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Structural Elucidation and Antibacterial Evaluation of Natural Products from the Nigric Section of *Aspergillus* Species against Sorbitol-Positive and -Negative *Escherichia coli*

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Abstract

Studies have shown that limited availability, supply shortages and pricing of conventional antibiotics including carcinogenic and mutagenic nature are serious global problems that restrict access to effective treatment for common bacterial infections, and this may not only worsen clinical outcomes but potentially accelerate the development of antibiotic resistance. Hence this study focused on the structural elucidation of antibacterial substances from Mycelia of Negric section of *Aspergillus* species using natural sources. The fungal isolates were isolated from garden soil samples using standard microbiological techniques. The fungi were grown in a submerged medium prepared from *Phoenix dactylifera* (PD) fruits (20g), *Chrysophyllum albidum* (C.A) fruits (10g), *Glycine max* (GM) peel (10g) and *Musa paradisiaca* (MP) peel (10g). The antibacterial substances were precipitated, eluted, purified and structurally elucidated using column chromatographic, thin layer chromatographic and C-S-MS techniques respectively. The *Aspergillus* species isolated were *Aspergillus niger* strain HUS1 (ANH1), *Aspergillus aculeatus* strain AN5 (AAA5) and *Aspergillus awamori* strain DN-SN2 (AAD2). The optical growth and production of antibacterial substances occurred when the pH, Temperature, Carbon Source and nitrogen source were 7.0, 25℃, and sugar extracted from PD and NOdz respectively. The purified fractions; oleic acid (N1) > 1-docosene (N2) > I-octadecene (N3) > 2,3 furanone-4-hydroxyl (N4) from ANH1 showed significant ($P<0.05$) inhibitory activities against sorbitol positive and sorbitol negative *Escherichia coli*; *Escherichia coli* O157: H7 strain MB4-1 (SECM41) and *Escherichia coli* HH35 (SECH35). Similarly trans (2-doscenyl) succinic acid (A2) > E-15-hepadecenal (A1) > silane ethyl-trimethoxy (A3) from AAA5 and oleic acid (D1) > hexacosanoic acid (D2) > n-hexadecenoic acid (D3) > 9-octadecenoic acid (Z) methyl ester (D4) from AAD2 also showed significant ($P<0.5$) pronounced activities against the Sor+ and Sor- *Escherichia coli*. From this study, the purified fractions from ANH1, AAA5 and AAD2 showed pronounced activities against uropathogenic *Escherichia coli* and formed the basis of newer antibiotics from natural sources.

Keywords: *Natural antibiotics, Aspergillus species, Structural elucidation, E. coli inhibition*

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Introduction

Aspergillus is a large genus of mold belonging to family Aspergillaceae family, which has about 492 species that have been studied. Its section Nigri is an important group of species (Akinfala *et al.*, 2020). *Aspergillus* species are ubiquitous and occupy a wide spectrum of habitats in animal and plant environments, and they are economically important both as harmful and beneficial microorganisms. Due to its ability to survive adverse environmental conditions, *Aspergillus* species also inhabit terrestrial soil, water, and space. It also occupies a wide spectrum of habitats in plants and animals such as herbs, shrubs, trees, lichen, shrimp, and marine sponges.

A. niger strain grows well in various media with different carbon sources, including glucose, maltose, xylose, sorbitol, and lactose (Ano *et al*., 2017). However, its metabolism is remarkably affected by culture conditions, such as medium composition and fermentation mode (Blin *et al*., 2019). Species within this genus often grow quickly and can sporulate within a few days of germination. A combination of characteristics unique to *A. niger* makes the microbe invaluable to the production of many acids, proteins and bioactive compounds. Characteristics including extensive metabolic diversity, high production yield, secretion capability, and the ability to conduct post-translational modifications are responsible for *A. niger's* robust production of secondary metabolites (Fang *et al*., 2016).

Several researchers have reported that *Aspergillus* species are famous for the biosynthesis of valuable natural products of nutritional, agrochemical, and pharmaceutical interest, while others have only documented that the black mold is capable of causing disease in humans via the production of potent mycotoxins (Akinfala *et al.*, 2020; Cairns *et al.*, 2018). *Aspergillus* species possess large storage of active genes, which take part in regulating primary and secondary metabolisms.

Based on the chemical structures of the metabolites produced by *Aspergillus* species, these chemicals are grouped into five types: pyranone, alkaloid, cyclopentapeptide, polyketide, and sterol, respectively. Research has shown that these structures such as Malformin A demonstrated antibacterial and anticancer activities, while malformin C exhibited a broad spectrum of biological properties including anti-HIV-1, cytotoxic, anticancer, and antibacterial. Some other valuable chemicals that have low molecular weight are also produced by *Aspergillus* species, such as 2-phenoxyethanol and benzoic acid derivatives.

Several chemical studies have also suggested that *Aspergillus* is one of the fruitful sources of functional biomolecules, including organic acids, vitamins, pesticides, valuable proteases, and therapeutic agents, which have potential applications in various fields including agriculture, the food industry, and medicine (Elissawy *et al*., 2019; Blin *et al*., 2019).

Several researchers have evaluated secondary metabolites produced by molds such as Akinfala *et al.* (2020), Blin *et al*. (2019) and Elissawy *et al*. (2019) but little had been documented on structural elucidation of some secondary metabolites from mycelia of the nigric section of *Aspergillus* species using natural products. Hence, this study is aimed at elucidating the structure of some secondary metabolites from the mycelia of the nigric section of *Aspergillus* species using natural products.

Materials and Methods

Collection of samples: A total of 300 soil samples from hospital waste dumping site were randomly collected from different sites in Ihiala L.G.A, Anambra State. This was carried out using the method described in the study published by Iheukwumere *et al*. (2021). The litter from the soil surfaces was carefully scrapped out using a sterile stainless spoon. The soil auger was derived to a plough depth of 15 cm in the farm land, and soil sample was drawn up to 10 samples from each sampling unit into a sterile tray. The samples were thoroughly mixed and foreign materials such as roots, stones, pebbles and gravel were carefully removed. The soil sample was then reduced to half by quartering the sample. Quartering was carried out by dividing the soil sample into four equal parts and the two opposite quarters were discarded and the remaining two quarters were mixed. The process was repeated for the rest of the soil samples used for this study. The samples were carefully labelled and then kept in a disinfected cooler, to maintain the temperature and stability of the number of the isolates. The samples were transported to the laboratory for analysis.

Isolation of the Fungal Isolates: The media used for this isolation was Sabouraud dextrose agar (SDA/BIOTECH). One gram of the soil sample was weighed into a boiling test tube, 5 mL of normal saline was added and shake thoroughly and then made up to 10 mL using the normal saline (10^{-1}dilution). One milliliter of the suspension was added to four milliliter (4 mL) of normal saline (0.85% NaCl), which was given 5^{-1} dilution. From 5^{-1} dilution test tube, a five-fold serial dilution was carried out to obtain 5^{-5} dilution. One milliliter aliquot from 10^{-1}, 5^{-1} and 5^{-5} test tubes were collected and aseptically plated onto solidified sabouraud dextrose agar plate (90 mm x 15 mm) which was prepared according to the manufacturer's instruction and the procedures described in Cheesbrough (2010) supplemented with chloramphenicol (0.05 %) and spread using a spreading rod. The SDA was incubated in an inverted position for 5-7 days at 30 ± 2^0C.

Identification of Fungal Isolates: The fungal isolates were identified to the genus/species level based on macroscopic, microscopic and molecular characteristics of the isolates obtained from pure cultures as described in the study published by Iheukwumere *et al*. (2020).

Screening the Fungal Isolates for Antibiotic production: For antibiotic production, Mueller Hinton Agar (MHA) medium was prepared according to the manufacturer's direction. This was allowed to cool and then poured in Petri dishes and kept in incubator at 37^0C for 24 h to check its sterility. Then the test organisms; *Escherichia coli, Staphylococcus aureus,* and *Salmonella species* were grown on broth culture at 37^0C for 24 h. After incubation, sterilized swab stick was dipped into the broth cultures and swabbed on

MHA plates and allowed for 1 h. Then wells were made on the MHA plates using a sterile cork borer. Then the broth culture of the fungal isolates were carefully centrifuged at 6000 rpm for 10 minutes and their supernatants were poured in the wells and incubated at 37^0C for 48 h. zones of inhibition were observed after incubation (Iheukwumere *et al*., 2025a and Iheukwumere *et al*., 2025b).

Designing of Medium (PCGM Medium)
Phoenix dactylifera (PD) fruits, *Chrysophyllum albidum* (CA) fruits *Glycine max* (GM) peel and Musa paradisiaca (MP) peel were air-dried and then ground into powdered form. Fifty grams comprising 20 g of PD, 10 g of each of the CA, GM and MP samples were weighed into 1000 mL Erlenmeyer flask, 200 mL of distilled water was added and then made up to 500 mL using the distilled water. This was sterilized and allowed to cool at room temperature (30±2°C).

Extraction of Antibiotics: The characterized fungal isolates were sub cultured in Sabouraud dextrose broth and incubated for 5 days. Then 20 mL of the broth culture was introduced into the 500 mL PCGM medium and then incubated at 30±2°C for 7 days.

Optimization of Carbon source, Nitrogen source, PH and Temperature for Production of the Antibiotics: The effects of carbon sources (glucose, sucrose, fructose, maltose, sugar extracted from *Phoenix dactylifera*/Date fruits) nitrogen source (peptone, beef extract, $NaNO_3$, $(NH_4)_2SO_4$, NodZ) was investigated by supplementing the PCGM medium with the above carbon and nitrogen sources at varying pH (4,5, 6, 7, 8, 9) and temperature (25°C, 30ºC, 35ºC, 40ºC, 45ºC) for antibiotics production (Suganthi *et al.*, 2014). NodZ was prepared from the mixture of *Rhizobium leguminosarum* (1 g of lyophilized mixture), soybean meal (10 g) and *Arachis hypogoea* nodule meal (10 g) (Iheukwumwere *et al*., 2025).

Extraction and Elution of Antibiotic: The culture medium was centrifuged at 8000 rpm for 15 min. This was filtered using What Man No1 filter paper (110 mm × 110 mm). The supernatant was eluted using a column chromatographic technique using ethyl acetate/hexane/methanol/dichloromethane at the ratio of 2:2:1:1 (Iheukwumere *et al*., 2025).

***In vitro* Antibacterial Activities of the Eluate using Agar Well Diffusion Method:** This was carried out by the modified method of Iheukwumere *et al*. (2017). Each labeled plate was uniformly inoculated with the test organism (*Escherichia col*i, *Staphylococcus aureus* and *Salmonella* specie) using spread plate method. A sterile cork borer of 5 mm diameter was used to make the wells on the medium. One tenth millilitre of the eluate was dropped into each labeled wells and then incubated at 35±2ºC for 24 h. Antibacterial activity was determined by measuring the diameter of the zones of inhibition (mm) produced after incubation

Purification and Elucidation of the Antibiotic: The eluate that inhibited the growth of the tested bacteria were subjected to Thin Layer Chromatographic technique using chloroform/methanol (24:1 v/v), chloroform/methanol/water (1:1:1 v/v/v), benzene/acetic acid/water (4:1:5 v/v/v) and acetonitrile/water (92.5/7.5 v/v). The successive bands seen on the plates were crapped off carefully, dissolved in methanol, and centrifuged at 10,000 rpm for 10 min to remove the silica. The supernatant was subjected to structural elucidation using gas chromatography coupled with mass-spectrophotometer (Iheukwumere *et al*., 2025).

Data Analysis: The data obtained in this study were presented in tables and figures. Their percentages were also calculated. Significance of the study was carried out using one way Analysis of Variance (ANOVA) at 95% confidence level. Pair wise comparism was carried out using student "t" test (Iheukwumere *et al*., 2018, Iheukwumere *et al*., 2020).

Results

The fungal isolates exhibited similar macroscopic and microscopic characteristics but differed slightly in the initial and final appearances of the isolates on the Sabouraud Dextrose agar (SDA). The growth rates and colony texture also differed as shown in Table 1 and 2. Isolate C was uniseriate whereas isolates B and D were biseriates as shown in Table 2. The molecular characteristics of the isolates revealed that isolate B was *Aspergillus niger* strain HUS1 (ANH1), isolate C was *Aspergillus aculeatus* strain AN5 (AAA5) and isolate D was *Aspergillus awamovi* strain DN-SN2 (AAD2).

The study also showed that the fungal isolates exhibited significant ($P<0.05$) optimal growth and production of antibiotics at pH = 7 and temperature of 25°C. The maximum production of antibiotics was isolated when sugar extracted from *Phoenix dactylifera* (Date fruit) and glucose, sodium nitrate ($NaNO_3$) and NOdZ were used most pronounced but the activities were not statistically significant ($P>0.05$) when compared to other sources of carbon and nitrogen employed in this study shown in figures 1,2,3 and 4.

The fractions (N1>N2>N3>N4) eluted from ANH1 showed pronounced activities against sorbitol positive *Escherichia coli* strain HH351 (SECH35) and sorbitol negative *Escherichia coli* 0157:H7 strain MB41-1 (SECM41) of which the activities were significantly ($P<0.05$) higher against SECH35 as shown in figure 5.

The study further revealed that N1, N2, N3 and N4 were Oleic acid, 1-Docosene, 1-Octadecene and 2 (3)-Furanone 4-hydroxy respectively. Similarly fractions (A2>A1>A3) eluted from AAA5 exhibited similar antibacterial patterns as the fractions eluted from ANH1 as shown in figure 6.

A1, A2 and A3 were E-15-hepadecenal, trans (2-Docosenyl) succinic acid and silane ethyl-trimethoxy respectively. Similar results were observed from the fraction (D1>D2>D3>D4) eluted from AAD2. D1, D2, D3 and D4 were Oleic acid, hexacosanoic acid, n- hexadecenoic acid, 9-octadecenoic acid (Z)-methyl ester respectively as shown in figure 7.

Table 1: Macroscopic characteristics of the fungal isolates

Parameter	Isolate P	Isolate Q	Isolate R
Initial Appearance on SDA(2-3 days)	White to pale	Colourless to pale	Colourless to creamy
Later Appearance on SDA (5 days)	Dark/Black with white edges	Dark brown	Chocolate black
Reverse Colour	Pale	Yellow	Dull yellow
Growth Rate	Rapid	Moderate to rapid	Moderate to rapid
Colony texture	Velvety	Rough	Rough/velvety
Colour of mycelium	Black	Dark brown	Choxolate black
Fungus	*Aspergillus* species	*Aspergillus* species	*Aspegillus* species

Table 2: Microscopic characteristics of the isolates

Parameter	Isolate B	Isolate C	Isolate D
Shape of vesicle	Globose	Globose	Globose
Metula covering	Entire	Entire	Entire
Shape of conidia	Ellipsoidal	Ellipsoidal	Ellipsoidal
Colour of conidia	Black	Dark brown	Chocolate
Conidia head	Radiate	Radiate	Radiate
Texture of conidia	Finely wrinkled	Spiny wrinkled	Finely wrinkled
Nature of hyphae	Septate	Septate	Septate
Colour of conidiosphore	Hyaline	Hyaline	Hyaline
Texture of conidiosphore	Smooth	Smooth	Smooth
Length of conidiosphore	Long	Long	Long
Seriation	Biseriate	Uniseriate	Biseriate
Fungus	*Aspergillus niger*	*Aspergillus aculeatus*	*Aspergillus* species

Table 3: Molecular characteristics of the isolates

Parameter	Isolate B	Isolate C	Isolate D
Max score	832	793	785
Total score	832	793	785
Query cover(%)	100	100	100
E-value	0.0	0.0	0.0
Identity (%)	100	100	100
Accession number	MF163441	KU527791	KY509551
Description	*Aspergillus niger* strain HUS1 (ANH1)	*Aspergillus aculeatus* strain AN5(AAA5)	*Aspergillus awamori* strain DN-SN2 (AAD2)

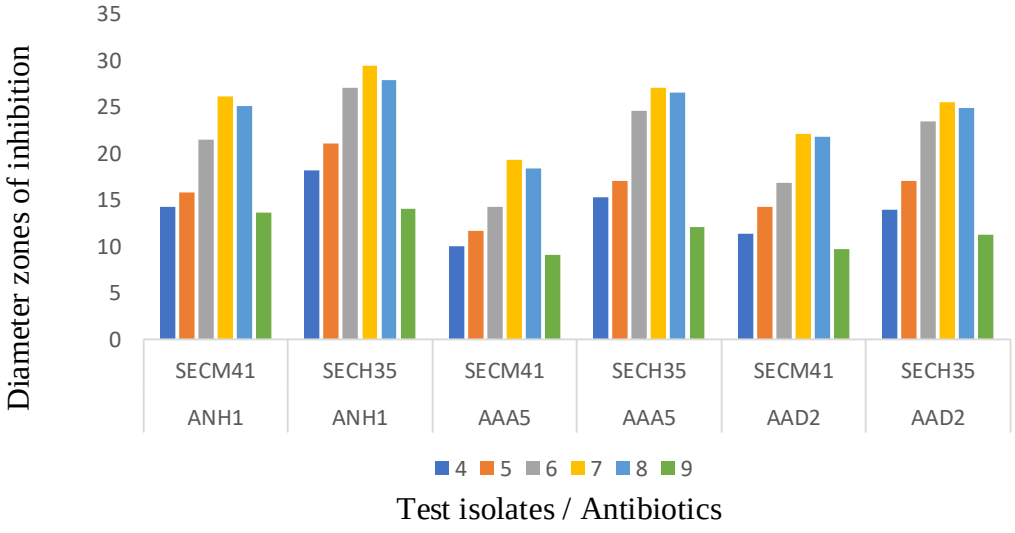


Figure 1: Effect of pH on the production of antibiotics
Sorbitol Negative Escherichia coli strain MB41-1 ----- SECM41
Sorbitol positive Escherichia coli strain HH35 ------ SECH35

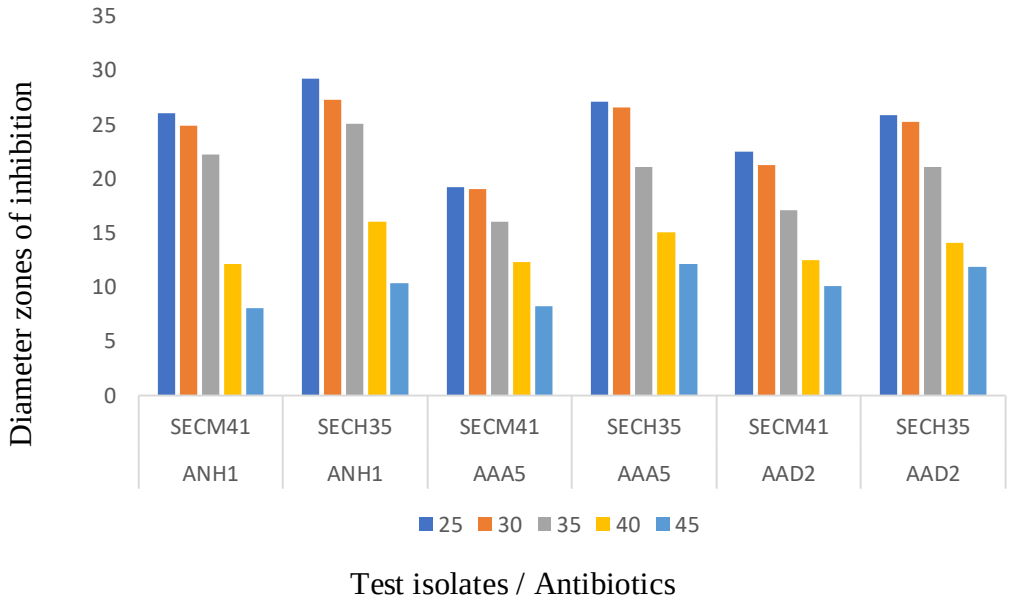


Figure 2: Effect of temperature on the production of antibiotics
Sorbitol Negative Escherichia coli strain MB41-1 ----- SECM41
Sorbitol positive Escherichia coli strain HH35 ------ SECH35

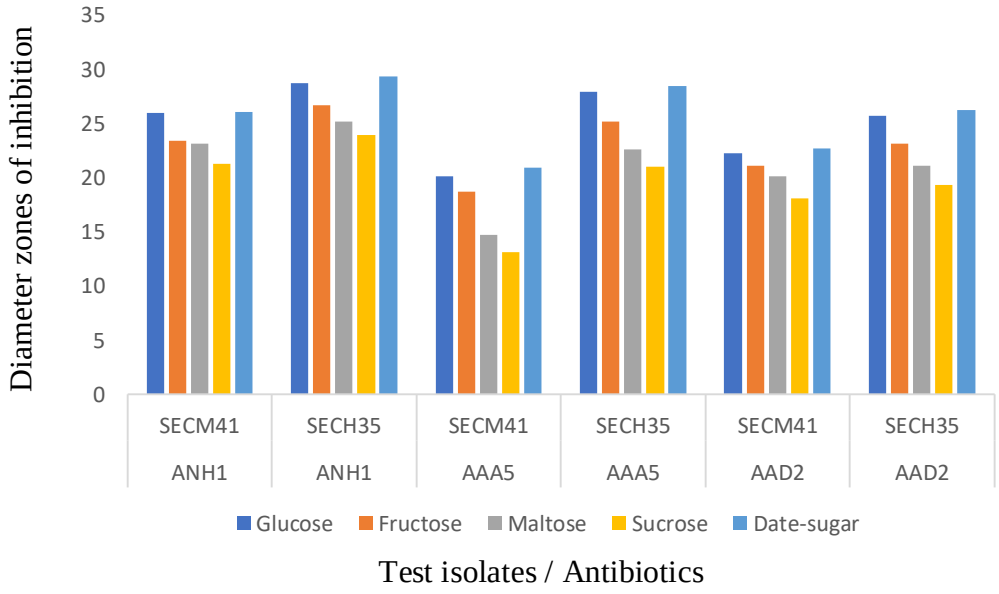


Figure 3: Effect of carbon source on the production of antibiotics
Sorbitol Negative Escherichia coli strain MB41-1 ----- SECM41
Sorbitol positive Escherichia coli strain HH35 ------ SECH35

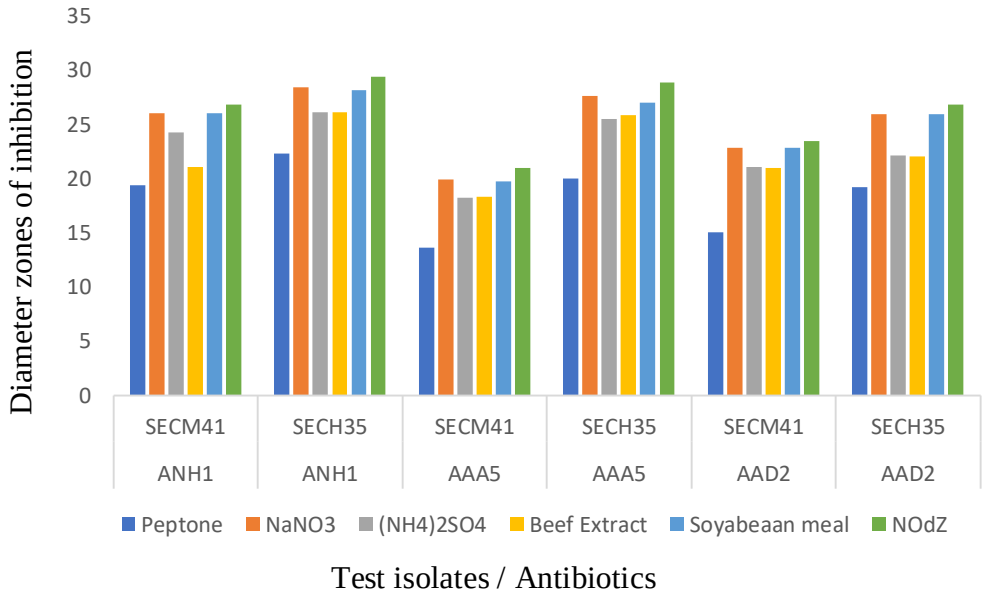


Figure 4: Effect of nitrogen source on the production of antibiotics
Sorbitol Negative Escherichia coli strain MB41-1 ----- SECM41
Sorbitol positive Escherichia coli strain HH35 ------ SECH35

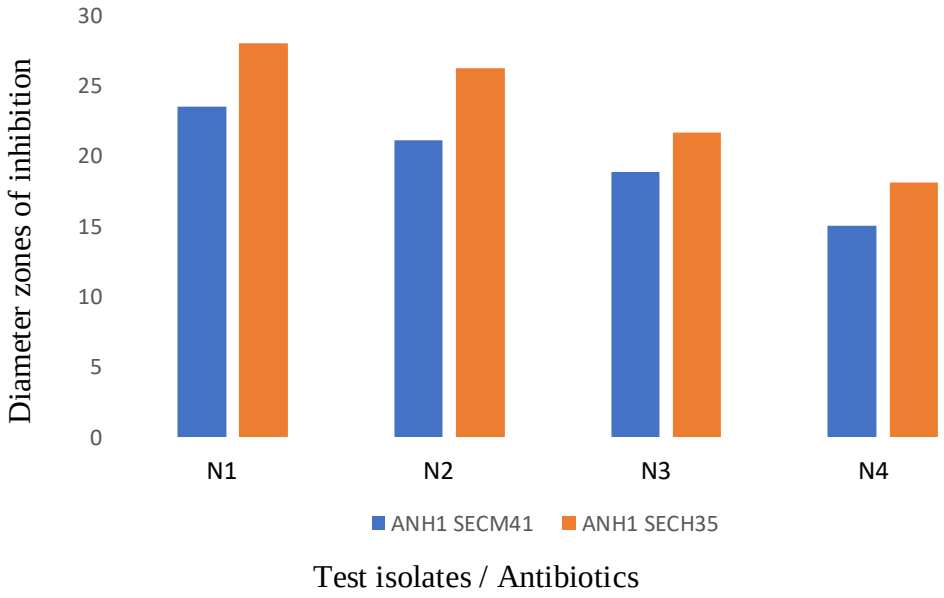


Figure 5: Diameter zones of inhibition of the fraction of the eluates from ANH1 against the test isolates.
Sorbitol Negative Escherichia coli strain MB41-1 ----- SECM41
Sorbitol positive Escherichia coli strain HH35 ------ SECH35

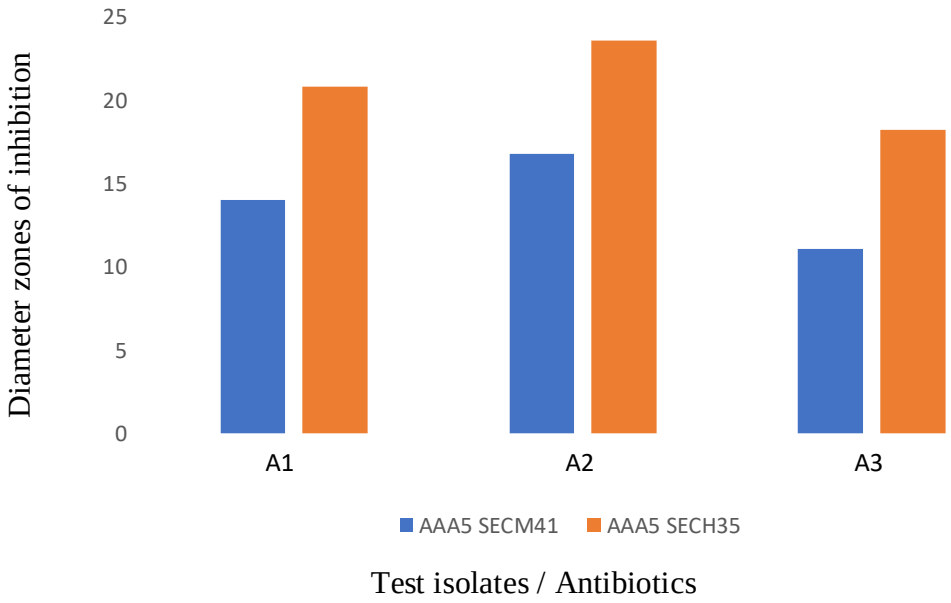


Figure 6: Diameter zones of inhibition of the fraction of the eluates from AAA5 against the test isolates.
Sorbitol Negative Escherichia coli strain MB41-1 ----- SECM41
Sorbitol positive Escherichia coli strain HH35 ------ SECH35

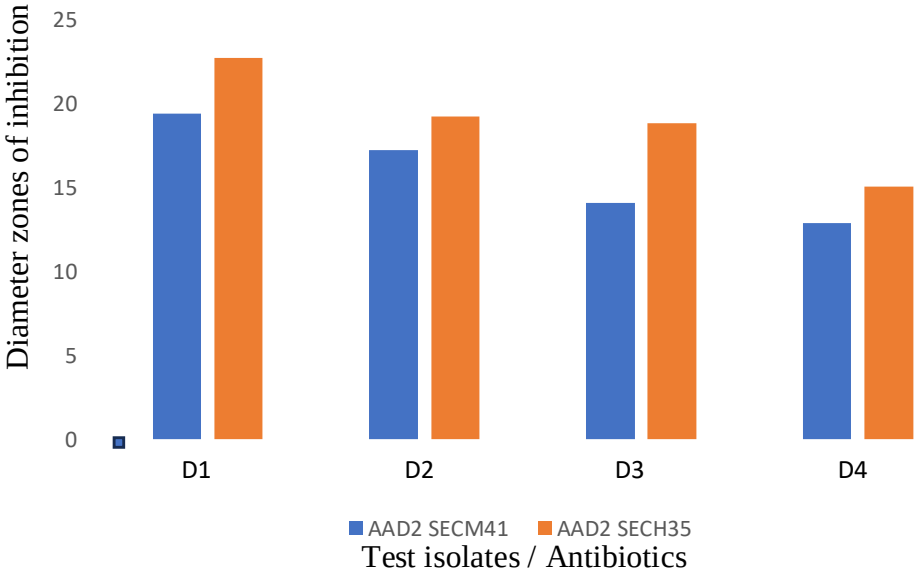


Figure 7: Diameter zones of inhibition of the fraction of the eluates from AAD2 against the test isolates.
Sorbitol Negative Escherichia coli strain MB41-1 ----- SECM41
Sorbitol positive Escherichia coli strain HH35 ------ SECH35

Table 4: GC/MC products from ANH1 eluates

Fraction	Product	Molecular Formula
N1	Oleic Acid	$C_{18}H_{34}O_2$
N2	1-Docosene	$C_{22}H_{44}$
N3	1-Octadecene	$C_{18}H_{36}$
N4	2(3H)-Furanone, dihydro-4-hydroxy-	$C_4H_6O_3$

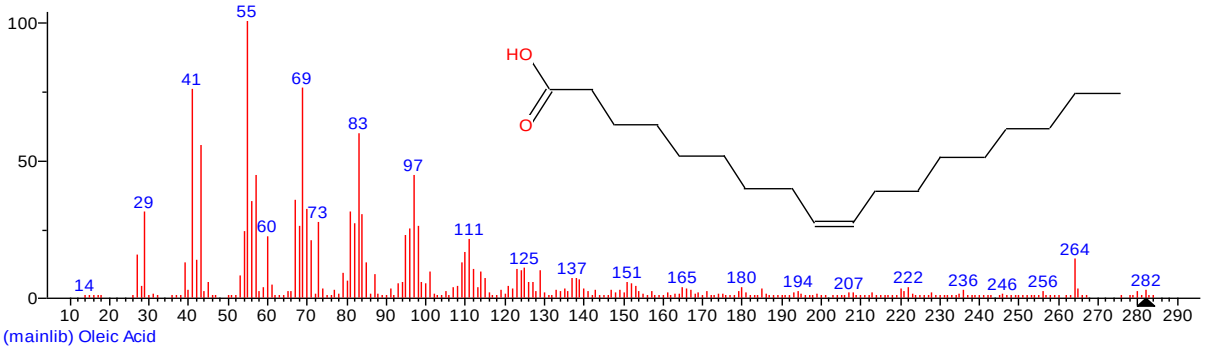
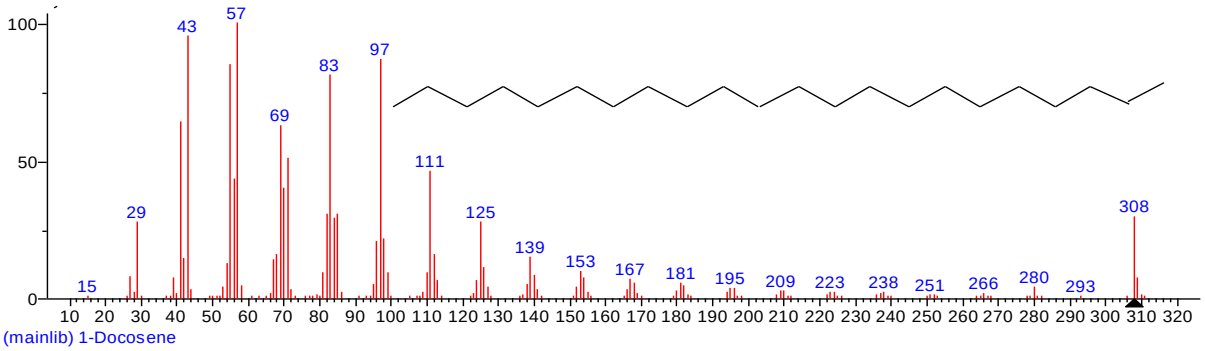
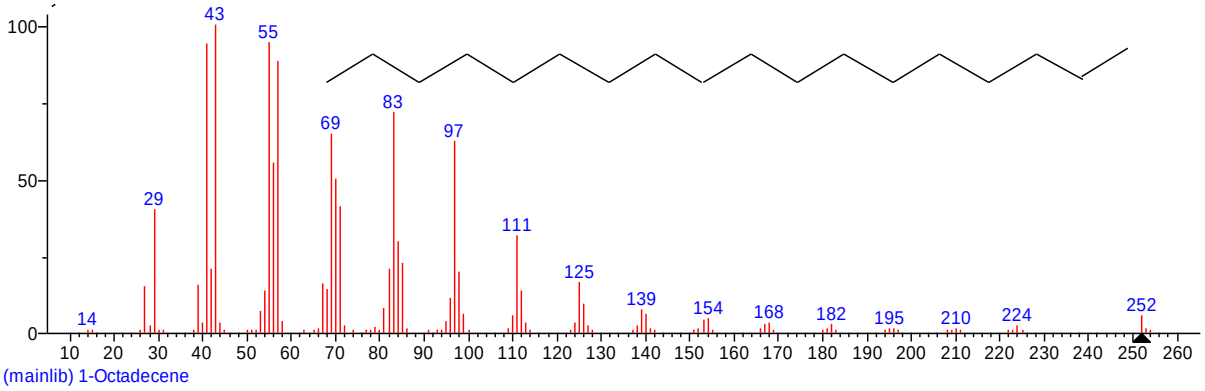


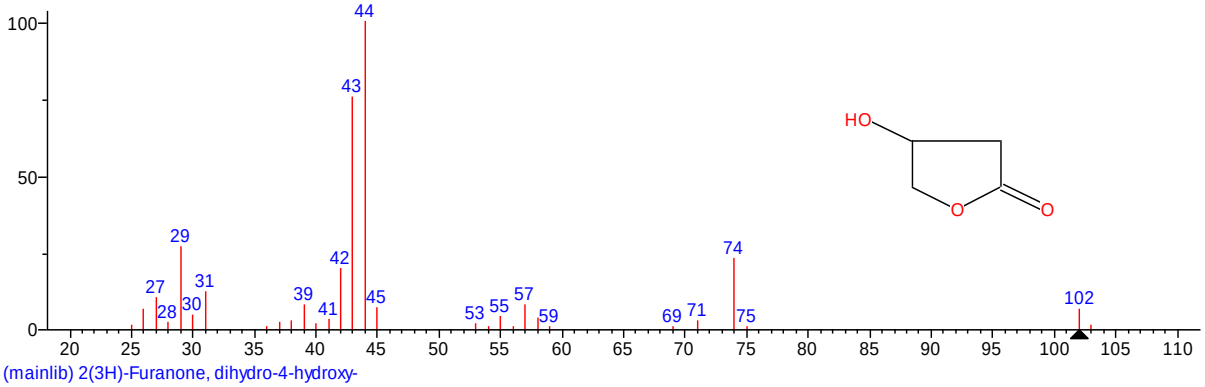
Figure 8: Structural elucidations of samples from gas chromatography coupled with mass-spectrophotometer (a)-(k)



(b)



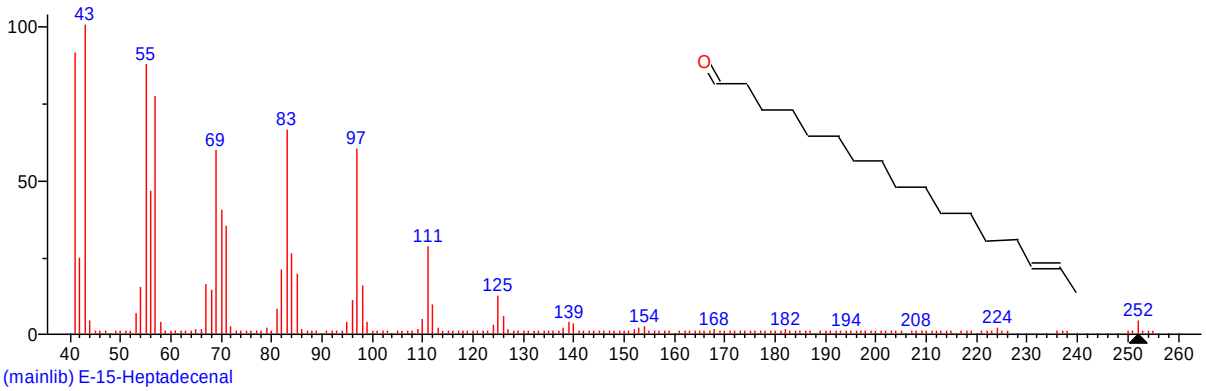
(c)



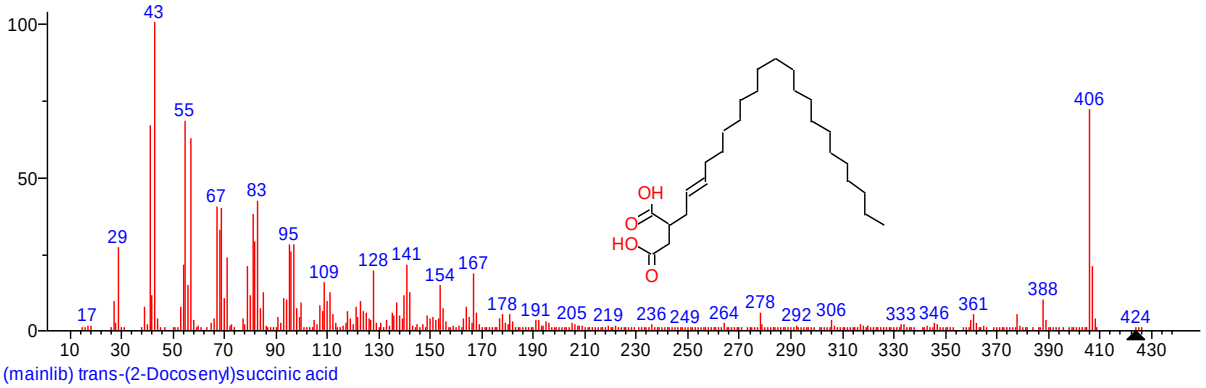
(d)

Table 5: GC/MC products from AAA5 eluates

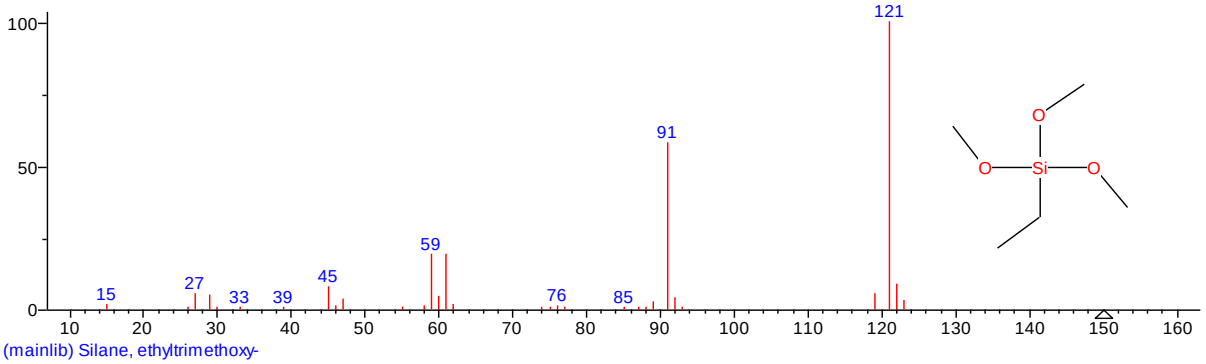
Fraction	Product	Molecular Formula
A1	E-15-Heptadecenal	$C_{17}H_{32}O$
A2	trans-(2-Docosenyl)succinic acid	$C_{26}H_{48}O_4$
A3	Silane, ethyltrimethoxy-	$C_5H_{14}O_3Si$



(e)



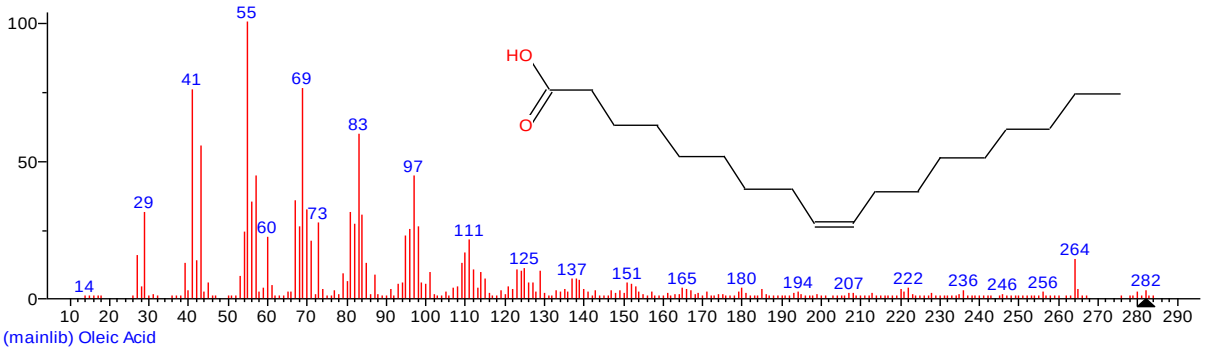
(f)



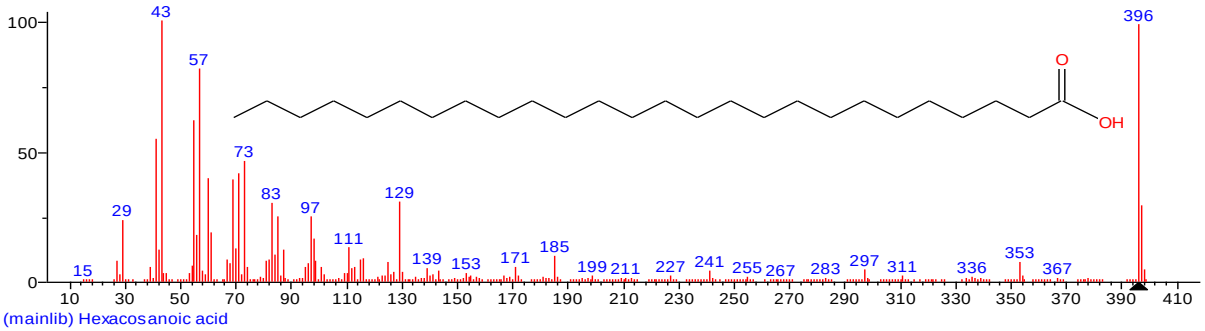
(g)

Table 6: GC/MC products from AAD2 eluates

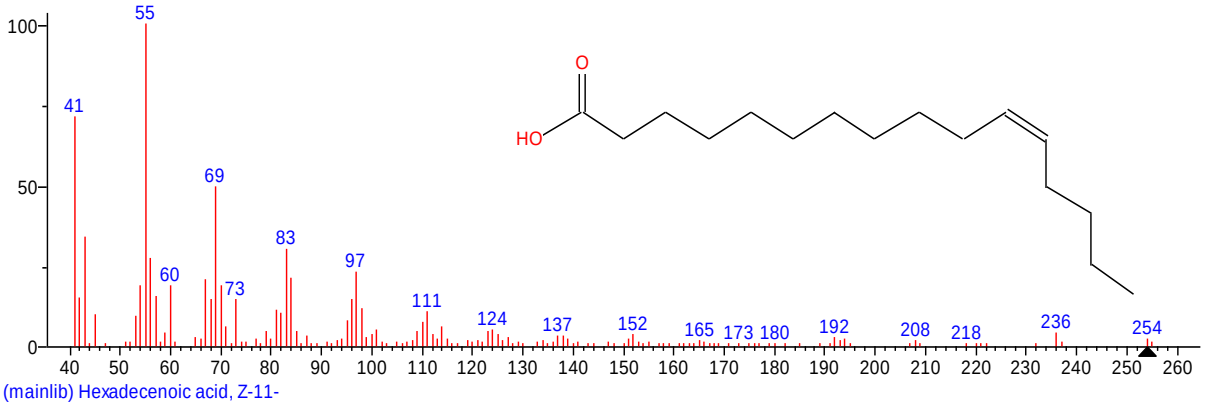
Fraction	Product	Molecular Formula
D1	Oleic Acid	$C_{18}H_{34}O_2$
D2	Hexacosanoic acid	$C_{26}H_{52}O_2$
D3	n-Hexadecanoic acid	$C_{18}H_{24}O$
D4	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$


HO
O
(mainlib) Oleic Acid

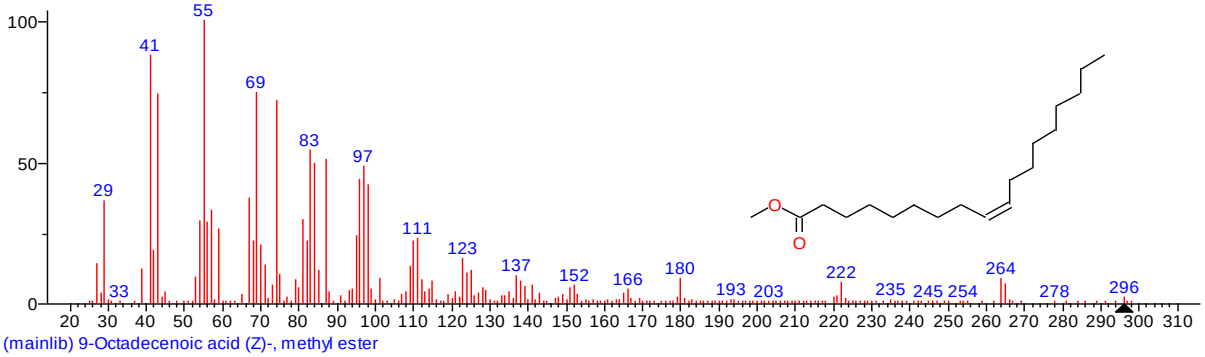
(h)


O
OH
(mainlib) Hexacosanoic acid

(i)


O
HO
(mainlib) Hexadecenoic acid, Z-11-

(j)


O
O
(mainlib) 9-Octadecenoic acid (Z)-, methyl ester

(k)

Discussion

The characteristics features of *Aspergillus niger* strain HUS1 (ANH1), *Aspergillus aculeatus* strain AN5 (AAA5) and *Aspergillus awamori* strain DN-SN2 (AAD2) isolated in the present study corroborated with the reports of many researchers (Al-shaibani *et al*., 2013; Fukuda *et al*., 2014; Ismaiel *et al*., 2016; Akinyemi, 2017; Amina *et al*., 2017; Iheukwumere *et al*., 2020).

The formulation of mycological medium from natural sources for the production of antibiotics corroborated with the reports of many researchers (Kwaguchi *et al*., 2013; Oledibe *et al*., 2023). In the present study, the mycological medium formation completely involved natural raw materials unlike those formulated by other researchers. Optimal production of the antibiotics at pH 7.0 and temperature 25℃ supported the reports of many researchers (Simin *et al*., 2011; Thamila *et al*., 2011; Kwoseh *et al*., 2012; Rao *et al*., 2012; Yahya *et al*., 2022). The maximum production of antibiotics when the carbon xxx

The production from the Negri section of *Aspergillus* species the findings of many researchers (Al-shaibani *et al*., 2013; Fukuda *et al*., 2014; Ismaiel *et al*., 2016; Akinyemi *et al*., 2017; Amina *et al*., 2017).

The inhibitory activities of the eluted and purified fractions against sorbitol-positive and negative *Escherichia coli*; *Escherichia coli* 0157:H7 strain MB41-1 (SECM41) and *Escherichia coli* strain HH35 (SECH35) supported the findings of Singh and Singh (2003), Alawode *et al*.(2020) Iheukwumere *et al*. (2022) and Albralty *et al*.(2023).

The elution and purification of oleic acid (Yoon *et al*., 2018; Kumar *et al*., 2020; Casillas-vargans *et al*., 2021) 1-octadecene (Alawode *et al*., 2012), Silane ethyl trimethoxy (Daood *et al*., 2020) and hexacosanoic acid agrees with the present study.

Conclusion

The *Aspergillus* species isolated were *Aspergillus niger* strain HUS1 (ANH1), *Aspergillus aculeatus* strain AN5 (AAA5) and *Aspergillus awamori* strain DN-SN2 (AAD2). The optical growth and production of antibacterial substances occurred when the pH, Temperature, Carbon Source and nitrogen source were 7.0, 25℃, sugar extracted from PD and NOdz respectively. From this study , the purified fractions from ANH1, AAA5 and AAD2 showed pronounced activities against uropathogenic sorbitol positive and sorbitol negative *Escherichia coli* ; *Escherichia coli* O157: H7 strain MB4-1 (SECM41) and *Escherichia coli* HH35 (SECH35)*i* , and this forms the basis of newer antibiotics from nature sources.

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