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ANTIBACTERIAL POTENTIAL OF MARINE *ASPERGILLUS AUSTROAFRICANUS*: METABOLITE OPTIMIZATION, GC–MS CHARACTERIZATION, *IN VITRO* ACTIVITY AND IN SILICO MECHANISTIC EVALUATION

Samuel Agim Abuh[1,3]*, Justine Akpanke[2], Adeboye Olusoji Lekan[3], Chidimma Ruth Chukwunwejim[4], Ifeayinchuchwu R. Iroha[4,5], Malachy C. Ugwu[4,6]

[1,3]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, University of Calabar, Cross River State.
[2]Department of Microbiology, Faculty of Biological Sciences, University of Calabar, Cross River, Cross River State.
[4]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences NnamdiAzikiwe University Awka, Anambra State.
[5]Department of Applied Microbiology, Ebonyi State University, P.M.B 053, Abakaliki, Nigeria.
[6]Tetfund Centre of Excellence for Biomedical, Engineering, Agriculturalal Translational Studies (TCE-BEATS), UNIIK.

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***Corresponding Author**

Samuel Agim Abuh

Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, University of Calabar, Cross River State.

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ABSTRACT

This study aimed to isolate fungi from marine stones collected from the Oron River near the Atlantic Ocean, identify the isolates, and evaluate their antibacterial potentials. Marine stone samples were brushed into 10 mL phosphate-buffered saline, serially diluted, and cultured on Czapek–Dox agar for fungal isolation. Pure isolates were screened for antibacterial activity and identified using molecular techniques. Metabolite production was optimized under varying cultural conditions. Selected fungi were cultured in Czapek–Dox broth, and crude extracts were evaluated using the agar well diffusion method. Active extracts were fractionated, and the most potent fractions were analyzed by GC–MS. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using standard methods. Metabolite production was optimized, and the mechanisms of action of bioactive compounds were investigated using molecular docking. Molecular docking and ADMET profiling were performed to assess target interactions and

pharmacokinetic properties. *Aspergillus austroafricanus* was isolated and exhibited broad-spectrum antibacterial activity, with mean inhibition zones of 25.6 mm against *Staphylococcus aureus*, 20.8 mm against *Escherichia coli*, and 15 mm against *Pseudomonas* spp. and *Bacillus cereus*. GC–MS revealed bioactive compounds with superior activity compared to standard antibiotics. Optimal metabolite production occurred at pH 8, temperature of 25 °C, using lactose and potassium nitrate supplements. Docking studies showed strong binding to bacterial DNA gyrase (up to −7.6 kcal/mol), while ADMET analysis predicted high oral absorption. Marine-derived fungal metabolites demonstrated potent antibacterial activity and favourable pharmacokinetic profiles, highlighting their potential as novel agents against drug-resistant bacteria.

KEYWORDS: Metabolites, *Aspergillus austroafricanus*, antibiotics, DNA gyrase, Metabolites, Antibiotics.

INTRODUCTION

Microorganisms have demonstrated to be resistant to available antibiotics thus resulting in prolong hospital stay of patients, increase in cost of treatment and ultimately death due to failure of treatment. Metabolites from natural sources have shown to be safe in the treatment of different ailment.[1,2] The biological actions of fungal secondary metabolites have led to the development of life-saving medications.[3] In medicine, records have shown the use of metabolites from organisms which dates back to about 2000 years ago or more. Systematic investigation of oceans began in the mid of 20th century.[4] The marine environment constitutes approximately two-thirds of the Earth's surface and remains largely untapped. This raises an important question: can these biological resources be systematically explored, with microorganisms isolated from marine ecosystems and studied for both human benefit and the advancement of fundamental biology? The field of marine biotechnology provides promising answers to this challenge.

Therefore, this study aims to isolate and evaluate bioactive metabolites produced by *Aspergillus austroafricanus* for antibiogram analysis, with the ultimate goal of identifying potential lead compounds for therapeutic development.

METHOD

Sampling and Isolation of Microorganisms

The map of sample collection point is presented in figure 1. Stone samples were obtained

from a depth of approximately 10cm using a stainless-steel water trap. The surfaces of stones were gently washed with sterile water using a smooth sterile brush and subsequently transferred into clean sample containers and processed within 24 hrs.[5]

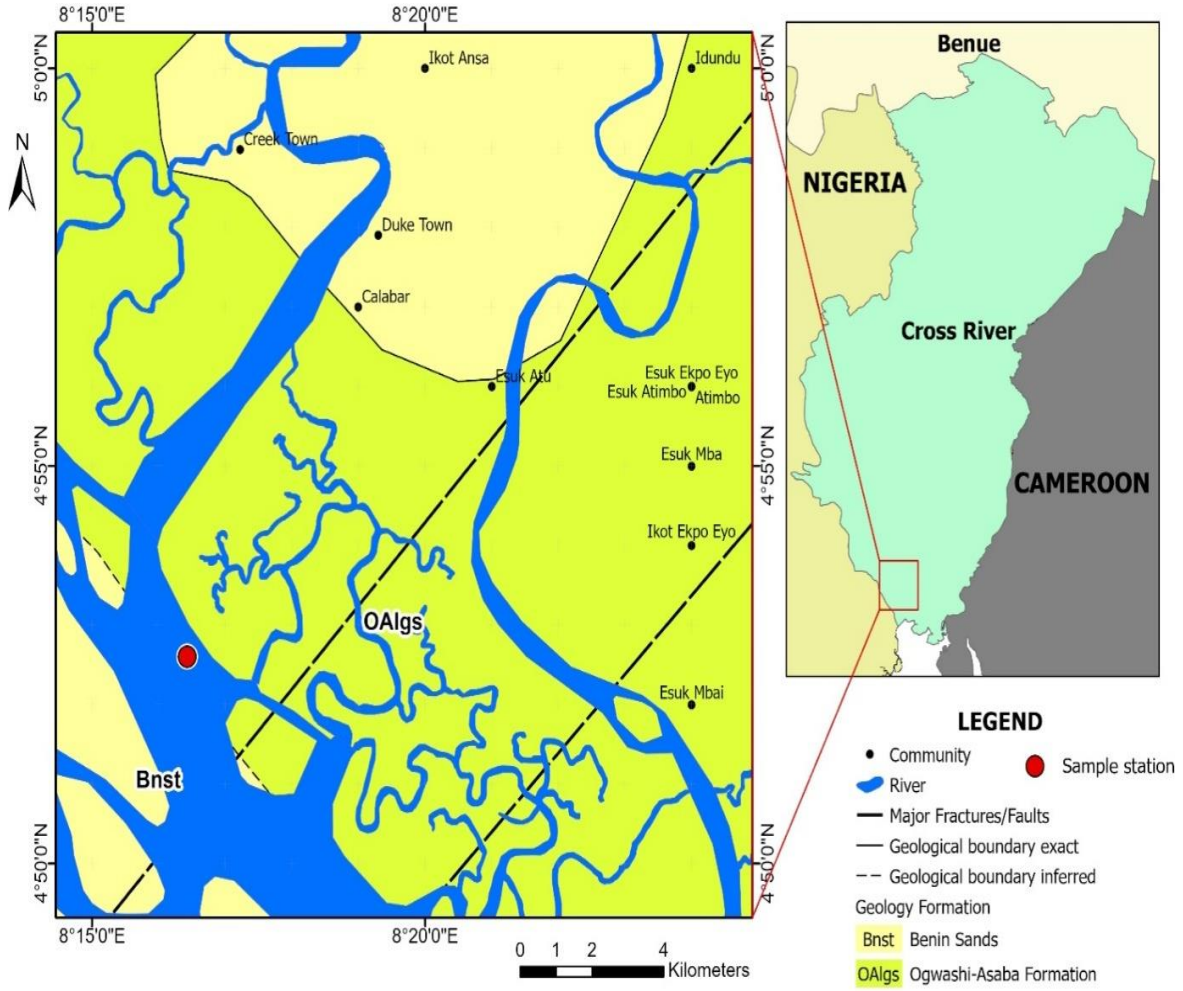


Figure 1: Map of Sampling Location.

Isolation of Fungi

The fungi was isolated and identified as reported by[6,7] with slight modification. The stones samples were gradually and distinctly brushed with sterile brush into 10 ml phosphate-buffered saline to form a stock solution. Briefly, a 10.0 mL serial dilution was prepared by transferring 1.0 mL of the stock solution to obtain 10^{-1}, 10^{-3}, and 10^{-5} dilutions level. From each dilution, 1.0 ml was transferred out and dispensed onto Czapekdox agar. The plates were incubated upside down in dark at 25 ± 1°C for 4 to 7 days. The isolated fungi were further subcultured on Czapekdox yeast Agar (CYA) to observe and identify the colonies on the basis of macro- and micro-morphology. Colony characteristics like growth rate, texture, extent of sporulation, mycelial colour, and reverse colony colour were observed. Molecular

identification based on 18S rDNA sequencing was done.

Molecular Identification

DNA Extraction

Fungal DNA was extracted with the help of ZR Fungal/Bacterial DNA MiniPrep Kit (Inqaba, South Africa) according to the manufacturers instruction .The dense growth of purified fungal isolate was suspended in 200 µL of isotonic buffer and transferred to a ZR BashingBead™ Lysis Tube. Briefly, 750 µL of lysis solution was added and processed at maximum speed for 5 minutes, the tubes were secured in a bead beater with a 2 mL tube holder assembly.

After cell lysis, the samples were centrifuged at 10,000 × g for 1 min. Subsequently, 400 µL of the clear supernatant was carefully transferred into a Zymo-Spin IV Spin Filter (orange cap) placed in a collection tube and centrifuged at 7,000 × g for 1 min. The filtrate obtained was mixed with 1,200 µL of fungal/bacterial DNA binding buffer to achieve a total volume of 1,600 µL.

An initial 800 µL of this mixture was loaded onto a Zymo-Spin IIC Column fitted in a collection tube and centrifuged at 10,000 × g for 1 min, after which the flow-through was discarded. The remaining sample volume was then applied to the same column and centrifuged under identical conditions.

The column was washed by adding 200 µL of DNA Pre-Wash Buffer in a fresh collection tube and centrifuging at 10,000 × g for 1 min, followed by a second wash with 500 µL of Fungal/Bacterial DNA Wash Buffer using the same centrifugation settings. The Zymo-Spin IIC Column was then transferred to a clean 1.5 mL microcentrifuge tube, and DNA was eluted by adding 100 µL of DNA Elution Buffer and centrifuging at 10,000 × g for 30 s. The extracted DNA was stored at −20 °C until further analysis.

3.4.2 DNA quantification

The concentration of the extracted genomic DNA was determined using a NanoDrop™ 1000 spectrophotometer. The instrument software was opened by selecting the NanoDrop application, after which the system was initialized with 2 µL of sterile distilled water and blanked with normal saline. Subsequently, 2 µL of the DNA sample was placed on the lower measurement pedestal, and the upper pedestal was lowered to ensure proper contact. DNA

concentration was then recorded by selecting the “Measure” option.[8]

3.43. Internal Transcribed Spacer (ITS) Amplification

The ITS region of the isolates was amplified using the ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3, primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 53ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a blue light transilluminator.[8]

3.44. Sequencing

DNA sequencing was conducted by Inqaba Biotechnological Industries (Pretoria, South Africa) using an ABI 3510 Genetic Analyzer with the BigDye® Terminator v1.1/v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were prepared in a final volume of 10 µL, comprising 0.25 µL of BigDye® Terminator (v1.1 or v3.1), 2.25 µL of 5× BigDye® Sequencing Buffer, 10 µM of the relevant PCR primer, and 2–10 ng of purified PCR amplicon per 100 bp. Thermal cycling was performed for 32 cycles, with denaturation at 96 °C for 10 s, primer annealing at 55 °C for 5 s, and extension at 60 °C for 4 min.

Fungal Metabolite Extraction and Purification

Fungal metabolites were extracted according to the method described (Rakesh, 2021)[9] with slight modification. Briefly, 100mL CzapekDox Broth was suspended with agar plugs from a 7-day-old culture in an Erlenmeyer flask and incubated in the dark for 10 days at 25°C. The mycelial mat was harvested shredded and dried at 40 ^{o}C in an oven for 72 hrs, the dried mycelia mat was pulverised suspended in a 1:1 chloroform: methanol solution in a rotary shaker overnight (120 rpm).[9] The mixture was filtered and concentrated in a BuchiRotavapor, and evaporated to dryness. The dried extract was then suspended in a mixture of 100 mL acetonitrile, 100 mL methanol, and 100 mL hexane and agitated (120 rpm, 1 h).[10] The different fractions were then collected separately and evaporated until it was completely dry. The extracts were then suspended in dimethyl sulfoxide and kept at 20 °C at a concentration of 1 mg/ml.

Screening of Extracted Fungi Metabolite for Inhibitory Activity of W and B Extract

The extracted metabolites were screened for antibacterial activity using the agar-well diffusion method as reported by[11] The fungi metabolites were then evaluated against clinical isolates like; *Escherichia coli*, *Staphylococcus. aureus*, *Pseudomonas Sp*and *Bacillus cereus*. Preparation of bacterial inoculants was done in nutrient broth at 0.5 McFarland standards. Each test organism (0.1 ml) was mixed with cooled Mueller–Hinton agar and poured into 80 mm petri dishes. A stock concentration 1mg/ml of the crude extract and the different fractions was prepared. By means of a sterile cork borer, wells (8 mm in diameter) were made on the agar; 25 µg/ml of test extracts was poured into the wells. This was done in triplicates and incubated at 37°C for 24 h. The procedure was repeated for other fractions of the extract. Antibiotic sensitivity disc 30 µg of amoxicillin clavulanic acid and ceftriaxone were used as controls. The diameters of zone of growth-inhibition produced was measured and the mean values calculated. The isolates that produced extracts with clear zones of inhibition were selected for the next level of studies.[12]

Minimum inhibitory and bactericidal concentrations determination of the metabolite

Minimum Inhibitory Concentration (MIC) was determined using both broth dilution and agar diffusion method as reported some where[13] with slight modification. Preparation of a stock solution 200 µg/ml was done for the two extracts, 1ml of the stock was transferred into1ml Mueller-Hinton Broth in a test tube to make 100 µg/ml. A two fold serial dilution was done to obtain a dilution in the range100 µgml, 50 µg/ml 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml and 3.125 µg/ml. The inoculum (100 µl) of the test microorganisms prepared from 18 h broth cultures (containing 10^5cfu/ml) was dispensed into the tubes in triplicates. The minimum inhibitory concentration (MIC) was determined as the least concentration that inhibited organism growth in the broth (clear solution). For the agar diffusion method, stock solutions of 1000 µg/mL, 500 µg/mL, 250 µg/mL, 62.5 µg/mL, and 31.25 µg/mL were prepared. Briefly, 1 mL of each drug concentration was added to sterile Petri dishes containing 100 µL of the test organism. The plates were gently swirled in clockwise and anticlockwise directions to ensure even mixing. Subsequently, 9 mL of molten agar was poured into each plate and gently agitated to ensure uniform distribution with concentration range between (100µg/ml-3.12µg/ml). The plates were left to solidify, labelled accordingly with the different concentrations (range100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml and 3.125 µg/ml.), and incubated at 37°C for 24 hours. The MIC was the least concentration that showed no growth. The minimum bactericidal concentration (MBC) test was performed by

transferring 100 µL of the test organism into the different concentrations(range: 200 µg/ml 100 µgml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml) of the extracts in Muller Hinton brothand incubated for 24hrs at 37°C, 1ml of the solvent (10%) DMSO was equally transferred into 1ml of the broth inoculated with 100 µL aliquots of the test organism which served as the control. After 24 hours of incubation, 100 µL of the broth was withdrawn from the different cultured concentration and inoculated into 15 ml nutrient agar plate. These was incubated at 37°C for 3 days and observed for signs of growth. The result was documented as either positive (+) growth or negative (-) for no growth. The minimum concentration showing no growth after three days was taken as the minimum bactericidal concentration.

Optimization of Culture Condition

3.7.1 Testing Thermal and pH Effect on Fungal Metabolites Production

The effect of pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0), temperature (0, 4, 10, 20, 30 and 40°C), and salinity percentage (0, 7.5, 15, 22.5 and 30% was studied to determine the optimum condition for fungal metabolite production.[14]

The optimum pH for maximum activity was assessed by fermenting the isolates in 100 mL CzapekDox Broth in an Erlenmeyer flask and incubated in the dark for 10 days to 14 days at 25°C (Arumugam*et al* 2014) at varying pH values (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) with agitation speed of 150 rev min^{-1}. The isolate with optimum metabolite and highest stability to changes in both pH, heat and salinity were used for further studies. pH 7 was taken as control pH.

Carbon and nitrogen sources

The source of carbon for optimum activity was assessed by conducting the experiment in 1000-ml Erlenmeyer flasks containing 24g CzapekDox Broth as reported.[14] The medium was sterilized via autoclaving at 121°C for 20 min and cooled to room temperature. Various carbon sources (5%): glucose, sucrose, mannitol, lactose, fructose and maltose were used. The extracted metabolite obtained were tested for antimicrobial activity against the test organisms. The procedure was repeated for nitrogen sources (asparagine, sodium nitrate, potassium nitrate, ammonium chloride, ammonium nitrate, ammonium phosphate and ammonium sulphate). The optimal conditions was used in cultivation of the fungi for further studies.

Determination of chemical composition of the metabolite

The chemical composition of the extract was done using Gas chromatography mass spectrometry (GC-MS) as reported.[14] The phytoconstituents of compounds present in the extracts of sample W and B were evaluated in Agilent Technologies 8890A gas chromatography (GC) coupled to a mass spectrum detector (MSD) (Agilent Technologies, USA). The carrier gas was helium with column velocity flow of 1.0 L/min, ion-source temperature was 250 C, interface temperature was 300 C, operating pressure was 16.2 psi, out time was 1.5 min, injection temperature was 300Cin split mode at1µL injector, while the temperature of the column was initially 50 C for 5 min and raised to 250 C at the rate of 20C/min for 5 min. Mass spectra-based identification of the components was carried out using the following software: Chromatec Analytic (Chromatec, Russia); NIST MS Search Program V.2.3 (NIST,USA) and NIST 20 (NIST, Mass Spectra Libraries, USA); Wiley Registry of Mass Spectral Data, 12th ed.

Molecular Docking

Elucidation of the potential mechanisms of antibacterial activity of the bioactive molecules and molecular docking was performed on the bacterial protein components and the bioactive molecules to analyze their potential interactions. Selected bioactive molecules that were identified by GC-MS analysis were analyzed to study their putative antibacterial activities through docking studies. The structure-based molecular docking was performed to study the protein-ligand interactions of the bioactive molecules with bacterial target proteins (Bacterial DNA Gyrase (PDB ID: 3G7E)) that was obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (https://www.rscb.org). DNA gyrase is a type II topoisomerase enzyme found exclusively in bacteria (and not in humans), making it an essential and attractive antibacterial drug target. It plays a crucial role in the replication, transcription, and overall maintenance of bacterial DNA.

The bioactive compounds were docked using Biovia Discovery Studio (version 2021) and Auto Dock Vina, with binding energies calculated for each interaction. Ligands and target proteins were prepared following standard protocols for protein and ligand preparation before submission to Auto Dock Vina. The resulting binding energies and interaction of each ligand were analyzed using Discovery Studio Visualizer.

Determination of ADMET

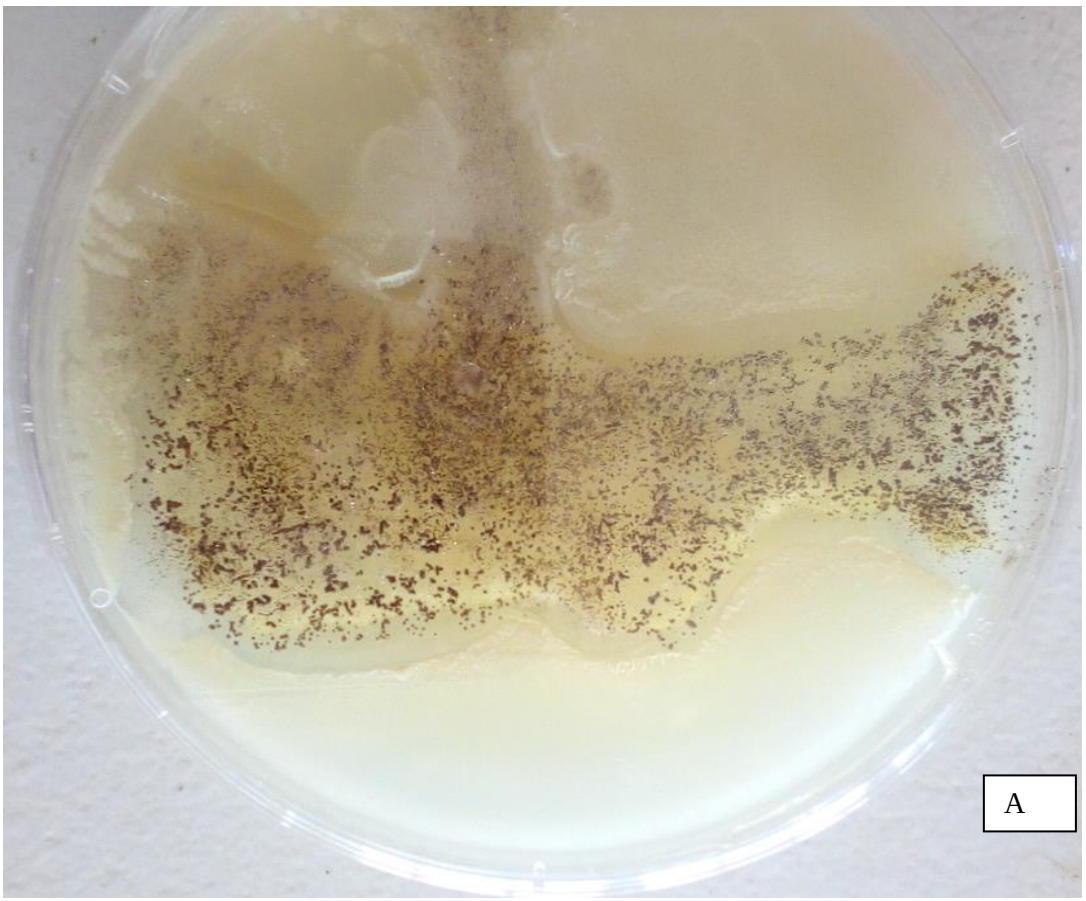
Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) properties of the

bioactive compounds, was evaluated using Swiss ADME. First the compound's structural information was prepared. ChemDraw was used to carry out the drawing of the compound structure. It was later converted into the SMILES (Simplified Molecular Input Line Entry System) format, which is a text-based representation compatible with SwissADME. With the SMILES representation in hand, the next step was accessing the SwissADME web platform at http://www.swissadme.ch. The SMILES strings were entered into the input box provided on the homepage. The compounds were entered into SMILES strings, each on a new line. Once the inputs were finalized, the analysis was initiated by clicking the "Run" button, which prompts the platform to process the data and generate a comprehensive set of pharmacokinetic and physicochemical properties.

RESULTS AND DISCUSSIONS

Isolation and Identification of *Aspergillusaustroafricanus*

The fungi used in this study were isolated following the method described by Rajpal *et al.*, (2016). The isolates were purified through sub-culturing and subsequently identified. Figure 2 presents an image of the isolated fungi.



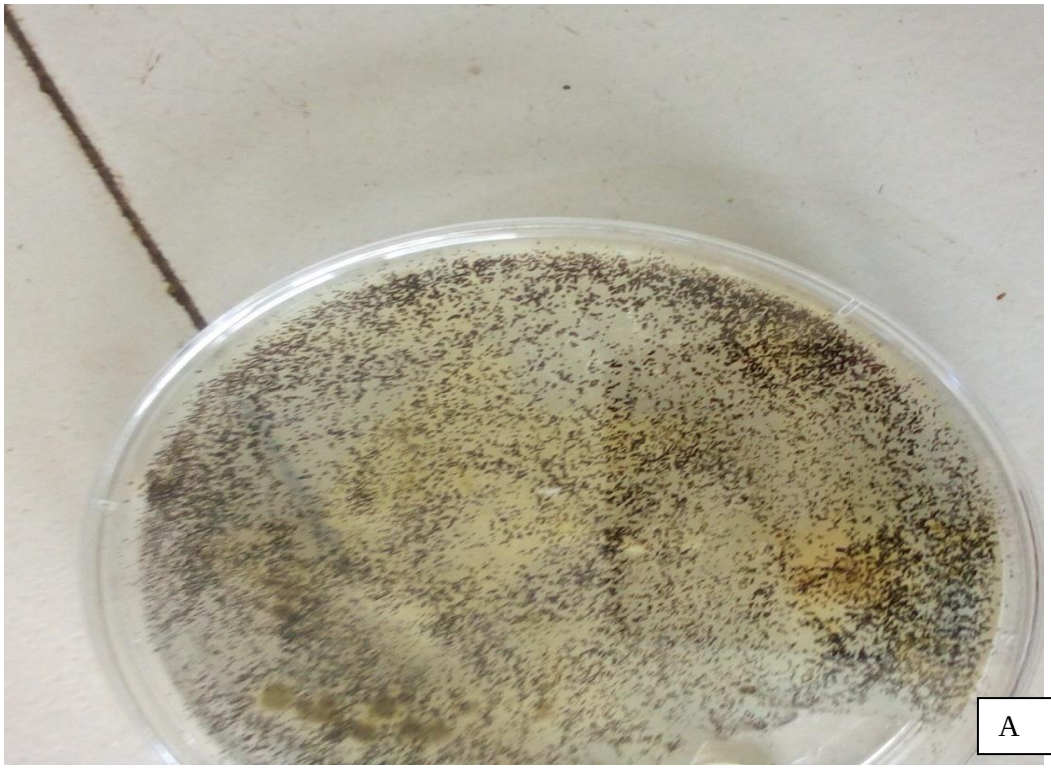
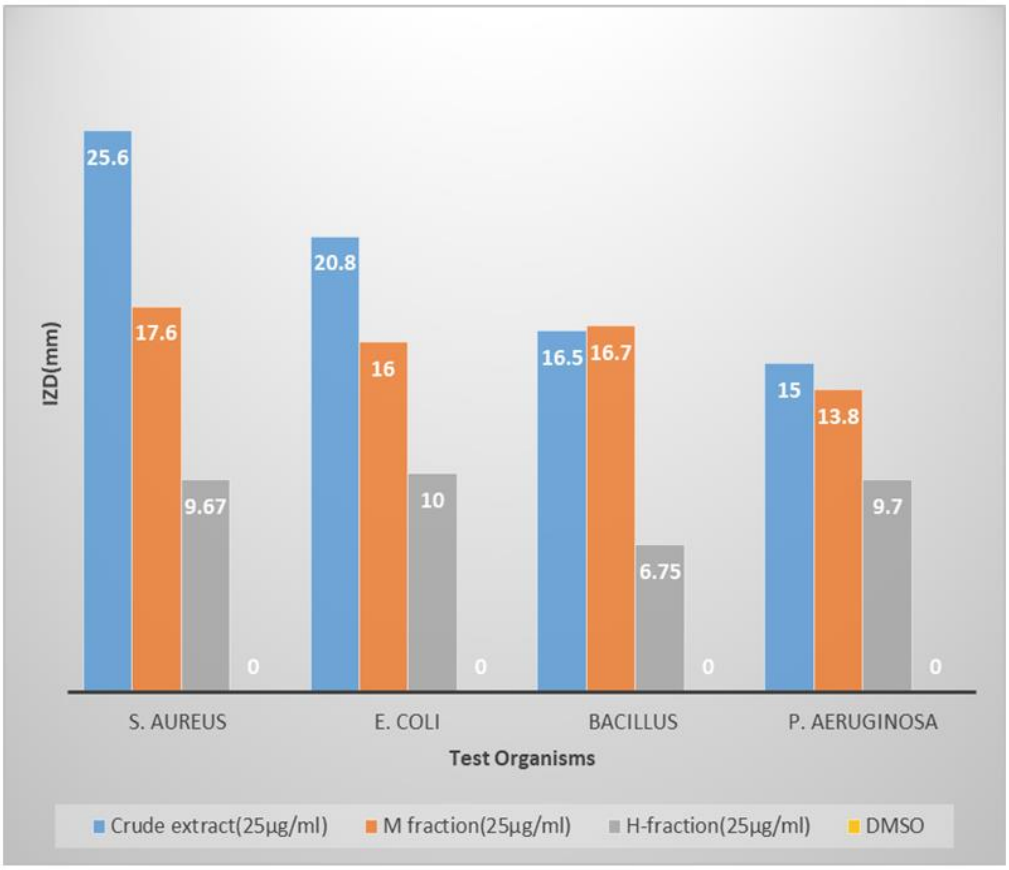


Figure 2: Isolates of *Aspergillus austroafricanus* from sample (A) and the purified isolates (B).

Antibacterial Activity of Extracts

The antibacterial results in figure 3A shows that metabolites derived from *Aspergillus austroafricanus* exhibit pronounced broad-spectrum activity. At 25 µg/mL, the crude extract produced the largest inhibition zones against *Staphylococcus aureus* (25.6 mm), *Escherichia coli* (20.8 mm), *Bacillus* spp. (16.5 mm), and *Pseudomonas aeruginosa* (15 mm). In contrast, figure 3B shows that the M-fraction retained moderate activity, particularly against *Bacillus* spp. (16.7 mm) while the H-fraction showed comparatively weak inhibition (6.75–10 mm). The absence of inhibition in the DMSO (control) confirms that the antibacterial effects were attributable solely to fungal metabolites.



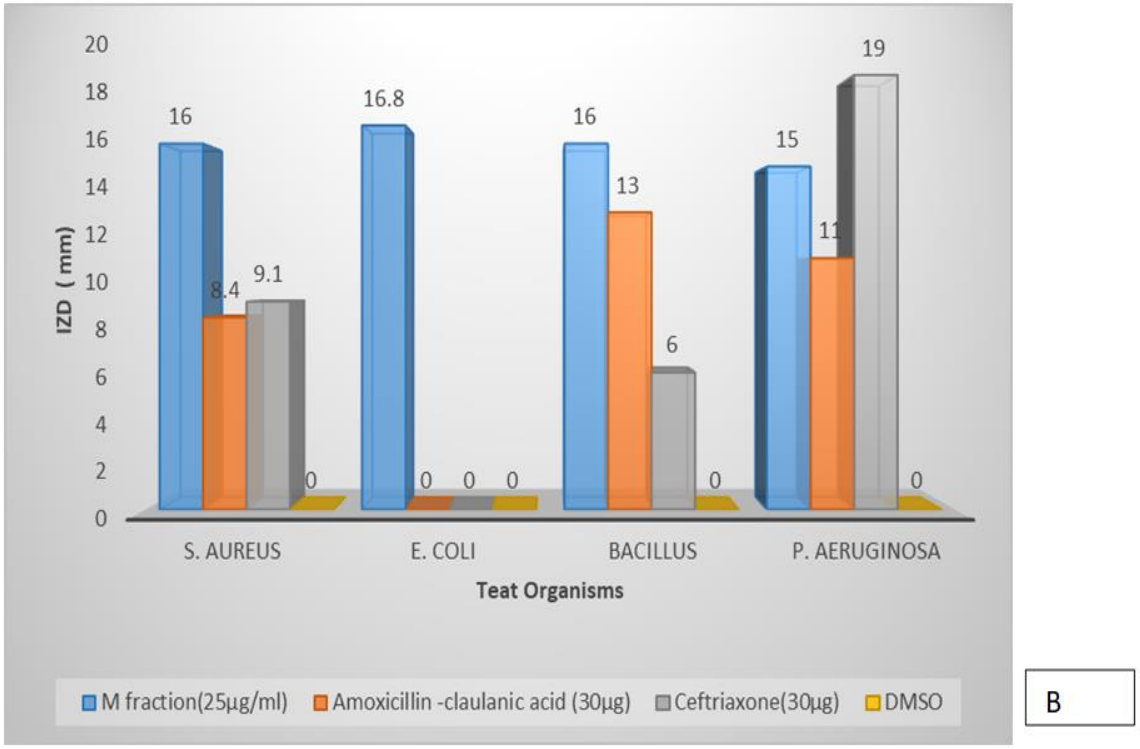


Figure 3: Antibacterial activity of the crude extract and the extract fractions on test Organisms (A) and antibacterial activity of M fraction of the metabolite compared to control (B).

Table 1: MIC and MBC of *Aspergillus austroafricanus* Extract against each Organism.

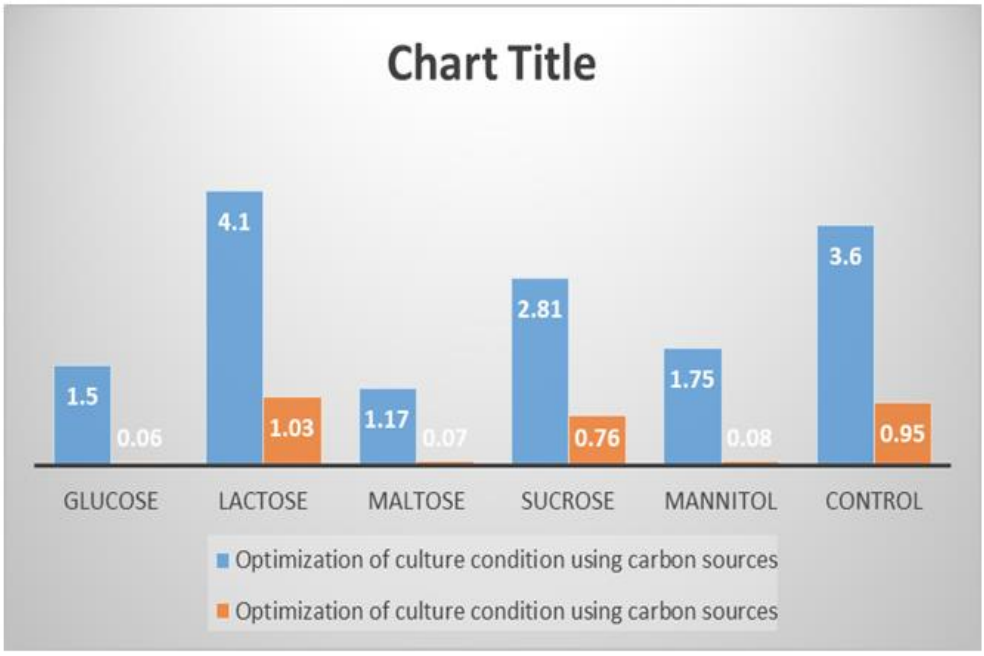
Organisms	*MIC*	*MBC*
E. coli	*12.5*	*50*
Bacillus cereus	*25*	*100*
Staphylococcus aureus	*25*	*100*
Pseudomonas aeruginosa	*100*	*100*

Pseudomonas aeruginosa exhibited higher MIC and MBC values (100), reflecting reduced susceptibility consistent with its multidrug-resistant phenotype; however, the equal values indicate a bactericidal effect once threshold concentrations are achieved. Importantly, all MBC/MIC ratios were ≤4, confirming killing rather than growth inhibition.

Optimization of Culture Condition

Figure 4 A and B present the effect of different carbon and nitrogen sources on metabolite production, the tested carbon sources produced the following; lactose (4.1 g dry weight)had the highest mycelial biomass, followed by control (3.6 g) and sucrose (2.81 g)**.** Glucose (1.5 g), mannitol (1.75 g), and maltose (1.17 g) supported comparatively lower biomass production.

Nitrogen source optimization in Fig 4 B showed that potassium and sodium nitrate were the most favourable for mycelial growth. This is likely due to the presence of nitrate reductase enzyme in the fungi, facilitating efficient assimilation.



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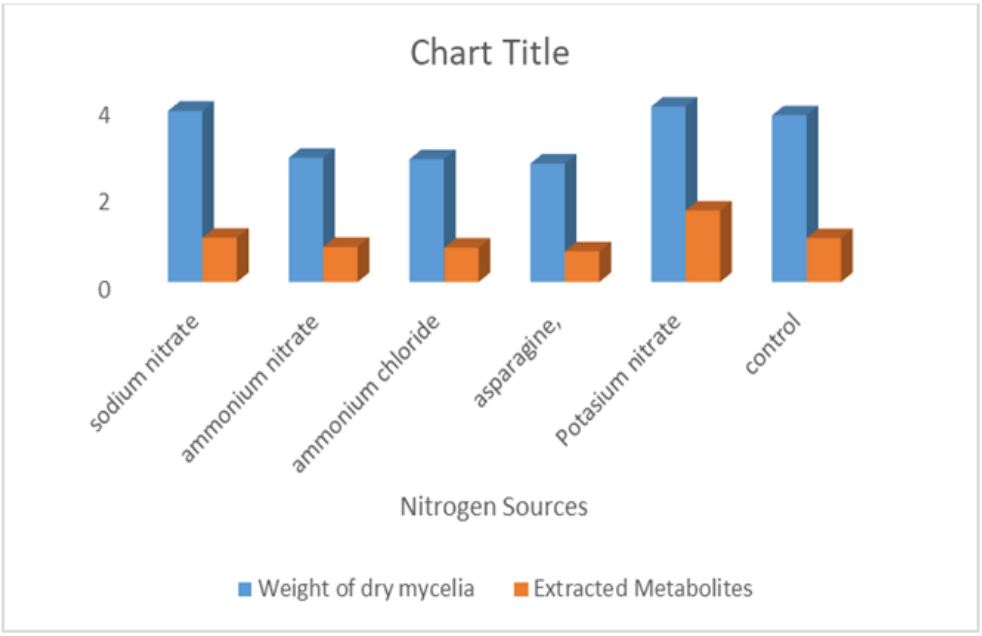
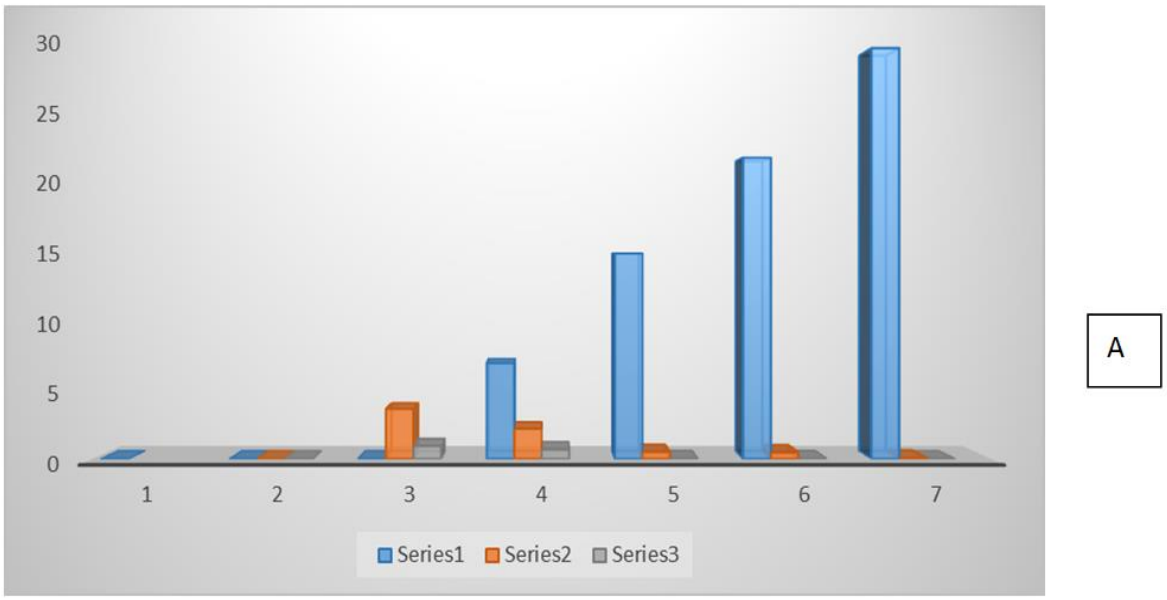


Figure 4: Optimization of culture condition using carbon (A) and Nitrogen sourcessources(B).

Salinity tolerance studies (Figure 5A) revealed that mycelial growth was optimal at 0% NaCl and drastically reduced beyond 7%. While viability persisted up to 30% salinity, biomass accumulation was minimal, indicating salt sensitivity. While figure 5B established an optimal growth range between 20–25°C for the fungi. Below 10°C and above 30°C, growth was substantially inhibited.



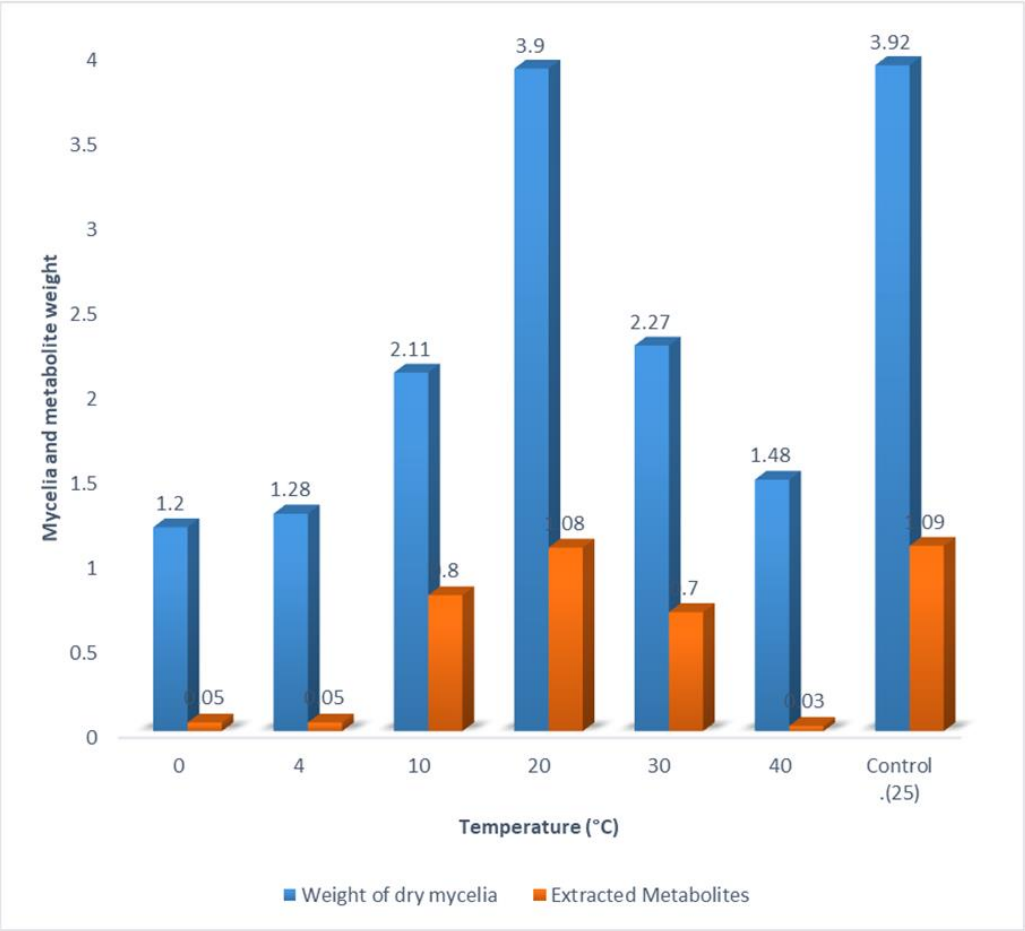


Figure 5: Optimization of culture condition using NaCl(A) and Temperature.

Fungal metabolite production was optimized under various environmental conditions. Table 2 indicate that pH 8 supported the highest mycelial biomass, though pH 6–7 also yielded considerable growth.

Table 2: Optimization of culture condition using Ph.

pH	Weight of dry mycelia	Extracted Metabolites
4	2.2	0.71
5	2.8	0.78
6	3.91	1
7	4.2	1.26
8	4.41	1.36
9	3.48	1.05
10	3.1	0.98

The Chromatogram in figure 6 showed a major peak identified as phytol, the compound showed an excellent spectral match with the NIST library (Match Factor = 999; Reverse Match Factor = 999**)**. Hexadecane, 2,6,10,14-tetramethyl was also a major volatile compound identified in the GC-MS result.

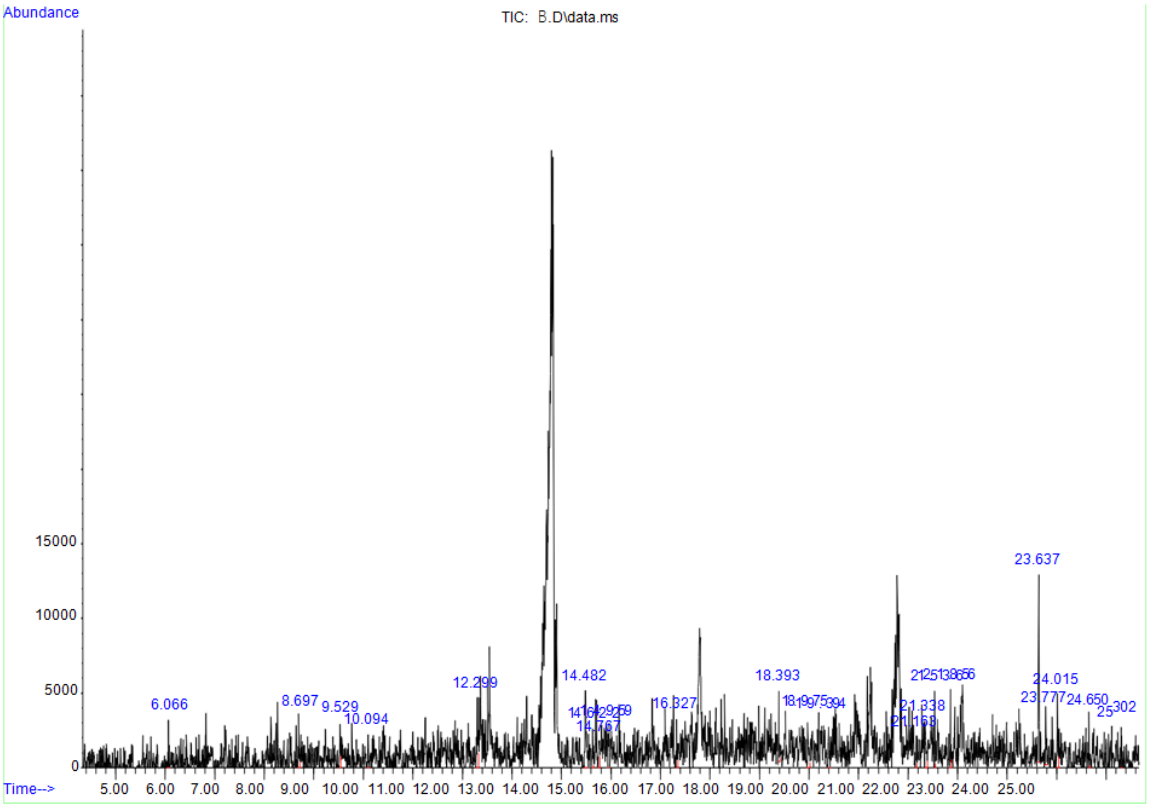
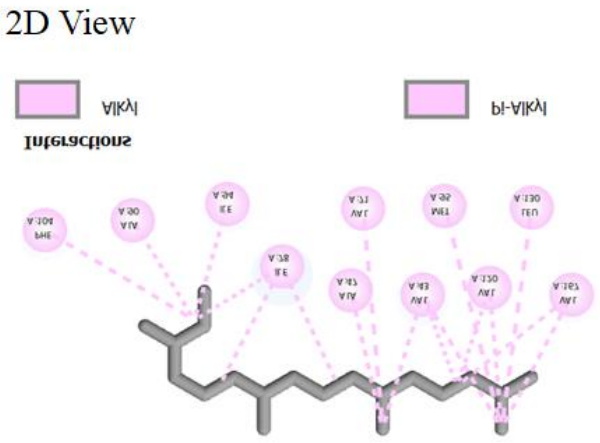


Figure 6: Chromatogram of Sample B.

Figure 7(A): illustrates the docking pose of Hexadecane, 2,6,10,14-tetramethyl, within the active site of bacterial DNA gyrase, a type II topoisomerase essential for DNA supercoiling and replication, surrounded by key amino-acid residues (PHE 104, ALA90,ILE 94, ILE 78, ALA 47, VAL 120, VAL 167, VAL 71,MET 95, LEU 130, LEU 130) that participate in stabilizing the ligand–protein complex. Figure 7B illustrates the docking pose of Phytol, a diterpene alcohol, within the active pocket of bacterial DNA gyrase, an essential enzyme required for bacterial DNA replication and supercoiling.



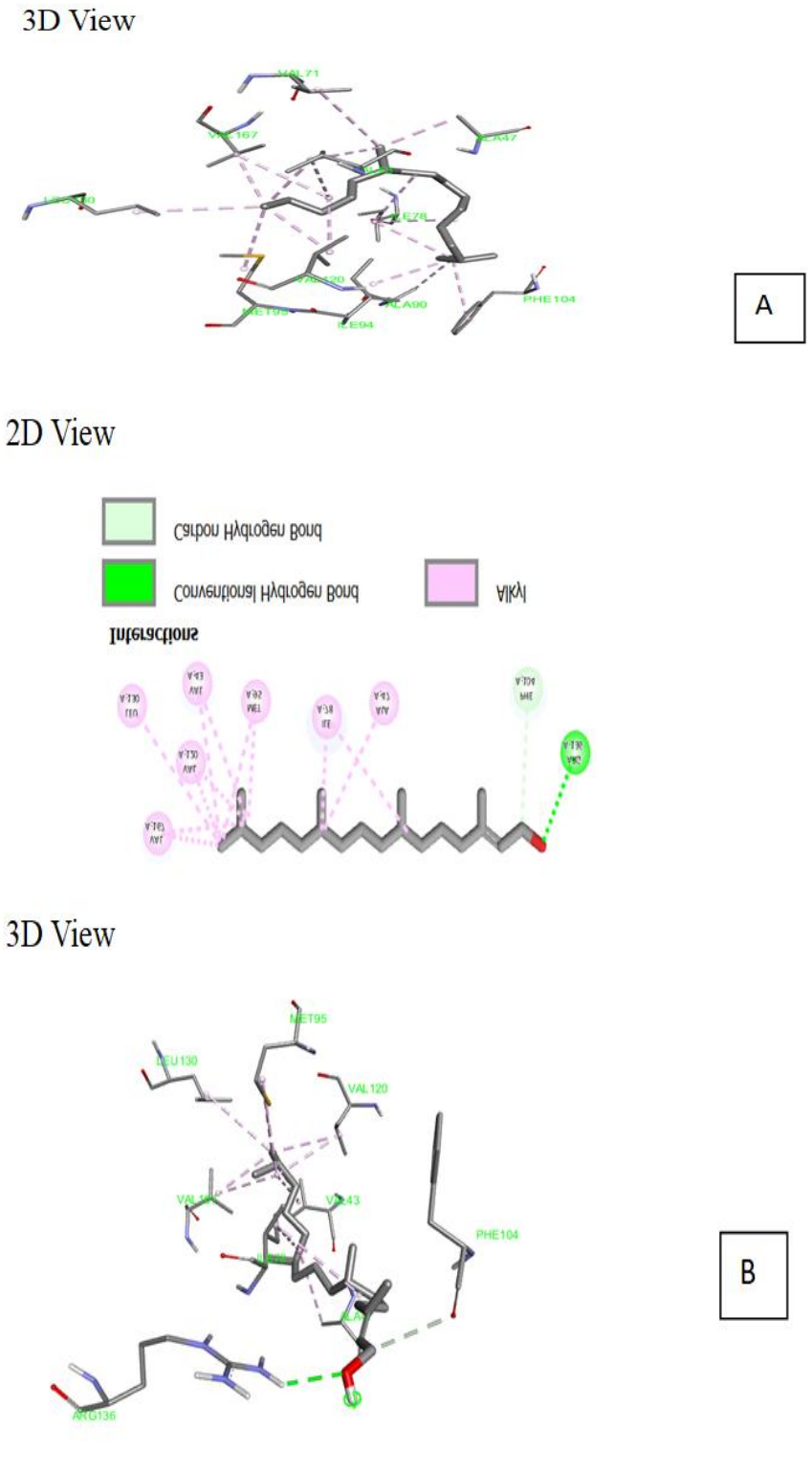
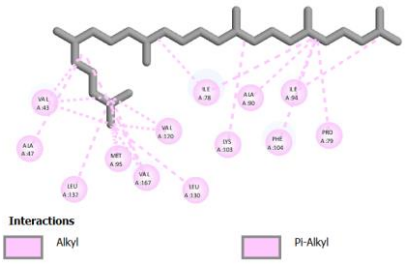


Figure 7: Molecular interaction of Hexadecane, 2,6,10,14-tetramethyl (A) and Phytol (B) with Bacterial DNA gyrase.

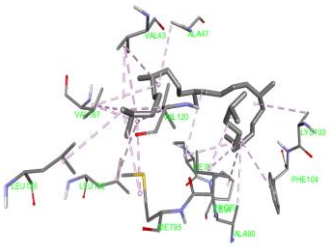
Figure 8Arepresents the 2D and 3 D docking pose of Squalane, a highly hydrophobic, branched triterpenoid hydrocarbon, within the active binding pocket of bacterial DNA gyrase.

Hydrophobic interactions are known to stabilize ligand, protein complexes by reducing solvent exposure and enabling tight molecular packing. These values were comparable to those of the standard antibiotic Amoxicillin-clavulanic (fig 8 B), suggesting that some of these natural compounds may be promising antibacterial candidates.

2D View



3D View



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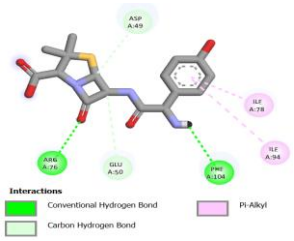

3D view

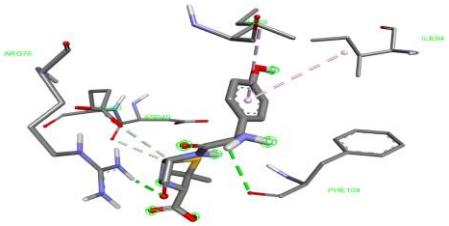

Figure 8: Molecular interaction of Squalane (A) and Amoxicillin and clavulanic acid (B) (Control) with Bacterial DNA gyrase.

Physicochemical properties of the compounds were analyzed in accordance with Lipinski's Rule of Five, which helps predict oral bioavailability. As shown in Table 3, none of the compounds violated the rule, and their molecular weights ranged from 136.23 g/mol to 422.81 g/mol, supporting their classification as small molecules.

Table 3: Basic physicochemical properties of the bioactive compound isolated from W and B.

Properties	Hexadecane, 2,6,10,14-tetramethyl	Squalane	Phytol	Amoxicillin Clavulanic acid (control)
Formula	$C_{20}H_{42}$	$C_{30}H_{62}$	$C_{20}H_{40}$	$C_{16}H_{19}N_3O_5S$
Molecular weight	282.55g/mol	422.81g/mol	296.53g/mol	365.40g/mol
Number of heavy atoms	20	30	21	25
Number of aromatic heavy atoms	0	0	0	0
FractionCsp3	1.00	1.00	0.90	0.44
Number of rotatable bonds	13	21	13	5
Number of hydrogen bonds acceptors	0	0	1	6
Number of hydrogen bond donor	0	0	1	4
Molar refractivity	98.25	146.32	98.94	94.59
Topologicl polar surface area(TPSA)	$0.00A^{02}$	$0,00A^{02}$	$20.23A^{02}$	$158.26A^{02}$

Table 4: ADMET Properties of Isolated compounds.

ADMET PROPERTIES	Hexadecane 2,6,10,14-tetramethyl	Squalane	Phytol	Amoxicilin Clavulanic acid(Control)
Absorption				
Water solubility (Log mol/L)	-8.673	-7.434	-7.592	-3.156
CaCO2 permeability (log papp in 10^{-6} cm/s)	1.424	1.163	1.487	-0.157
Intestinal absorption (human) (% absorbed)	92.227	88.453	90.182	39.83
Skin permeability (Log kp)	-2.53	-2.746	-2.648	-2.735
P-glycoprotein substrate (yes/no)	No	No	No	Yes
P-glycoprotein 1 inhibitor (yes/no)	No	No	No	No
P-glycoprotein 11 inhibitor (yes/no)	Yes	Yes	Yes	No
Distribution				
VDss (human) (Log L/kg)	0.7	0.149	0.398	-0.793
Fraction unbound (human) (Fu)	0	0	0.016	0.671
BBB permeability (Log BB)	0.969	1.109	0.788	-1.458

CNS permeability (Log Ps)	-1.299	-0.644	-1.583	-3.578
Metabolism				
CYP2D6 substrate (yes/no)	No	No	No	Yes
CYP3A4 substrate (yes/no)	Yes	Yes	Yes	Yes
CYP1A2 substrate (yes/no)	Yes	Yes	Yes	No
CYP2C19 substrate (yes/no)	No	No	No	No
CYP2C9 substrate (yes/no)	No	No	No	No
CYP2D6 substrate (yes/no)	No	No	No	No
CYP3A4 substrate (yes/no)	No	No	No	No
Excretion				
Total clearance (Log ml/min/kg)	1.612	1.51	1.686	0.279
Renal OCT2 substrate (yes/no)	No	No	No	No
Toxicity				
AMES toxicity (yes/no)	No	No	No	No
Maximum tolerated dose (human) (Log mg/kg/day)	0.284	-0.443	-0.377	0.474
LERG 1 inhibitor (yes/no)	No	No	No	No
LERG 11 inhibitor (yes/no)	Yes	Yes	Yes	No
Oral Rat Acute Toxicity (LD50) (mol/kg)	1.519	1.89	1.708	2.11
Oral Rat Chronic toxicity (LOAEL) (Log mg/kg-bw/day)	1.177	0.889	1.02	1.545
Hepatoxicity (yes/no)	No	No	No	Yes
Skin sensation (yes/no)	Yes	Yes	Yes	No
T.Pyriformis toxicity (Log µg/L	1.621	0.312	1.601	0.285
Mininow toxicity (Log mm)	-2.161	-4.198	-1.322	1.836

DISCUSSIONS

The obtained ITS sequences from the isolates showed an exact match in the Mega BLAST search against highly similar sequences in the NCBI non-redundant nucleotide (nr/nt) database, with 100% similarity to other species. Evolutionary distances, computed using the Jukes-Cantor method, aligned with the phylogenetic placement of the isolates within the *Aspergillus* genus, showing close relatedness to *A. austroafricanus*.[8]

The superior activity of the crude extract (figure 3) relative to its fractions suggests synergistic interactions among multiple bioactive constituents, a phenomenon widely reported in natural product research where combined metabolites enhance antimicrobial potency beyond that of isolated compounds.[15]

The crude extract demonstrated greater efficacy against Gram-positive organisms, which may be explained by their exposed peptidoglycan layer that facilitates penetration of antimicrobial compounds. However, notable activity was also observed against Gram-negative bacteria, including *E. coli* and *P. aeruginosa,* indicating the presence of metabolites capable of

overcoming outer membrane permeability barriers. Gram-negative resistance is typically associated with lipopolysaccharide-rich outer membranes, multidrug efflux systems, and enzymatic degradation.[16] The observed inhibition therefore suggests that the fungal metabolites may possess amphipathic or lipophilic properties enabling membrane destabilization or interference with essential intracellular processes.[17,18]

Further evaluation of the M-fraction (fig 3 B) revealed consistent broad-spectrum activity against both Gram-positive (*S. aureus*, *Bacillus* spp.) and Gram-negative (*E. coli*, *P. aeruginosa*) pathogens. From the obtained result, inhibition zones exceeded those of amoxicillin–clavulanate and ceftriaxone (the positive controls). The relatively weak activity of ceftriaxone(6 mm) against *Bacillus* spp. may be due resistance mechanisms in the bacteria.[19] These findings suggest that the fungal metabolites may operate through mechanisms distinct from classical β-lactam antibiotics, potentially involving membrane disruption, metabolic inhibition, or attenuation of virulence pathways.

Particularly noteworthy is the inhibition of *P. aeruginosa*, a pathogen recognized by the World Health Organization as a priority antimicrobial-resistant organism. The ability of the fungal fraction to inhibit this intrinsically resistant species underscores its therapeutic potential. Emerging evidence indicates that microbial natural products may exert multi-target effects, simultaneously interacting with membranes, proteins, and regulatory networks, thereby reducing the likelihood of resistance development.[11]

Table 1 demonstrates organism-dependent antibacterial potency of *Aspergillusaustroafricanus* extracts. The strongest activity was observed against *Escherichia coli* (MIC 12.5; MBC 50; MBC/MIC = 4), indicating bactericidal action despite the intrinsic resistance typically associated with Gram-negative outer membrane barriers. This suggests the presence of amphipathic, membrane-active secondary metabolites. *Bacillus cereus* and *Staphylococcus aureus* showed identical MIC (25) and MBC (100) values (ratio = 4), also confirming bactericidal activity, though at higher concentrations.

Extreme acidic or alkaline conditions (table 2) inhibited biomass accumulation, likely due to enzyme inactivation and cellular stress responses. These findings are consistent with those of[19], who identified a growth optimum for *Aspergillus* species between pH 5.5 and 8.

The superior performance of lactose in figure 4 A suggests that it may favour sustained

growth, possibly due to slower assimilation and reduced catabolite repression compared to glucose. Recent studies have shown that lactose can enhance fungal biomass in *Aspergillus* species by minimizing rapid glycolytic flux and allowing prolonged metabolic activity.[21]

Sucrose also supported substantial biomass accumulation, likely due to its efficient hydrolysis into glucose and fructose, which enter central carbon metabolism. However, simple sugars such as glucose often trigger carbon catabolite repression (CCR), which may limit overall growth efficiency and downstream metabolite biosynthesis in filamentous fungi.[22]

Metabolite production followed a similar but not identical trend. Lactose again produced the highest metabolite yield (1.03), followed by control (0.95) and sucrose (0.76). Glucose (0.06), maltose (0.07), and mannitol (0.08) yielded minimal metabolite production.

The low metabolite yield in glucose-containing media can be attributed to CCR, where easily metabolizable sugars repress genes involved in secondary metabolism. In filamentous fungi such as *Aspergillus* spp., glucose-mediated repression of polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) pathways has been well documented.[22] This explains why, despite moderate biomass formation, glucose did not significantly enhance metabolite production.

Lactose, in contrast, is known to alleviate CCR effects and can act as a favorable carbon source for inducing secondary metabolite pathways. Several reports indicate that disaccharides like lactose support enhanced biosynthesis of bioactive compounds in filamentous fungi by promoting a metabolic shift from primary growth to secondary metabolism.[21] Mannitol and maltose produced relatively low metabolite yields, suggesting limited activation of biosynthetic gene clusters under these conditions.

These findings in figure 4B align with those of[23] and contrast with[2], who reported ammonium and asparagine as optimal for *Aspergillus* growth. In the present study, these organic nitrogen sources yielded suboptimal results, possibly due to stress induction or less efficient metabolism.

Phytol in figure 6 is a bioactive compound recognized to have antimicrobial and antifungal activities and cytotoxic effects.[25] Hexadecane, 2,6,10,14-tetramethyl is also a major metabolite identified from the extract fraction. This branched hydrocarbon is known to be produced as fungal secondary metabolites and may contribute to the biological activities

associated with the crude extract.[26] The GC–MS analysis provides a strong foundation for further chemical and bioactivity investigations of this isolate.

Molecular docking is a computational technique that predicts the most favourable orientation and binding affinity of a small molecule (ligand) when interacting with a biological target, typically a protein, enzyme, or receptor. This approach facilitates a detailed understanding of molecular interactions at the atomic level, and is widely applied in drug discovery, structural biology, and computational chemistry.[27]

Hexadecane, 2,6,10,14-tetramethyl in fig 7A fits deeply into a hydrophobic cavity formed by the residues. The ligand's flexible alkyl chain adopts a curved conformation that matches the shape of the binding pocket, increasing binding stability despite the absence of classical hydrogen bonds. This is consistent with studies showing that lipophilic volatile organic compounds and branched alkanes can bind to protein hydrophobic clefts and disrupt enzyme function (Hydrophobic interactions in protein – Basics and Structure, 2024). Phytol is a hydrophobic, flexible molecule with a terminal hydroxyl group and these chemical properties are reflected in the interaction pattern observed in the figure.

In this study, selected bioactive compounds isolated from fungal extracts were subjected to molecular docking analysis against bacterial DNA gyrase, an essential enzyme in prokaryotes responsible for introducing negative supercoils into DNA during replication, transcription, and recombination. DNA gyrase, a type II topoisomerase found exclusively in bacteria, serves as an ideal and selective target for antibacterial drug development due to its absence in humans.[28]

All bioactive compounds demonstrated interactions with bacterial DNA gyrase. Among them, hexadecane, 2,6,10,14-tetramethyl and squalene exhibited the highest binding affinities (-7.6 kcal/mol), followed by phytol (-7.4 kcal/mol),

To further evaluate their drug-likeness and pharmacokinetic profiles, ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) analysis was conducted. ADMET profiling is essential in early-stage drug development to assess a compound's interaction with biological systems and potential for adverse effects.[29]

According to[30,31], compounds with molecular weight within the range of 136.23 g/mol to 422.81 g/mol,(figure 3) are generally more favourable as drug candidates.

Table 4 highlights the ADMET profiles, revealing high predicted absorption rates for all compounds, ranging from 88.45% to 95.90%, suggesting excellent potential for oral bioavailability.[29] The volume of distribution (VD) values further indicated that the compounds could efficiently reach various tissue compartments, higher VD correlates with broader tissue penetration, while lower VD suggests plasma confinement.[32]

CONCLUSION

The study shows *A. astroafricanus* as promising producers of bioactive compounds with superior antibacterial efficacy. The combined application of microbial isolation, growth optimization, bioactivity screening, compound identification, antibiogram assessment, and molecular docking offers a comprehensive approach toward the discovery of new antimicrobial agents. Future in vivo studies and compound purification are recommended to advance these findings toward pharmaceutical application, especially in the fight against multi-drug-resistant bacterial pathogens.

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Contributions

Samuel Agim Abuh conceived and designed the study, participated in data collection, analysis, and interpretation, and contributed to manuscript drafting and revision. **Malachy C Ugwu** supervised the research, guided the experimental design and assisted in the final proofreading of the manuscript.

IR Iroha: Co-supervised the research work.

Apkanke Justine: Assisted in sample collection and laboratory investigation.

ChidimmaRuthChukwunwejim assisted in laboratory investigations and manuscript organization.

Olusoji Lekan Adeboye contributed to data interpretation, laboratory investigations literature review. All authors read and approved the final version of the manuscript.

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