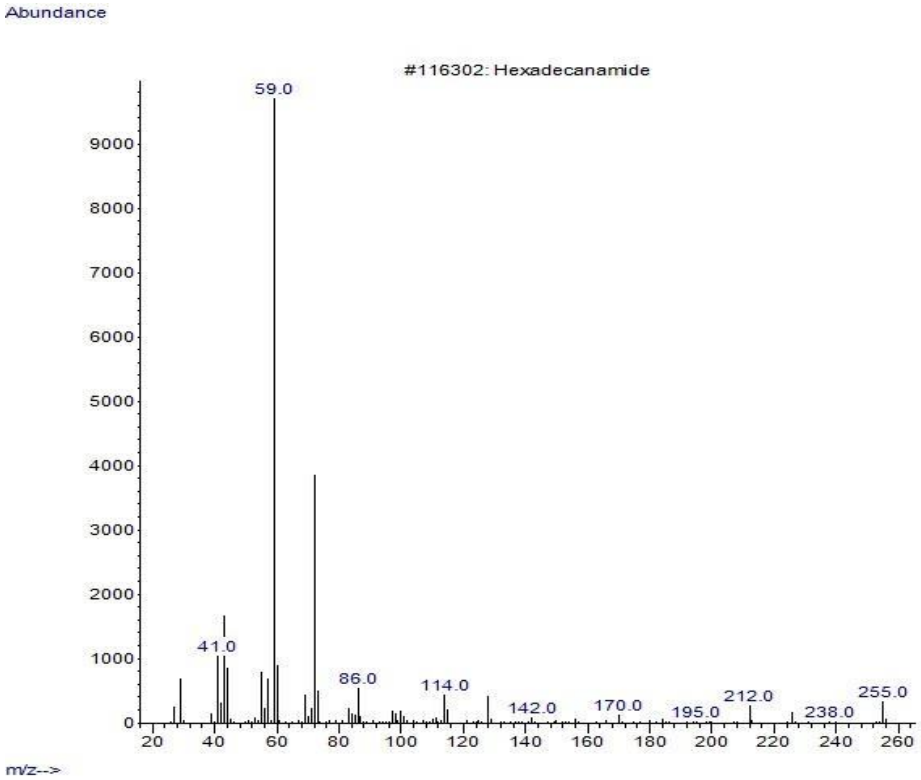

Figure 12: Mass spectrum of hexadecanamide

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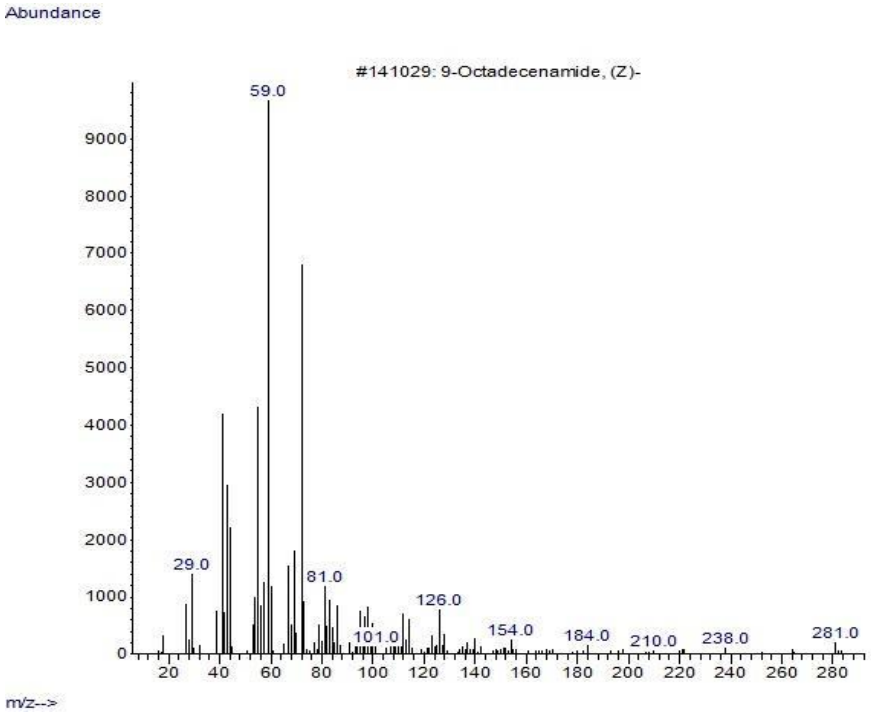

Figure 13: Mass spectrum of z-9-octadecenamide

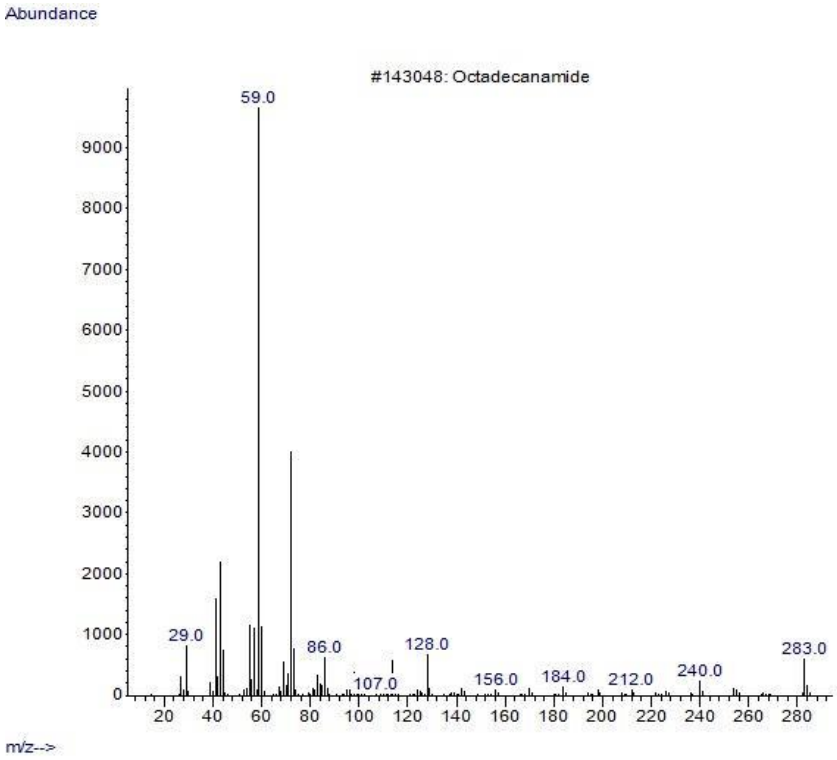

Figure 14: Mass spectrum of octadecanamide

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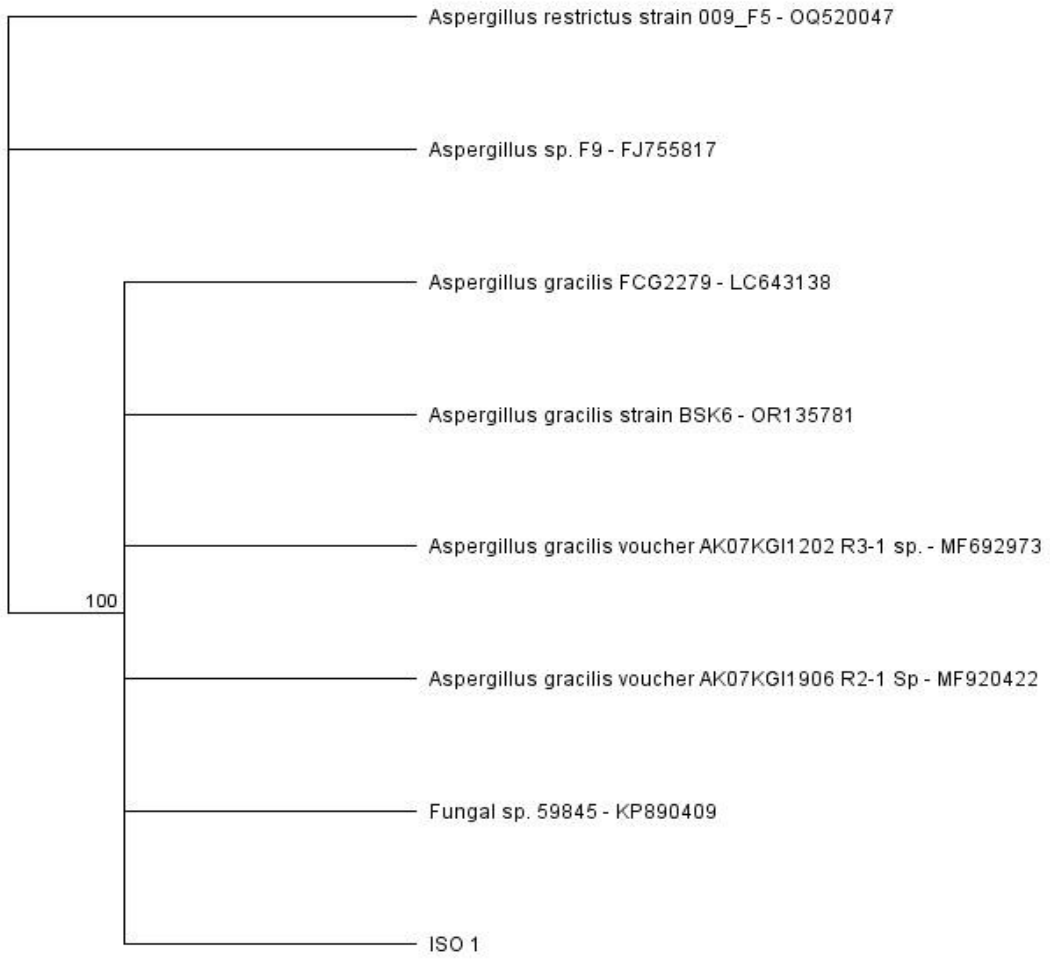

Figure 15: Phylogenetic tree of Isolate 1 (*Aspergillus* sp)

DISCUSSION

The results from this work showed the production of a cocktail of organic compounds mainly of fatty acids and amides by a newly discovered *Aspergillus* species isolated from sewage dump sites. The result of BLASTn for Isolate 1 stated that its sequence is similar to fungi species strain 59845 with accession number KP89040 in the same linkage as *Aspergillus gracilis*. In terms of percentage relatedness, Isolate 1 was found to have 99.6% relatedness to *Aspergillus* sp 59845 KP89040 with an E-value of 0. Therefore, Isolate 1 is a confirmed *Aspergillus* species. The results also showed that all the test organisms were inhibited by the fractionated extract with zones of inhibition greater than 45 mm.

Many of the organic compounds produced by this organism, which are methylene chloride, heptanoic acid, octanoic acid, methyl hydrogen phthalate, 2,4-di-tert-butylphenol, dodecanoic acid, z-10-tetradecen-1-ol acetate, tetradecanoic acid, 2-tetradecyloxy-ethanol, n-hexadecanoic acid, hexadecanamide, (9z)-octadecenamide and octadecanamide, have been established in the past as having antibiotic capabilities.

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Methylene chloride, methyl hydrogen phthalate and 2-tetradecyloxy-ethanol have not been linked to any antibiosis function in the past (Chirumamilla *et al.,* 2022; Huang *et al.,* 2021; Onyegbule *et al.,* 2011; Cunha *et al.,* 2009). Their functional contribution in this observed inhibition is doubtful. On the other hand, Elaidic acid has in the past been associated with antibiosis in relation to erythromycin (Fung et al., 2017). Park *et al.* (2022) found that the antibiotic capacity of tobramycin increased when combined with heptanoic acid in conjunction with other fatty acids such as lauric acid, myristic acid, palmitic acid and oleic acid. Tung *et al*. (2021) also identified heptanoic acid and octanoic acid in conjunction with other fatty acids from Tamarillo seed that showed antibiotic activity. The work of Sultan *et al.* (2009) also established octanoic acid from *Prunus japonicum* as having antibiotic activity.

This work detected another compound named 2,4-di-tert-butylphenol (2,4-DTBP). This is a phenolic compound produced by many organisms – bacteria, fungi and plants – and it is a compound known to be a toxic secondary metabolite (Zhao *et al.,* 2020). It was established by these authors that this compound has an inhibitory action on bacteria and fungi. The authors also established that the mechanism via which this compound exact inhibition on bacteria is by inhibition of formation of biofilm and quorum sensing while it affects spore germination and hyphal growth in fungi. The work of these authors on this compound showed that it has a vital role to the degree of inhibition noted on the test organisms in this work. Again, the work of Varsha *et al.* (2015) also showed that 2,4-di-tert-butylphenol is a strong antifungal. They showed that the compound isolated and purified from *Lactococcus* sp inhibited common fungi crop pathogens such as *Aspergillus niger*, *Penicillium chrysogenum* and *Fusarium oxysporum* (which had the highest inhibition recorded). This organic compound is definitely a factor in the microbial inhibition success recorded in this work.

Again, another compound, z-10-tetradecen-1-ol acetate (an alcoholic compound), was also detected in the biological extract of this work. Its inhibitory activity is documented (Subvathy & Thilaga, 2016). This compound has been noted to be among organic compounds detected in the whole body extract of *Cypraea arabica*, a marine mollusc, and shown to have antimicrobial capacity (Subavathy & Thilaga, 2016). It is believed via the research above that this compound also played a role in the inhibitory action noted among the test organisms used in this work.

A list of fatty acids and their derivatives comprising dodecanoic acid, tetradecanoic acid, n-hexadecanoic acid, hexadecanamide, z-9-octadecenamide and octadecanamide were also detected in this work. They have also been associated with inhibitory actions on microorganisms following their detection from biological extracts that showed microbial inhibitions (El Shoubaky *et al.,* 2014; Karimi *et al.,* 2015; Ma *et al.,* 2014). The above research works also gave credence to this work as the compounds they detected in their work as being responsible for the bacterial inhibition noted were also detected in this work.

Furthermore, hexadecanoic acid methyl ester and oleic acid (both detected in this work) were among the organic fatty acids also detected in the works of Ghareeb *et al.* (2022) and Nakaziba *et al*. (2022). They assayed the extracts of *Paratapes undulatus* clam (a marine mollusc) and *Corchorus olitorius* L leaves (Egyptian spinach) respectively for bacterial inhibition of *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Escherichia coli* with success. This shows that these compounds possess microbial inhibition capacity. Again, oleic acid has been recorded to have a good inhibition on methicillin resistant *S. aureus* when combined with erythromycin (Fung *et al.,* 2017). These research works also support the results recorded in this work.

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Also, in the distant past, fatty acid compounds – myristic acid, caprylic acid, lauric acid (all detected in this work) – among others and their glycerides had been noted to have a deleterious effect on organisms such as *Helicobacter pylori*. This is a revelation from the work of Sun *et al* (2003). They found out that a little increase in the concentration of the extract of these compounds had a deleterious effect on bacterial burden.

CONCLUSION

This research has once again established the fact that products of microorganisms (fatty acids and their derivatives – octanoic acid, myristic acid, hexadecanoic acid, hexadecanamide, oleic acid, octadecenamide, elaidic acid, 2,4-tetradecen-1-ol acetate, etc) are good prospects in the development of antibiotics for use in the management of infections. Thus, they are capable of strengthening and/or replacing the existing drugs in the fight against infectious agents if well tapped.

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