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Research Article

# Effect of Culture Media on Secondary Metabolites from Aspergillus Terrus from Soil of Osun Osogbo Grove

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## ABSTRACT

Fungi belonging to the families; Hypocreaceae, Ascomycota and Basidiomycota are known to produce different secondary metabolite, that are biologically active and have been found to play significant beneficial roles to man. However, the culturing condition as well as the constituent of the media plays significant roles on the quantity and diversity of metabolites produced. In this study, validated pure Aspergillus terrus characterized from the soil of Osun Osogbo Grove was conditioned to produce secondary metabolite using four (4) secondary metabolite induction media: Wickerham medium (WSP); Modified Wickerham medium (MoWSP); 3% Malt Extract broth (MEB) and Tubaki in a 250ml conical flask. Purified A. terrus inoculum was cultured in flasks containing 100ml culture media of varying compositions, u same physiological culturing parameters. Cultures were incubated in a stationary position in the dark for twenty-one (21) days prior extraction of extracellular (Mycelium) and intracellular (Broth) metabolites. Physical evaluation of culture revealed the morphology of organism grown on MEB, WSP and MoWSP has similar fungal growth pattern while Tubaki produce fungal with different morphological pattern. Gas Chromatography Mass Spectrophotometer (GC-MS) analysis of intracellular extract revealed eight (8) metabolites were consistent in all three media while Furo [2,3-c] pyridine, 2,3-dihydro-2,7-dimethyl- was also consistent in the extracellular extract of the three 0media. Three nucleotide analogues; {1,2,4} Triazolo {1,5-a} pyrimidine-6-carboxylic acid,7-amino ethyl esters, 1,2,4}Triazolo{1,5-a}pyrimidine-6-carboxylic acid,7-imino ethyl esters and 2,3-dihydro-2,7-dimethylFuro[2.3-c]pyridine were detected. Structural and functional elucidation of secondary metabolites from MEB revealed A. terrus is a potential novel source for anticancer, immunosuppressor, anti-helminths, antioxidant, antibiotics as well as pesticide.

**Keywords:** Aspergillus terrus, Antimetabolites, ethyl oxalate, lithostat, intracellular and extracellular secondary metabolites.

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# INTRODUCTION

Fungi are complete heterotrophic organism unable to perform photosynthesis and must obtain their nutrients from the environment, living, dying or dead organism as well as soil. They are more closely related to animal than the plant but are different from both groups. They are characterized by rapid growth and abundant production of conidial spores as well as capacity to produce sclerotia. Fungi are extremely useful organisms in biotechnology, they construct unique complex molecules using established metabolic pathways and metabolites formed along these metabolic pathways may also be biologically active. Different species produce sets of related molecules, each with slightly different final products. Under modified metabolic conditions some fungi have been observed to produce diverse secondary metabolites with several importance to humans, animals and plants, which could be harness industrially through modification of physiologic parameters; such as temperature, pH, light and nutrient sources of carbon and nitrogen. Prominent among the biologically active component producing fungi are the Ascomycetes; which include penicillum spp, Aspergillus spp and basidiomycete; Trametes

Trichoderma are fungi in the family Hypocreaceae that is present in all soils, where they are the most prevalent culturable fungi. Many species in this genus can be characterized as opportunistic avirulent plant symbionts. The rhizocompetent nature of Trichoderma spp. allows it to colonize roots, stimulates the plant immune system (induced systemic resistance; ISR), and pre-activation (priming) of the molecular mechanisms of defense against several potent phytopathogens (Hermosa et al., 2012; Pieterse et al., 2014; Martínez-Medina et al., 2017). Aspergillus terrus is a saprophytic fungus found worldwide in tropical and subtropical regions, soil, decomposing vegetation and dust. It is brownish in color and get darker has its ages on culture media (Samson et al., 2004), colonies on malt extract agar grow faster and sporulate than any other media (Samson et al., 2004). They are thermotolerant specie, with optimal growth temperature between 35-400C and maximum growth within 45-480C (Anderson et al., 1980). It has been found as plant pathogen by disrupting the male sexual reproductive cycle in plant through the production of aspterric acid and 6-hydroxymellen (Shimada et al., 2002). It can also cause opportunistic infection in human and animals; inhalation of fungal spores causes respiratory infection in immuno-compromised patients (Molinaa, 2013). Aspergillus terreus produce several metabolites such as lovastatin; a potent drug for lowering blood cholesterol (Lopez et al., 2003). It is the only fungal that has been utilized and commercialized to produce lovastatin (Lai et al., 2007; Bizukojc et al., 2011). Biosynthesis of lovastatin depends not only on composition of culture media like carbon and nitrogen sources, but also on the strain used and culture conditions (Lopez et al., 2003). In this present report, A. terrus from the soil of the conserved UNESCO heritage site; the Osun-Osogbo Grove, was purified and classified taxonomically to the subclass level using ITS genes prior secondary metabolite induction and characterization on Mass Spectroscopy enhanced Gas Chromatography.

# Method

## Media Preparation

The media were dissolved in distilled water and autoclaved at 121oC, 2% Malt Extract Agar (MEA) (2% malt extract, 1.5% agar); Czapek agar (CZA) (Sucrose 3% Sodium nitrate 0.3, Dipotassium phosphate 0.1%, Magnesium sulphate 0.05%, Potassium chloride 0.05% Ferrous sulphate 0.001); Yeast Extract Agar (YES) (20 g soy peptone, 20 g dextrose, 5 g yeast extract, 1L H2O); 3% Malt Extract broth (MEB) (Malt extract 3%, Peptone 0.5%, pH-8.0). Wickerham medium (WSP) (1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract and 3% sodium chloride (pH = 6.8); Modified Wickerham medium (MoWSP) (1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract and 3% commercial common salt) (pH = 6.8) The medium initial pH was adjusted using hydrochloric acid. The solutions were sterilized at 121oC for fifteen minutes in an autoclave. The medium was allowed to cool prior plating and the store overnight prior subsequent fungi inoculation.

## Study site and location

The Grove of Osun River in Osun State situated adjacent Fountain University, Osogbo in the tropical rain forest zone of South West Nigeria, West Africa. The Osun River covers 14,875 sq km and lies between latitude 7° 30′ 0″ N and longitude 4° 30′ 0″ E. The sampling area involved three (3) locations within the grove.

## Isolation of fungi from the soil samples

The soil micro fungi were enumerated using two methods, namely Soil Dilution (Waksman, 1922) and soil plate method (Warcup, 1950) on different media; Malt Agar Extract, Potato Dextrose Agar and Czapek Dox Agar.

## Soil dilution plate method

One (1) g of soil sample was suspended in 10 mL of double distilled water to make microbial suspensions (10-1 to 10-5). Dilution of 10-3, 10-4 and 10-5 were used to isolate fungi. One (1) mL of microbial suspension of each concentration was added to sterile Petri dishes (triplicate of each dilution) containing 15 mL of sterile Malt Agar Extract, Potato Dextrose Agar and Czapek, Dox Agar. One percent streptomycin solution was added to the medium before pouring into petri plates for preventing bacterial growth. The Petri dishes were then incubated at 28 ± 2°C in dark. The plates were observed every day until third days.

## Soil plate method

About 0.005g of soil was scattered on the bottom of a sterile petri dish and molten cooled (40-450C) agar medium (MAE), (PDA) & (CZA) was added, and rotated gently to disperse the soil particles in the medium. The Petri dishes were incubated at 28 ± 20C in dark for three days.

## Morphological Identification of the soil fungi

Fungal morphology was studied macroscopically by observing colony features (Colour and Texture) and microscopically by staining with lacto phenol cotton blue and observed under compound microscope for the conidia, conidiophores and arrangement of spores as described by Aneja (2001).

## Molecular and Taxonomical Characterization of Fungi

### Isolation of genomic DNA

Mycelium was crushed to a pasty consistency using mortar and pestle prior genomic DNA isolation using commercial fungal DNA Extraction Kit according to manufacturer specification. The extracted DNA was electrophoresed on 1.2% agarose gel and quantified using Nanodrop Spectrophotometer.

### PCR Amplification of ITS gene

The gene responsible for ITS region of the DNA extracted were amplified using ITS 1 (Gardes and Burns 1993) as forward and ITS 2 (White et al., 1990) for the reverse reaction in a 25㎕ reaction with an initial denaturation at 940C for 30 seconds, followed by thirty cycles of denaturation at 940C for 30 seconds, annealing at 52.80C and an extension at 680C for 60 seconds and a cycle of final extension at 680C for 5 minutes. The resulting amplicons were electrophoresed on 1.2% agarose.

### Sequencing

The amplicons were sent for sequencing using the forward primers as sequencing primer.

### Bioinformatics Analysis

Obtained sequence were compared with sequence in database on www.ncbi.nih.gov and www.ebi.ac.uk

### Secondary metabolites production

Confirm Aspergillus terreus species were subjected to secondary metabolite production using liquid media stationery fermentation process. The fungi were inoculated on plates, and inoculum of the fungus incubated in the dark for twenty-one days.

### Liquid Media Cultivation

Several liquid media were chosen to represent a broad range of nutrient sources, including both defined and undefined broths. Fungal strains were first cultivated on 2% malt extract agar. Plugs from these cultures were used to inoculate 50 mL of four different culture broths in 250 mL Erlenmeyer flasks as described by Vandermolen et al., 2013.

## Extraction and Purification Secondary Metabolites

### Mycelium Extraction

The fungal intracellular secondary metabolites were extracted as described by Bin et al., (2014). The mycelium in the media was separated from the culture broth using aseptic techniques, mycelia were washed in sterile distilled water three times to remove adjourning spores. The mycelium was homogenized in 150ml of ethanol before centrifugation at 10000rpm for 10mins. The supernatant was collected, and the ethanol allowed evaporating. The remaining aquatic phases were extracted twice with 20ml ethyl acetate in a separatory funnel; the organic phase was collected in a beaker and expose in a laminar flow to evaporate. The resulting residue and intracellular ethyl acetate contents were dissolved in 5 ml methanol and store at 40C until analysis.

### Broth extraction

The fermentation broths were extracted with 27ml ethyl acetate per flask using a separating funnel. After which 15ml of deionized water was added to the organic phase in the separating funnel. The upper layer was collected in a beaker and expose in a laminar flow to evaporate. The residues were dissolved in 5ml methanol to obtain the extracellular ethyl acetate soluble content.

### Separation of Extract Using Column Chromatography

Extract was reconstituted with ethyl acetate and column chromatography was done using mixtures of ethyl acetate and n-hexane. Slurry was made using silica gel and ethyl acetate; this was then transferred into the column avoiding bubbles. The gel was allowed to pack, the compound to be separated was added gently on the packed gel and mixture of mobile phase (ethyl acetate and n-hexane) in different ratios were continuously added making sure that the column was never dry. The separated fractions were collected in separate tubes for further analysis.

## Evaluation of Biological Activities of Secondary Metabolites

### Anti-Inflammatory Activity of Separated Compounds

Procedures from (Anosike et al., 2012) were followed with slight modification. Extract samples were dissolved in distilled water (hypotonic solution) and put in centrifuge tubes. Isotonic solution also containing extracts were put in another set of centrifuge tubes and distilled water (vehicle) to be used as control tubes. Diclofenac sodium (standard drug) was added to the control tubes and erythrocytes suspension was added to each of the tubes (hypotonic, isotonic and control) and mixed gently. The mixtures were incubated at room temperature for 3 minutes before centrifuging at 1300 g at 3mins. Absorbance of the hemoglobin content of the supernatant was read at 540 nm. The percentage hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water to be 100%. The percentage inhibition of hemolysis by extract was calculated thus:

% inhibition of hemolysis = 1- (OD2-OD1) / (OD3-OD1) * 100

OD1 = absorbance of test sample in isotonic solution

OD2 = absorbance of test sample in hypotonic solution

0D2 = absorbance of control sample in hypotonic solution

### Anti-Arthritic (Inhibition of Protein Denaturation) Property of Separated Fractions

Procedures from (Chowdury et al., 2015) were used with slight modification. Diclofenac sodium was used as standard for the test. Total volume of test solution was 0.5 ml containing 0.45ml of bovine serum albumin and 0.05ml of extract. Control solution contained 0.45ml of bovine serum albumin and 0.05mlml of distilled water. Product control contained 0.45ml of distilled water and 0.05ml of extract. Standard solution contained 0.45 ml of bovine serum albumin and 0.05ml of Diclofenac sodium. All samples were kept in the incubator at 57 degrees for 3 minutes. Samples were then cooled to room temperature before adding 2.5ml of phosphate buffer to all the solutions and absorbance was read at 416 nm. The percentage inhibition of protein denaturation was calculated as:

% inhibition = [100- (OD of test solution – OD of product control)] * 100

Where OD = Optical Density.

**Anti-Microbial Activity of Separated Fractions**

Medium containing nutrient agar was prepared and kept in a sterile cupboard overnight to ascertain sterility and make sure there is no foreign growth. Organisms were then inoculated using the spread plate method and then six holes were bored per plate using a cork borer. Standard, gentamycin and vancomycin were put into two of the bored holes and extracts to be tested were put into the other holes. Plates were incubated for 24 hours and distance of growth inhibition was measured afterwards.

## Identification of Separated Fractions

**Separated fractions were subjected to Gas Chromatography-Mass Spectrometer (GC-MS) and spectra analysis.**

Column purified extract of the culture broth and mycelium were subjected to GCMS. The chromatography was condition towards a Column oven temperature set at 500C for 5 min, then increased to 2600C (ramp: 4°C/min) and held for 5 min. The injection volume was 1µL and all the injections were performed in a split-less mode. Temperature of the injector and detector was 250 and 2800C, respectively. In addition, an HP-5MS fused silica capillary column (Hewlett-Packard, 30 m* 0.25 mm i.d., 0.25 µm films, cross-linked to 5% phenyl methyl siloxane stationary phase) was applied. The entire system was controlled by Chemstation software (Hewlett-Packard, version A.01.01). Electron impact mass spectra were recorded at 70-electron voltage and ultra-high pure He (99.999%) gas was used as the carrier gas at flow rate of 1 mL/min.

**Spectra Analysis**

Compounds suggested from spectra obtained from GC-MS analysis were elucidated on https://pubchem.ncbi.nlm.nih.gov/ and compared with literature for probably functions and novelty.

# RESULT

## Media Preparation

Pure media were obtained with no contaminants after 24 hours of autoclaving and plating, plate 4.1 shows photograph representing the pure media.

## Sample Collection and Preparation

Figure 2a shows images of soil sample on Potato Dextrose Agar (PDA).

## Culturing of sample

The images of cultured organisms on fungi selection media PDA are show in Figure 2B, C and D Figure 2B shows the fungi A. terreus with other fungi (mixed culture). Figure 2C show A. terreus subcultured with limited contamination. Pure A. terreus was obtained after four subcultures as shown in photograph in on Figure 2D. Microscopic view of A. terreus at 100x and 200x is represented on Figure 2E and 2F respectively.

*Figure 1: Sterile Liquid Media*

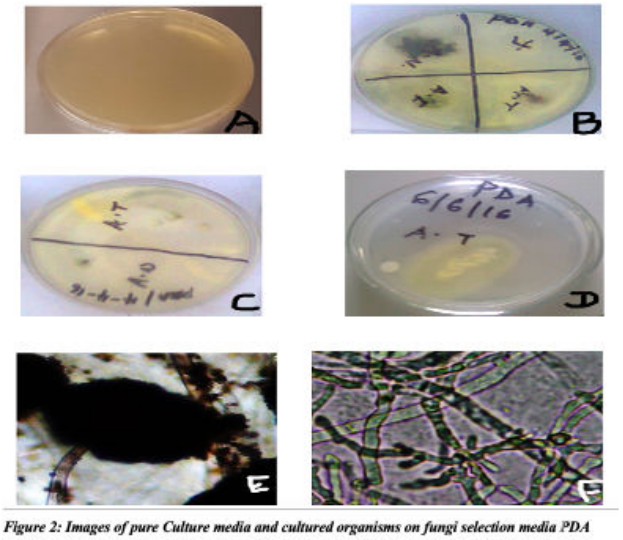



*Figure 2: Images of pure Culture media and cultured organisms on fungi selection media PDA*

**Figure 3:** Muscle WS alignment of the Sequence obtained from blastn of Dr NBAB_OSG_A10 on blast.ncbi.nlm.nih.gov

## Polymerase Chain Reaction and Sequence Bioinformatic Analysis

Sequence obtained from the spectra of the sequence analysis was named DrNBAB_OSG_A10 consisting of 530 bp, nucleotides blast indicates high similarity with Aspergillus terreus spp, Multiple alignment of DrNBAB_OSG_A10 indicates the sequence varied with those in the database with substitution as well as nucleotide deletions, the variations are represented in Figure 3. Phylogenetic analysis show that sample DrNBAB_OSG_A10 is most closely related to Aspergillus terreus strain MF12 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence with GenBank accession no. JF431429.1 the relationship is represented in Figure 4. Principal component calculation depleting the spatial representation of the similarities within all of the sequences in the alignment also indicated A. terrus MF12 is the closest in lineage with the obtained sequence as shown in figure 5.

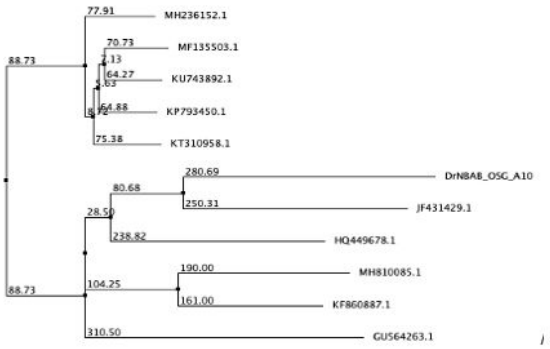



*Figure 4: Neighbour Joining Using Sequence obtained from blastn of DrNBAB_OSG_A10 on blast.ncbi.nlm.nih.gov*

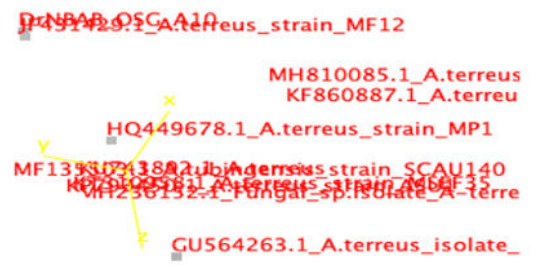



*Figure 5: Principal Component Analysis Sequence obtained from blastn of DrNBAB_OSG_A10 on blast.ncbi.nlm.nih.gov*

## Secondary metabolites production

Liquid medium cultivation

Fungal strain grew well on MEB, WSP and MoWSP has similar fungal growth pattern, Tubaki produce fungal with different morphology. Photograph of the organism in solid medium is represented in Figure 2. The growth rate of A. terreus mycelia on the four media was also observed to be in a media dependent manner with highest rate observed in MEB, Figure 2.

## Extraction and Purification Secondary Metabolites

Mycelium Extraction

Mycelia extract of fungi has variable color intensity in different media. Extraction of the secondary metabolite constituents in the mycelia of malt extract appears as triphasic in the Separating funnel.

Broth Extraction

Extract from culture broth also have differences in color intensity and particles constituent, appearing to be biphasic in the Separating funnel.

## Identification of Organic Solvent Soluble Secondary Metabolite from Organism

### GC-MS conditions

Samples analyzed on GC-MS produced several peaks of compounds with variable retention time for the fungal extract in different medium. Twenty-two intracellular metabolites were identified from mycelium of A. terreus cultivated in MEB while the extracellular metabolites in culture broth were sixteen (16);

the broth from the WSP and MoWSP produced extracellular 100 metabolites each. The spectra obtained from GC-MS are represented in Figure 6. Table 1 show the bioactive components produced by A. terrus in different media. The common compounds detected in organism per media is represented in Table 2. Constituent of intracellular extract on MEB is represented on Table 3 while Tables 4,5 and 6 contained the extracellular constituents on MEB, WSP and MoWSP respectively.

| S/N | Organisms | Media | Extract Sample | Amount Of Constituent |
|---|---|---|---|---|
| 1 | Aspergillus terreus | Malt Extract Broth | Mycelium & | 22 |
| | | | Broth | 16 |
| | | Wickerham Medium | Broth | 100 |
| | | Modified Wickerham Medium | Broth | 100 |

**Table 1:** The bioactive components Identified by GC-MS

| | | MEB Mycelium | | MEB Cul. Media | | WSP | | MoWSP | |
|---|---|---|---|---|---|---|---|---|---|
| S/N | Library/ID | RT | Area % | RT | Area % | RT | Area % | RT | Area % |
| 1. | 2-Propen-1-amine | 6.8091 | 0.1116 | - | - | 6.861 | 0.1642 | - | - |
| 2. | Aminoacetonitrile | 7.593 | 0.1007 | 6.6317 | 0.4349 | 6.466 | 0.5852 | 5.6361 | 2.4199 |
| 3. | Methylacrylonitrile | 9.8131 | 0.1158 | 36.0083 | 0.3823 | 10.32 | 0.2181 | 6.9006 | 0.6512 |
| 4. | Propargylamine | 10.6142 | 0.2158 | 9.321 | 0.382 | 35.72 | 0.4665 | 38.2398 | 2.4303 |
| 5. | N-Ethylformamide | 11.77 | 0.1556 | 36.1857 | 0.0897 | - | - | 36.6606 | 1.2074 |
| 6. | Pyrrole | 37.6562 | 0.127 | 6.8091 | 0.1387 | 7.433 | 0.2369 | 38.5946 | 0.4508 |
| 7. | Chlorine dioxide | 37.9309 | 0.1265 | - | - | 7.055 | 0.1817 | 40.2998 | 0.8723 |
| 8. | 2-Butanamine, (S)- | 39.2412 | 0.1843 | - | - | 40.24 | 0.8216 | 39.0295 | 1.1438 |

**Table 2:** Compounds detected in Aspergillus terreus cultured on MEB, WSP and MoWSP Identified by GC-MS.

| S/N | RT | Compound | Mol. formular | Mol. Weight | Area % |
|---|---|---|---|---|---|
| 1 | 32.175 | Ethyl oxamate | C4H7NO3 | 117.1 | 0.0988 |
| 2 | 32.518 | N- (3-Methylbutyl) acetamide | C7H15NO | 129.2 | 0.1971 |
| 3 | 32.65 | 4-Fluorohistamine | C5H8FN3 | 129.14 | 0.1712 |
| 4 | 32.758 | dl-3-Aminoisobutyric acid, methyl ester | C5H11NO2 | 117.14 | 0.1244 |
| 5 | 32.913 | Acetohydroxamic acid | C2H5NO2 | 75.066 | 0.1152 |
| 6 | 34.229 | Cyanogen chloride | CClN | 61.47 | 0.1279 |

| | | | | | |
|---|---|---|---|---|---|
| 7 | 34.767 | Methanamine, N-hydroxy-N-methyl- | C2H7NO | 67.083 | 0.1282 |
| 8 | 39.705 | Thiocyanic acid, methyl ester | C2H3NS | 73.117 | 0.1742 |
| 9 | 39.911 | 4-Dodecanol | C12H26O | 186.3342 | 0.417 |
| 10 | 50.96 | [1,2,4] Triazolo [1,5-a] pyrimidine-6-carboxylic acid, 4,7-dihydro-7-imino-, ethyl ester | C10H12N4O3S | 268.29 | 96.05 |

**Table 3:** Intracellular Compounds Identified from Aspergillus terreus cultured on MEB by GC-MS.

| S/N | RT | Compound | Mol. formular | Mol. Weight | Area % |
|---|---|---|---|---|---|
| 1. | 7.1124 | 2-Butenenitrile | C4H5N | 67.08 | 0.118 |
| 2. | 32.5179 | Furo[2,3-c]pyridine, 2,3-dihydro-2,7-dimethyl- | C9H11NO | 149.19 | 1.2055 |
| 3. | 34.4062 | Ethyl isocyanide | C3H5N | 55.0785 | 0.1776 |
| 4. | 34.5206 | 3(2H)-Thiophenone, dihydro-, oxime, 1,1-dioxide | C4H6OS | 102.15 | 0.1338 |
| 5. | 39.3099 | Ethenamine, N-methylene- | C3H5N | 55.078 | 0.1146 |
| 6. | 40.4657 | 1-Propenylaziridine | C5H9N | 83.13 | 0.2369 |
| 7. | 50.9484 | [1,2,4] Triazolo [1,5-a] pyrimidine-6-carboxylic acid, 4,7-dihydro-7-imino-, ethyl ester | C10H12N4O3S | 268.29 | 95.09 |

**Table 4:** Extracellular Compounds Identified from Aspergillus terreus cultured on MEB by GC-MS.

| S/N | RT | Compound | Mol. formular | Mol. Weight | Area % |
|---|---|---|---|---|---|
| 1. | 6.6948 | 1,6-Heptadiene | C7H12 | 96.17 | 0.2829 |
| 2. | 7.021 | Ethyl isocyanide | C3H5N | 55.0785 | 0.1053 |
| 3. | 7.2727 | Aziridine, 2-methyl- | C3H7N | 57.0944 | 0.2958 |
| 4. | 8.0795 | Ethenamine, N-methylene- | C3H5N | 55.0785 | 0.1822 |
| 5. | 32.5352 | Furo [2,3-c] pyridine, 2,3-dihydro-2,7-dimethyl- | C9H11NO | 149.19 | 2.2055 |

| | | | | | |
|---|---|---|---|---|---|
| 6. | 33.6682 | 1-Propanol, 2-methyl- | C4H10O | 74.1216 | 3.1411 |
| 7. | 35.0586 | 2-Propynenitrile, 3-fluoro- | C3FN | 69.0372 | 0.7388 |
| 8. | 37.273 | 9-Octadecenoic acid (Z)-, methyl ester | C19H36O2 | 296.4879 | 38.4947 |
| 9. | 37.7766 | Methyl stearate | C19H38O2 | 298.5038 | 31.1559 |
| 10. | 40.134 | 1H-1, 2,4-Triazole | C2H3N3 | 69.0653 | 0.5419 |
| 11. | 40.5288 | Furazano [3,4-b] pyrazin-5 (4H)-one, 6 -(1-pyrrolidinyl)- | C6H9N5O2 | 207.19 | 0.7301 |
| 12. | 41.6789 | Chloromethyl 6-chloroundecanoate | C12H22Cl2O2 | 269.208 | 2.0453 |

**Table 5:** Extracellular Compounds Identified from Aspergillus terreus cultured on WSP by GC-MS.

The compounds listed in the table matched the same compounds in the NIST library.

| S/N | RT | Compound | Mol. formular | Mol. Weight | Area % |
|---|---|---|---|---|---|
| 1. | 6.9464 | 2-Butenenitrile | C4H5N | 67.08 | 0.7116 |
| 2. | 7.427 | Methanamine, N, N-difluoro- | CH3F2N | 67.038 | 0.6685 |
| 3. | 30.7555 | 2-Propynenitrile, 3-fluoro- | C3FN | 69.0372 | 0.9172 |
| 4. | 32.535 | Furo[2,3-c] pyridine, 2,3-dihydro-2,7-dimethyl- | C9H11NO | 149.19 | 6.8365 |
| 5. | 35.6707 | Acetyl cyanide | C3H3NO | 69.069 | 1.672 |
| 6. | 37.742 | Formamide, N, N-dimethyl- | C3H7NO | 73.097 | 0.494 |
| 7. | 39.5731 | Oxazole | C3H3NO | 69.069 | 0.8844 |
| 8. | 39.6017 | 3-Butenenitrile | C4H5N | 67.089 | 1.3784 |

**Table 6:** Extracellular Compounds Identified from Aspergillus terreus cultured on MoWSP by GC-MS.

NB: Highlighted compounds are found in other media.

Spectra Analysis

Compounds suggested based on GC-MS library is represented in Figure 7. Eighteen intracellular compounds were predicted for A. terreus on MEB of which five (5) were detected at extracellular level in MEB and other media, 2-propen-1-amine was only detected in WSP while Chlorine dioxide and 2-Butanamine, (S) was detected in culture broth of WSP and MoWSP, (Table 2). Ten (10) compounds were detected only in MEB at intracellular level (Table 3), seven (7) detected in MEB culture broth of which four (4) were restricted to MEB, Furo [2,3-c] pyridine, 2,3-dihydro-2,7-dimethyl-was detected in WSP and MoWSP, 2-Butenenitrile was detected in MEB and MoWSP and Ethyl isocyanide was detected in MEB and WSP (Table 4). Twelve (12) Extracellular compounds were detected in in WSP of which nine (9) were restricted to WSP, 2-Propynenitrile, 3-fluoro- was also detected in MoWSP (Table 5). MoWSP produced eight (8) compounds, of which three are present in other media (Table 6).

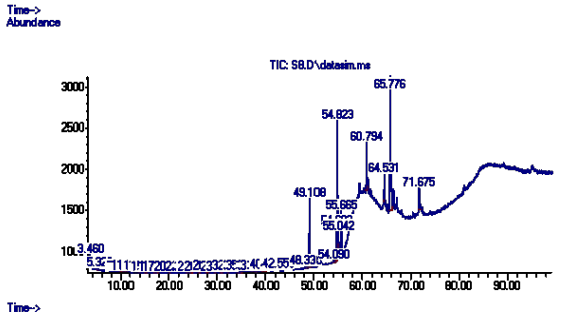



**Figure 6a:** Spectra obtained from GC-MS analysis of fraction 7

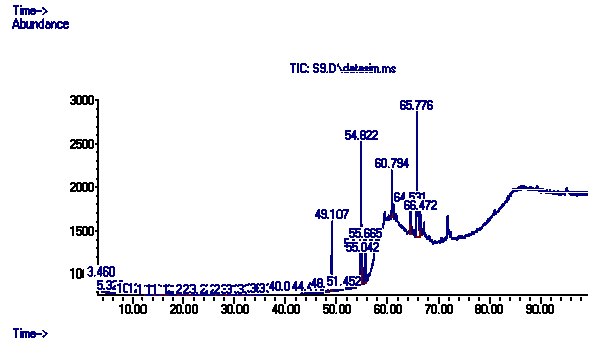
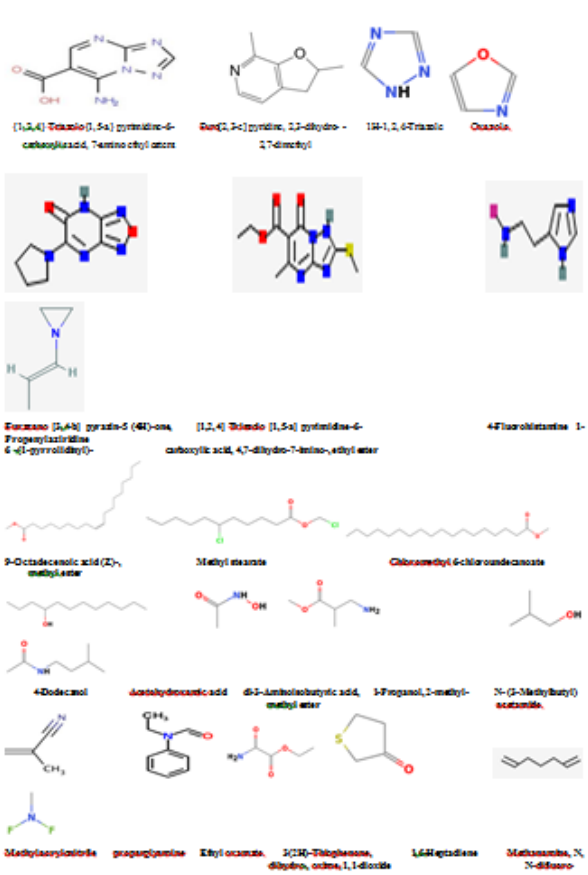



**Figure 6b:** Spectra obtained from GC-MS analysis of fraction 8



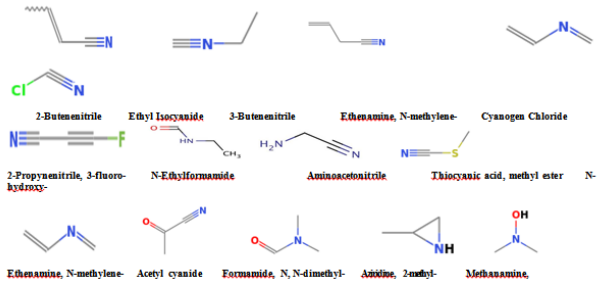



**Figure 7:** Structure of identified Compounds from Secondary Metabolites

## Biological Activities of Secondary Metabolites

### Anti-Inflammatory Activity of Separated Fractions

Percentage anti-inflammatory activity of each fraction is represented in figure 8. Fractions 3 had the highest antinflammatory effect.

### Percentage Inhibiton of Protein Denaturation (Anti-Arthritic Activity)

Percentage anti-arthritic activity of each separated fraction is shown in Figure 9.

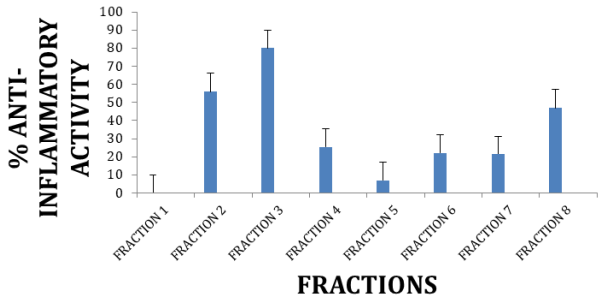



**Figure 8:** Chart Showing % Anti-Inflammatory Activity of Each Column Purified Extract

Fraction

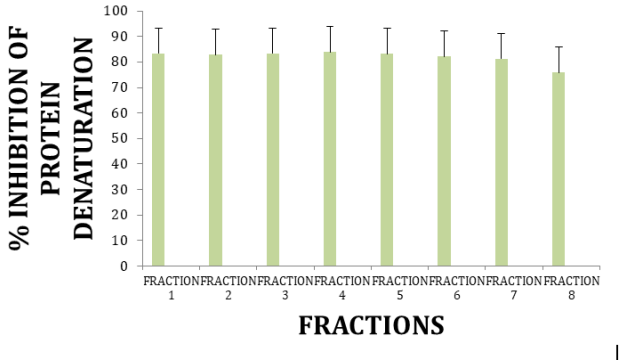



**Figure 9:** Chart Showing % Inhibition of Protein Denaturation of Each Column Purified Extract Fraction

## ANTI-MICROBIAL ACTIVITY OF SEPARATED FRACTIONS

Plates 1a, 1b and 1c shows zones of organism (E. coli and Staphylococcus) growth by standards ni6tmicrobial agent while fractions 1-6 showed no inhibition zone. Fractions 7 and 8 shows inhibition of 1.9cm and 0.5cm inhibition zone respectively for Staphylococcus alongside the standards as shown in Plate 1d.

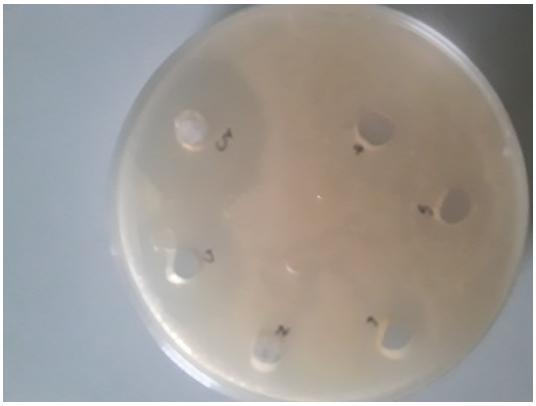


**Plate 1a:** Only standards showing inhibition zone

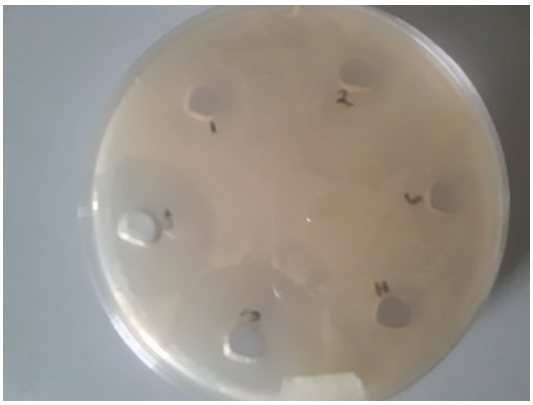


**Plate 1b:** Only standards showing inhibition zone

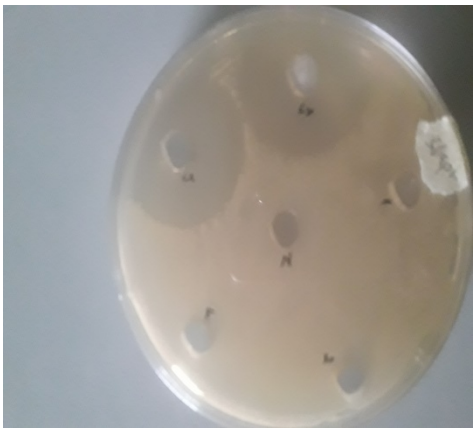


**Plate 1c:** Only standards showing zone of inhibition

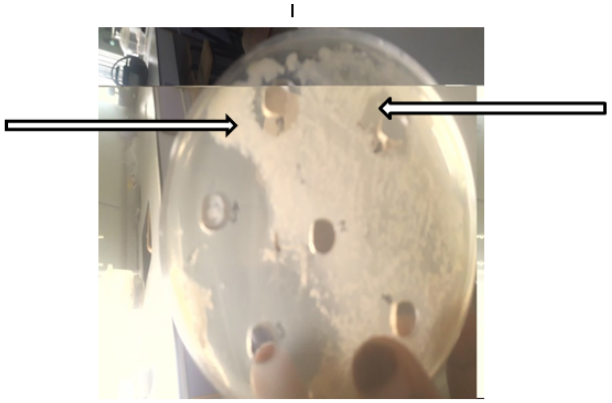


**Plate 1d**: Standards showing inhibition zones alongside fractions 7 and 8

## DISCUSSION

Aspergillus terreus was successfully isolated from soil samples of the Osun grove. Macroscopic evaluation of the organism on potato dextrose agar plate revealed a creamy brownish colony, this physical characteristic is similar to the known morphology of Aspergillus terreus. Colonies appears as a small, round, hyaline conidia ('accessory' conidia) attached to the vegetative hyphae, cinnamon-brown colour on Czapek solution agar and a wood-brown colour on malt extract agar similar the description Rath et al, 1999. In their report, Manna et al.; 2020, confirmed the ability of Aspergillus terreus and other soil fungi to utilize liquid medium contaminated with Cu and other heavy metals.

The ITS region has been used as targets for phylogenetic analysis because they generally display sequence variation between species but only minor variation within strains of the same species (Henry et al., 2000). Amplified ITS region yielded amplicon of 580bp. Sequence analysis of amplicon in its FASTA format obtained from the spectra was named DrNBAB_OSG_A10 consisting of 560bp represented in Figure 5, nucleotides blast indicate 98% similarity with Aspergillus terreus spp as shown in Figure 6 (blastn), Multiple alignment of DrNBAB_OSG_A10 indicates the sequence varied with those in the database with insertion, substitution as well as nucleotide deletions, the variations are represented in Figure 7. Phylogenetic analysis show that sample DrNBAB_OSG_A10 is most closely related to Aspergillus terreus isolate SJCFBKe01 internal transcribed spacer with GenBank accession no. GU564260.1 the relationship is represented in Figure 8.

Considering fungi growth on all cultivation media, czapek agar produced high mycelia growth compared to YES and MEA. However, the desired media for fungi subculture is MEA, as innoculom on MEB has short generation time and increase mycelia growth mass compared to growth on MOWSP and WSP. Aspergillus terreus exhibit morphological change on Tubaki, and this research report doesnot include metabolites detected in Tubaki. Ethyl acetate soluble metabolite from the mycelia (Intracellular metabolites) analysis on Gas-Chromatography Mass Spectrosphotometer revealed a variation in intracellular and exttracellular (culture broth) metabolite. A similar variation was observed in constituent abundance and retention time; this

research complement Vandermolen et al., (2013) where variation of metabolites is recorded in respected to culture broth and mycelium. The production of marker compounds varied widely between all cultures.

In this study, it was observed that the culture media in which the organism shows maximum mycelia growth doesn't necessarily turn out to produce key metabolites. What may be the best medium for one fungus could often prove unproductive for other strains, (Vandermolen et al., 2013) and may not be the optimized media for drug- discovery studies. Metabolite production followed a media content-dependant pattern, however, some of the metabolites were detected in the organism at intracellular level in all culture media (Table 2). Here we report the detection of 2- propan-1-amine a lachrymatory agent at 6.8091 in MEB at intracellular level but not in the culture broth however, it was detected at extracellular level in WSP but not in MoWSP, the implication of this is that enzyme catalysing the metabolic pathway for the biosynthesis of lachrymatory agent is produced intracellularlly when cultured on MEB while same enzyme is produced at extracellular level on WSP. We also observed the quantity of the agent produced at extracellular level when WSP is used is (0.1642) more than the quantity produced at intracellular level when MEB (0.1116), this made WSP a media of choice for the production of 2-propan-1-amine. In all media tends to produce Aminoacetonitrile, Methylacrylonitrile, Propargylamine, N-Ethylformamide, 2-Butanamine (S)-dioxide Pyrrole Chlorine, and at extracellular level but the highest quantity was obtained using MoWSP.

Some compounds were observed to be conserved to specific media as shown in Table 3-6 while some nucleotide antimetabolites such as [1,2,4] Triazolo [1,5-] pyrimidine-6-carboxylic acid, 4,7-dihydro-7-imino-, ethyl ester and [1,2,4] Triazolo [1,5-] pyrimidine-6-carboxylic acid, 4,7-dihydro-7-amino-, ethyl ester were detected at intracellular and extracellular level respectively. The intracellular enzyme produced the imino ester while the extracellular ester produced the amino ester. Furo [2,3-c] pyridine, 2,3-dihydro-2, 7-dimethyl- was detected in WSP and MoWSP where the highest quantity was in MoWSP.

Based on the findings of the study, it would be logical to conclude that the Tramete spp studied classically demonstrated the ability to yield secondary metabolites in media-dependent pattern. Structural elucidation and function comparison with literatures ascertained the metabolites produced are bioactive with medicinal properties as antitumour/anticancer and immuno-suppressant.

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