Archive of Science & Technology
An official Journal of Precious Cornerstone University Ibadan Nigeria
ISSN:0189-3548
Journal Homepage: https://pcujournals.org.ng

Molecular Characterization, Antimicrobial and Larvicidal Potentials of Fungi Isolated from Soil Samples of LAUTECH Botanical Garden Ogbomoso, Nigeria.

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ARTICLE INFO

Article history:
Received March 2020
Received and revised May 2020
Accepted June 2020
Available online June 2020

Keywords:
Soil fungi,
Metabolite
Larvicidal
FTIR
Bioinformatics tools.

ABSTRACT

The menace of malaria infection has necessitated the need for an eradication strategy either directly against Plasmodium or indirectly against its host, the mosquito. Eradication of mosquito can be more easily achieved by attacking the larva stage, this require the availability of an effective larvicide. This research work is focused on the production of biologically synthesized larvicide by exploring microorganisms isolated from the environment. In this study, soil fungi were isolated and inoculated on Potato Dextrose plate. Pure colonies were collected and identified by morphological and molecular characterization. The evolutionary relatedness was established using bioinformatics tools. The fungi colonies were subjected to submerged fermentation in Potato Dextrose broth and incubated for 21 days in a rotary shaker incubator for the production of the intracellularly metabolites. The metabolites were extracted and varying concentrations were used in challenging mosquito larva. Time of action and lethal dose (LD50) of each fungal metabolite were determined. Fourier Transform Infrared (FTIR) was used to monitor the bioactive compounds in the metabolites at the range of 4000-500 cm-1with an IR Affinity-1 Shimadzu Spectrometer. The results showed that metabolites of *Penicillum chrysogenum*, *Aspergillus aculeatus*, *Talaromyces albobiverticillus* and *Aspergillus carbonarius*possess potent larvicidal activities against the mosquito larvae at different concentrations and duration of lethal action. The band of the biomolecules present monitored with FTIR showed that they possesses: (C-O), (C≡C), (C≡N), (C-C=C), ((N-H), (OH), (C-I), (H-C-H), (N=O) and aromatic rings.

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https://doi.org/10.xxx.

1. INTRODUCTION

Fungi are microorganisms, which are found in their ecological niche as decomposers, mutualists, or pathogens of plants and animals. Their activities drive the carbon cycle in forest soils, facilitate plant mineral nutrition, and lessen carbon limitations of other soil organisms (Dengler et al., 2014; Zifcakova et al., 2016).There are about a 100,000 described species of fungi known to man, but the actual number of global fungal diversity is estimated to be about 0.8 million to 5.1 million species (Blackwell, 2011). Fungi in their diversity have found many important uses in everyday life of man, besides their utilization in baking and fermentation of alcoholic beverages that have been known for thousands of years, they are now being used in industrial processes, agriculture, medicine, textiles, bioremediation, natural cycling as bio-fertilizers and so on (Thilagar and Bagyaraj, 2015; Bagyara and Ashwin, 2017). According to Chapman and Hall (2015) fungal biotechnology is now making wave as an integral part of the human welfare. Fungal metabolites have been found to have various potentials in everyday life, some of which include larvicidal functions, antimicrobial properties, enzymes production, etc. (Strobel, 2002; Strobel, 2003; Strobel and Daisy, 2003). In recent time, the ever-growing importance of secondary metabolites in pharmaceuticals, agrochemicals, food additives, and as ingredients in cosmetics have become undeniable (Devi *et al.,* 2012; Csernatoni *et al.,* 2013). The search for effective mosquito bio-control agents that can help in the eradication or reduction of malaria by killing such vectors has been on-going for several decades. Both laboratory and field studies have been carried out on fungi species that have shown potential as mosquito pathogens capable of quickly killing them (Gopalakrishnan *et al., 2012*).

2. MATERIALS AND METHODS

2.1. Collection of Soil Sample

Samples of soil were collected randomly in five different spots at the Ladoke Akintola University of Technology Botanical Garden, Ogbomoso. The soil surface was scrapped and samples were collected using a sterile hand-trowel into a polythene bag and immediately transferred to Microbial Resources Research Laboratory for further work.

2.2. Isolation of Soil Fungi

To prepare stock solution, 1 g of soil sample was added aseptically into 9 ml of sterile water and thoroughly shaken to homogenize the mixture. 9 ml of sterile water was measured into five different sterile test tubes and labeled according to the dilution factor (e.g. 10^{-1}, 10^{-2}, 10^{-3} etc.). 1 ml of the stock solution was transferred aseptically into test tube labeled 10^{-1}, and shaken thoroughly. Again, 1 ml solution was taken from the test tube labeled 10^{-1} and transferred into test tube 10^{-2}. From the 10^{-2} labeled test tube, 1 ml of the diluted solution was taken and transferred into the 10^{-3} labeled tube, and this process was repeated until 10^{-5} dilution was reached. Diluent 10^{-3} and 10^{-4} were inoculated into an already prepared Potato Dextrose Agar (PDA) plates which have been pre-treated with antibacterial (Chloramphenicol) to inhibit bacteria growth. A negative control was prepared by streaking a plate with sterile forceps. The inoculated plates were incubated at 28^{o}C for 48 – 72 hours. The fungi colonies that developed were sub-cultured on fresh plates, and this was repeated until pure colonies were obtained. A total of eight (8) colonies were isolated after repeated sub-culturing, these were transferred into slant bottles for storage and labeled as OT, AS, OS, GT, AT, GS, BT, and BS. The isolates were identified by morphological, biochemical and molecular characterization.

2.3. Culture Media Preparation

2.3.1. Potato Dextrose Agar (PDA) Preparation

To prepare a liter of PDA, 39 g of PDA powder was dissolve in 1 litre of distilled water contained in a conical flask following the manufacturers' instruction ((name manufacturer). Chloramphenicol, 62.5 mg was added into the medium, and the solution was thoroughly mixed and homogenized for 1-3 minutes on a heating coil. The solution was dispensed into appropriate 500 ml conical flasks and transferred into an autoclave for sterilization at 121^{o}C for 15 minutes. The media was set aside to cool to about 50^{o}C before

pouring aseptically under laminar flow cabinet into sterile Petri dishes. The plates were allowed to solidify then inverted and stored for further use.

2.3.2. Potato Dextrose Broth (PDB) Preparation

A 24 g of PDB powder was dissolved in a conical flask containing 1 liter of distilled water. This is in accordance with the manufacturers' instructions (name manufacturer). The mixture was agitated thoroughly and heated on a heating coil to homogenize it. The homogenized mixture was dispensed into appropriate 500 ml flasks and sterilized at 121ºC for 15 minutes.

2.3.3. Muller Hilton Agar (MHA) Preparation

19.5gof Muller Hilton Agar (MHA) was dissolved in 500ml distilled water according to the manufacturer's instruction. The mixture was homogenized for 1 minute then sterilized at 121ºC for 15minutes.

2.4. Submerged Fermentation (SMF) of Soil Fungi Isolate

Potato Dextrose Broth (PDB) was prepared in eight (8) 500 ml Erlenmeyer flask. These were used as media for the submerged fermentation systems. The eight fungi isolates were aseptically inoculated into the broth medium and incubated at 28ºC in a rotary incubator. The growth of the biomass and its pH was monitored for seven days for metabolite production.

2.5. Extraction of the Fungi Metabolite

Metabolites produced are intracellular therefore the extraction process involved the use of a solvent. The biomasses of the fungi were harvested after seven days by filtering the fermentation medium through sterile muslin cloth into a sterile Erlenmeyer bottle to obtain the biomass. Wet weight of the biomass were recorded before they were transferred into a mortal. The biomass was crushed and 20 ml absolute ethanol was added to the crushed biomass to extract the intracellular metabolite. The mixture was filtered using sterile muslin cloth, and the crude extract was transferred into plain EDTA bottle for storage.

2.6. Molecular Characterization

Fungal isolates grown on cellophane membrane placed on potatoes dextrose agar (PDA) were incubated at room temperature. The fungal isolates weighing between 50-100 mg (wet weight) were placed in a sterile ZR Bashing Lysis Tubes containing 750 µl of lysis buffer. The mixture was secured in a bead fitted 2 ml tube holder assembly and crushed at maximum speed for $\geq$ 5 minutes. The solution was swirled briefly in the ZR Bashing Bead^{TM}Lysis Tube then centrifuged at 10,000 rpm for one minute in a micro-centrifuge. Resultant supernatant of about 400 µl was transferred into a Zymo-Spin^{TM} IV Spin Filter (orange top) in a Collection Tube and centrifuged for one minute at a speed of 7,000 rpm. A 1200 µl measure of fungal DNA binding buffer was added to the filtrate in the collection tube then 800 µl of the mixture was transferred to a Zymo-Spin^{TM} IIC Column in a Collection Tube and centrifuged at a speed of 10,000 rpm for 1-minute period. The flow through was discarded then 200 µl DNA pre-wash buffer was added to the Zymo-Spin ^{TM} IIC Column in new Collection Tube, this was centrifuge at 10,000 rpm for 1 minute. After centrifugation, 500 ul fungal DNA wash buffer was added to a Zymo-Spin^{TM} IIC Column and centrifuged at 10,000 rpm for one minute. Zymo-Spin ^{TM} IIC Column was transferred to a clean 1.5 ml micro-centrifuge tube and 100 µl (35µl minimum). DNA elution buffer was added directly to the column matrix and centrifuged at 10,000 rpm for 30 seconds to elute the fungal DNA, which was then used for PCR and other downstream applications.

2.7. Determination of functional groups of molecules present in the metabolite using (FTIR)

To determine the functional groups present in the fungal metabolites, Fourier Transform Infrared Spectroscopy (FTIR) was used. This was done by measuring the metabolite-containing intracellular samples at different interval without moisture at ambient condition using a transmission node. The measurements were carried out in the range 4000–500cm^{-1} with an IR Affinity-1 Shimadzu spectrometer (Olk *et al.*, 1999; Mermut *et al.*, 2001).

2.8. Larvicidal Potentials of the metabolites

Different species of mosquito larvae were collected from pool of stagnant water. 0.1ml of the metabolite was inoculated into a beaker containing 100ml of water and the mosquito larvae were observed for over a period of time. The LD_{50} of the metabolites were noted for each of the metabolite synthesized from the fungal species. The time that it took the larvae to die and percentage larvicidal activity of the metabolites were determined. Data were obtained in triplicate and the average values were determined.

3. RESULTS AND DISCUSSION

3.1. Molecular Identification and Characterization of Soil Fungi

The extracted DNA of the fungal isolates were sequenced and the sequences were blasted in the NCBI Genbank and aligned with sequences of submitted organisms, this suggested the name of the isolates and a phylogenic tree was drawn, this is seen in Table 1, the phylogenic tree is shown inFigures 1-5. Evolutionary analyses were conducted using MEGA X. (Kumar *et al.*, 2018).

Table 1. *Molecular identity of fungal isolates using evolutionary relationships*

CODE	Molecular Identity
LAU-MAJ-ASF	*Penicillium verrucosum*
LAU-MAJ-GTF	*Aspergillus aculeatus 1*
LAU-MAJ-OTF	*Aspergillus aculeatus II*
LAU-MAJ-OSF	*Aspergillus carbonarius*
LAU-MAJ-GSF	*Talaromyces albobiverticillius*

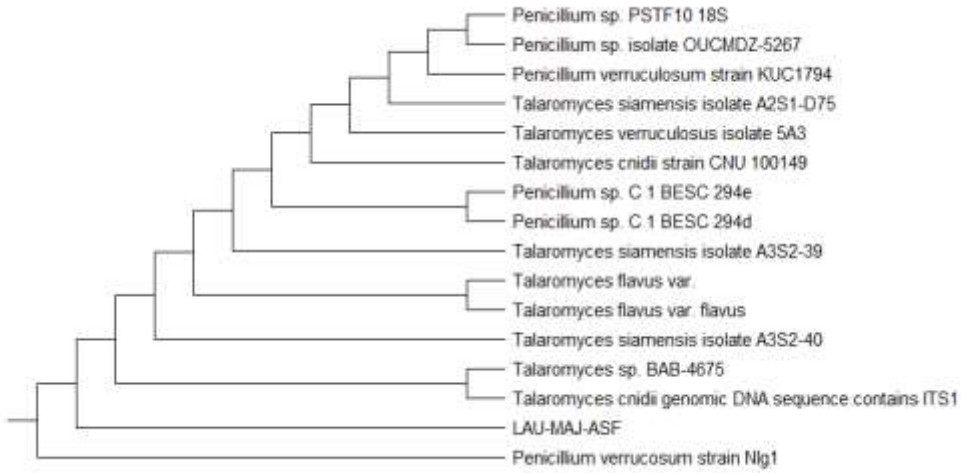

Figure 1. *Phylogenetic tree showing evolutionary relationships between LAU-MAJ-ASF and Pencillium verrucosum*

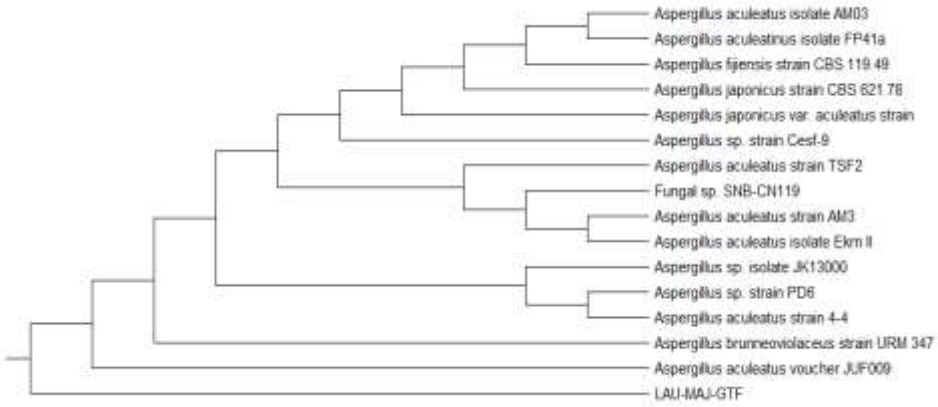

Figure 2. *Phylogenetic tree showing evolutionary relationships between LAU-MAJ-GTF and Aspergillus species*

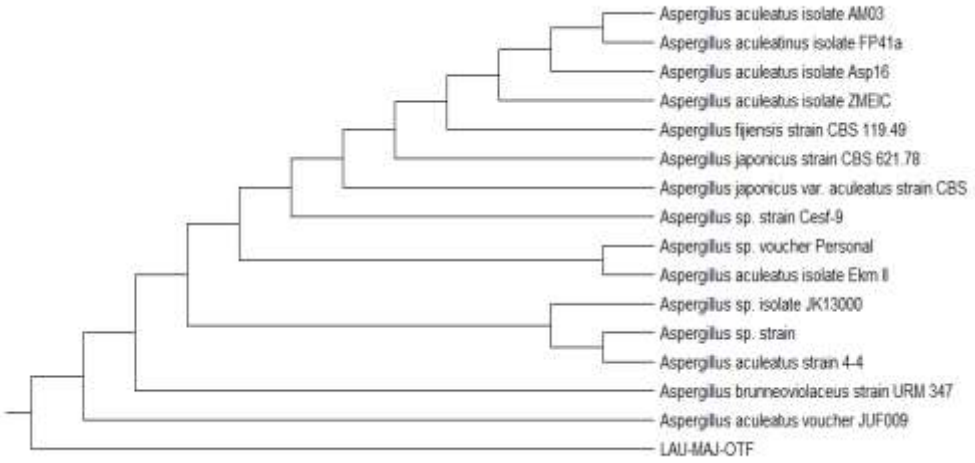

Figure 3. *Phylogenetic tree showing evolutionary relationships between LAU-MAJ-OTF and Aspergillus species*

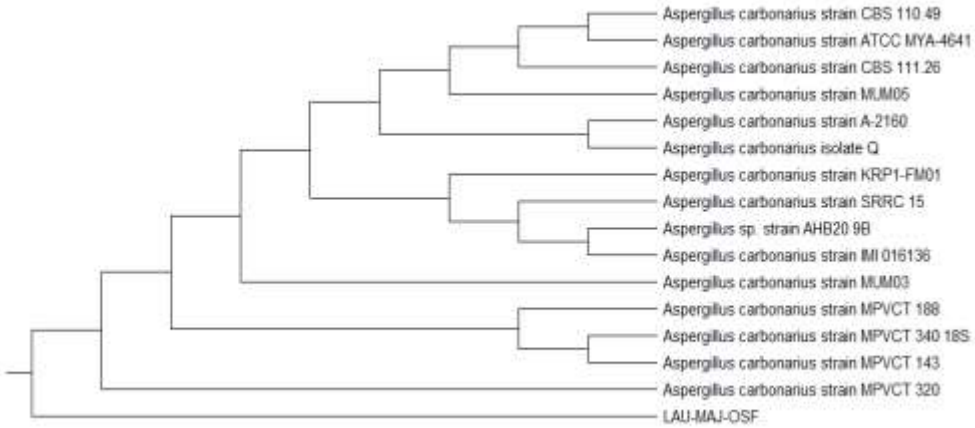

Figure 4. *Phylogenetic tree showing evolutionary relationships between LAU-MAJ-OSF and Aspergillus species*

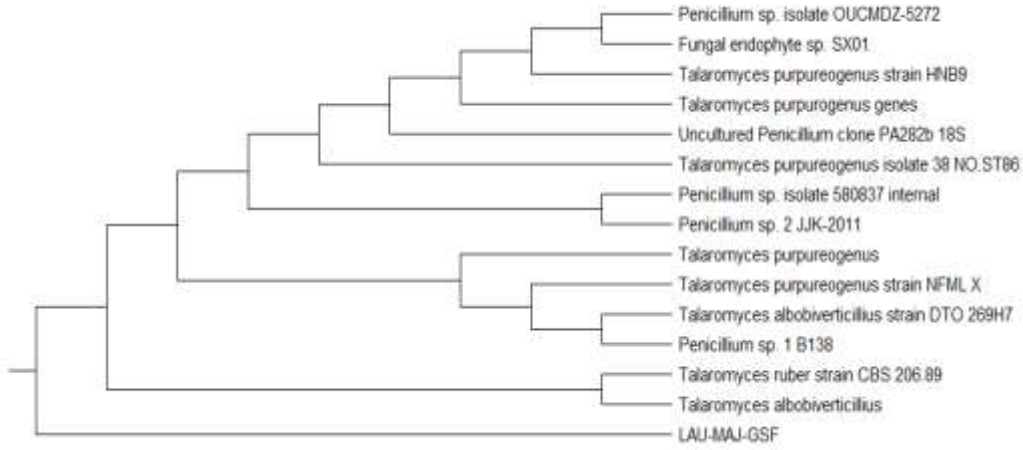

Figure 5. *Phylogenetic tree showing evolutionary relationships between LAU-MAJ-GSF and Talaromyces species.*

The evolutionary history was determined by the UPGMA method (*Sneathet al.*, 1973). The optimal tree with the sum of branch length = 0.61519578 is shown. Maximum Composite Likelihood method was sued to compute the evolutionary distances (Tamura*et al.*, 2004) they are in units of the number of base

substitutions per site. This analysis involved 15 to 16 nucleotide sequences. All ambiguous positions were taken out for each sequence pair (pairwise deletion option).

Fungi are ubiquitous, they are found in soil, on plant debris with the soil representing growth media for both non-pathogenic and pathogenic fungi (Devi *et al.,* 2012). Previous works showed that there are so much data on fungal flora of soil and a large number of the investigations focused on occurrence and isolation of keratinophilic fungi (Kachuei *et al.,* 2012; Shoham *et al.,*2013; Yazdanparast *et al.,* 2013).

Aspergillus have been used for industrial fermentation processes in the production of a number of important industrial enzymes, such as cellulases, hemicellulases and proteases all of which are broadly used in the food and feed industries.

3.2. FTIR Analysis of Metabolite

FT-IR Spectroscopy is an important method used in the identification and elucidation of functional groups of chemical constituents (Gopalakrishnan *et al.,* 2012; Kiran *et al.,* 2016). FTIR use infrared spectrum to simultaneously providing speed and accuracy in measuring whole a range of biological specimens (Griffiths *et al.,* 1986), FTIR have gained application as a requisite method for identifying bioactive molecule in compounds (Liu *et al.,* 2011).

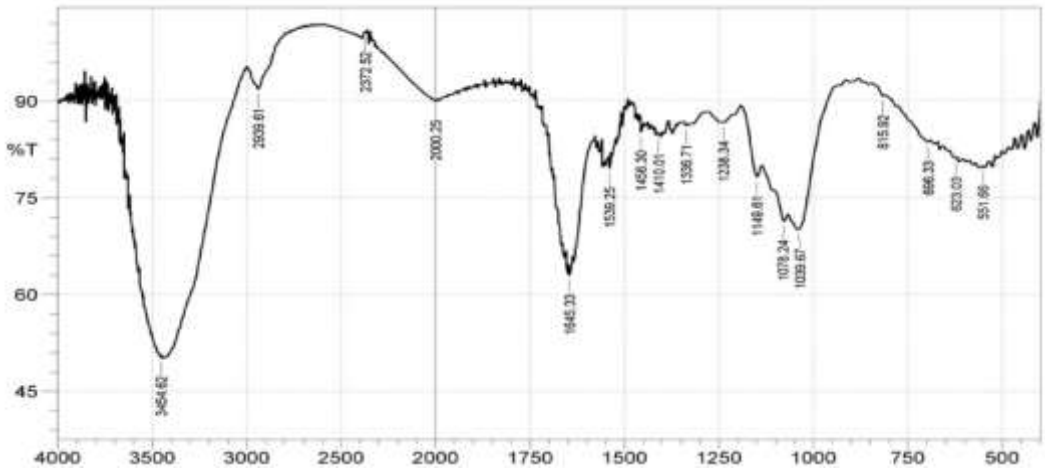

Figure 6: *FTIR spectra of Penicillum verrucosum metabolite (PV-m)*

Table 2. *Functional groups present in Penicillum verrucosum metabolite (PV-m)*

S/No	Bands (Cm^{-1})	Band Assignment/Possible functional groups
1	3454.62	OH Stretching
2	2939.61	CH Stretching
3	2372.52	C≡C Stretching
4	2000.25	C≡C, C≡N Stretching
5	1645.33	C=C Symmetric Stretching
6	1539.25	N-H Bending
7	1456.30	H-C-H Bending
8	1410.01	NO_2 Bending
9	1336.71	N=O Bending
10	1238.34	C-O Stretching
11	1149.61	C-O Stretching
12	1078.24	C-O Stretching
13	1039.67	C-O Stretching
14	815.92	Aromatic
15	696.33	Aromatic
16	623.03	Alcohol, OH out-of-plane Bending
17	551.66	C-I Stretching

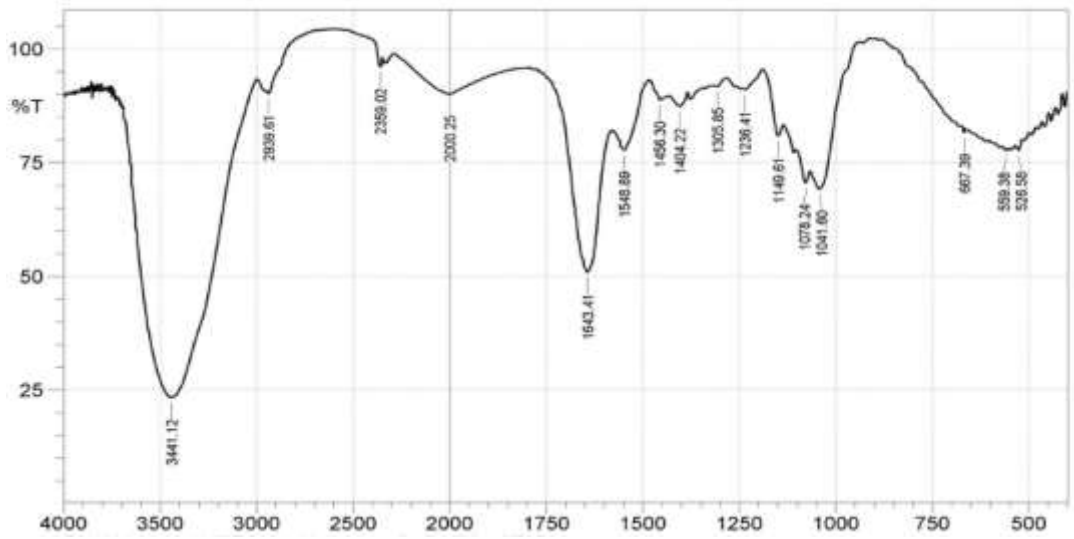

Figure 7: *FTIR spectra of Aspergillus aculeatus I metabolite (AA-m1)*

Table 3: *Functional groups present in Aspergillus aculeatus I metabolite (AA-m1)*

S/No	Band (Cm^{-1})	Band Assignment/Possible functional groups
1	3441.12	-OH Stretching
2	2939.61	-C-H Stretching
3	2359.02	C≡C Stretching
4	2000.25	C=C, C=N
5	1643.41	C-C=C Symmetric Stretching
6	1548.87	N-H Bending
7	1456.30	H-C-H Bending
8	1404.22	$-NO_2$
9	1305.85	N=O Bending
10	1236.41	C-O Stretching
11	1149.61	C-O Stretching
12	1078.24	C-O Stretching
13	1041.60	C-O Stretching
14	667.37	Alcohol, OH out of plane bend
15	559.38	C-I Stretching
16	526.58	C-I Stretching

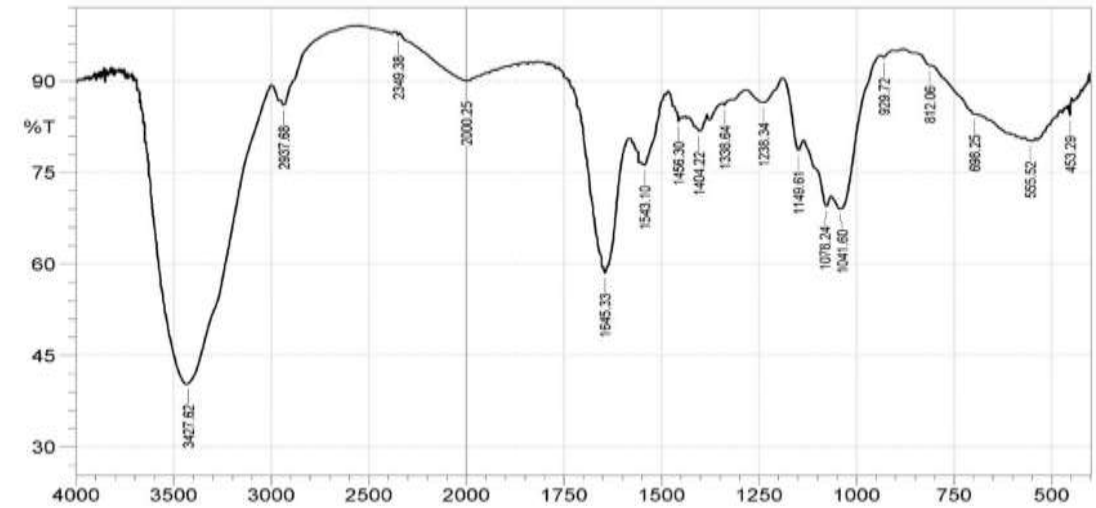

Figure 8. *FTIR spectra of Aspergillus aculeatus II metabolite (AA-m2)*

Table 4. *Functional groups present in Aspergillus aculeatus II metabolite (AA-m2)*

S/No	Bands (Cm^{-1})	Bands Assignment/Possible functional groups
1	3427.62	OH Stretching
2	2937.68	OH Stretching
3	2349.38	C≡C Alkyne Stretching
4	2000.25	C=C, C=N
5	1645.33	C-C=C Symmetric stretching
6	1543.10	N-H Bending
7	1456.30	H-C-H Bending
8	1404.22	NO_2
9	1338.64	N=O Bending
10	1238.34	C-O Stretching
11	1149.61	C-O Stretching
12	1078.24	C-O Stretching
13	1041.60	C-O Stretching
14	929.72	C-C Skeletal vibration
15	812.06	Aromatic ring
16	698.25	Aromatic ring
17	555.52	C-I Stretching
18	453.29	C-I Alkyl Halides

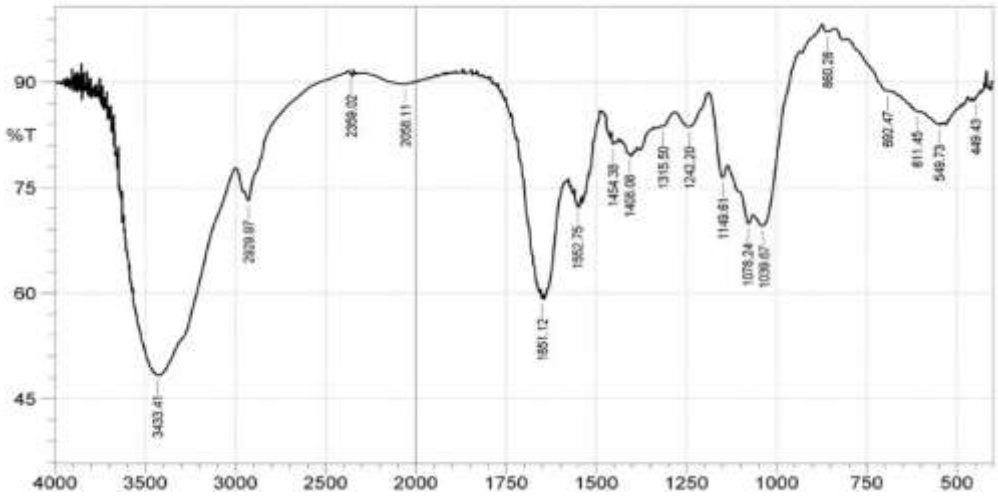

Figure 9: *FTIR spectra of Aspergillus carbonarius metabolite (AC-m)*

Table 5: *Functional groups present in Aspergillus carbonarius metabolite (AC-m)*

S/No	Band (Cm^{-1})	Band Assignment/Possible functional groups
1	3433.41	OH Stretching
2	2929.97	CH Stretching
3	2359.02	C≡C Alkyne Stretching
4	2058.11	C≡C, C≡N
5	1651.12	C-C=C Symmetric stretching
6	1552.75	Aromatic Ring
7	1454.38	H-C-H Bending
8	1408.08	$-NO_2$
9	1315.50	N=O Bending
10	1242.20	C-O Stretching
11	1149.61	C-O Stretching

12	1078.24	C-O Stretching
13	1039.67	C-O Stretching
14	860.28	Aromatic Ring
15	692.47	Aromatic Ring
16	611.45	Alcohol, OH out of plane ring
17	549.73	C-I Stretching
18	449.43	Alkyl Halide

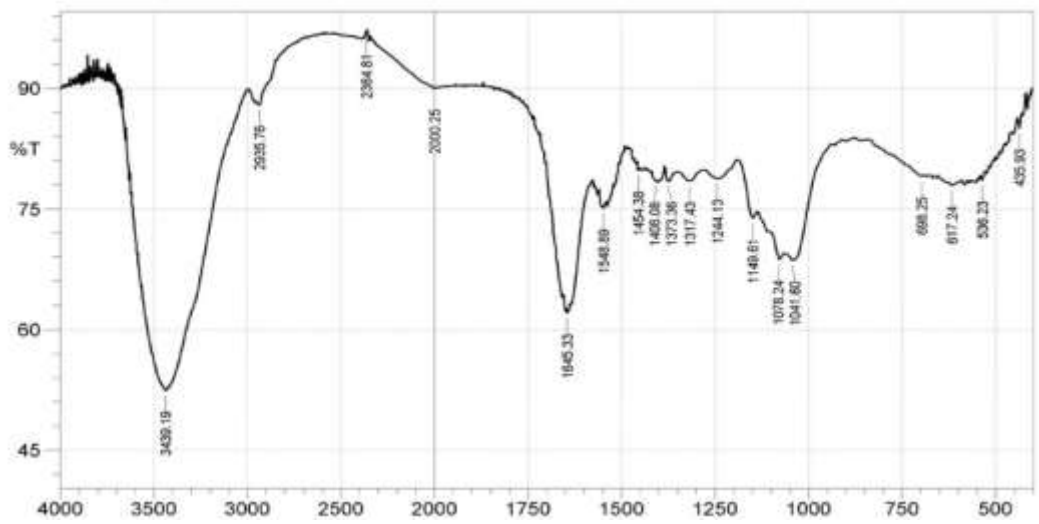

Figure 10. *FTIR spectra of Talaromyces albobiverticillius metabolite (TA-m)*

Table 6: *Functional groups present in Talaromyces albobiverticillius metabolite (TA-m)*

S/No	Band (Cm^{-1})	Band Assignment Possible functional groups
1	3439.19	OH Stretching
2	2935.76	CH Stretching
3	2364.81	C≡C Stretching
4	2000.25	C=C, C=N Stretching
5	1645.33	C-C=C Symmetric Stretching
6	1548.89	N-H Bending
7	1454.38	H-C-H Bending
8	1408.08	$N0_2$ Bending
9	1373.36	N=O Bending
10	1317.43	N=O Bending
11	1244.13	C-O Stretching
12	1149.61	C-O Stretching
13	1078.24	C-O Stretching
14	1041.60	C-O Stretching
15	698.25	Aromatic ring
16	617.24	Alcohol, OH out-of-plane Bend
17	536.23	C-I Stretching

The functional groups were separated on the basis of their peak ratio (Figures 6-10 and Table 2-6). The bands between 3400-3500 cm^{-1}shows the presence of an alcohol (O-H stretching) which is present in soil

fungi metabolites of *Aspergillus aculeatus, Aspergillus carbonarius, Penicillium verrucosum, Penicillium chrysogenum* and *Talaromyces albobiverticillius.* The C-H stretching from 2929.97-2939.61cm^{-1} indicate the alkane compound which is present in soil fungi metabolites of *Aspergillus aculeatus, Aspergillus carbonarius, Penicillium verrucosum, Penicillium chrysogenum, Talaromyces albobiverticillius.*The band between 2349.38- 2372.52cm^{-1} assigned to C≡C Stretching confirm the presence of alkyne compounds and the peak at 2605.92cm^{-1} was observed in the soil fungi metabolite *Aspergillus aculeatus,* but the functional group was unknown. The C≡C, C≡N stretches from 2000.25-2088.98cm^{-1} indicates the alkynes and methane nitriles are present in *Penicillium verrucosum, Penicillium chrysogenum, Aspergillus carbonarius and Aspergillus aculeatus*metabolite. The peak assigned2000.25cm^{-1} assigned to the C=C, C=N stretch which means alkene and are present in *Aspergillus aculeatus and Talaromyces albobiverticillius* metabolite.TheC-C=C symmetric stretches from 1651.12-1645.33cm^{-1} indicates alkane and alkene compounds. The peak at 1548.89-1539.25cm^{-1} assigned to N-H bend confirm primary amine in *Penicillium verrucosum, Penicillium chrysogenum, Aspergillus aculeatus* and *Talaromyces albobiverticillius* while the peak at 1552.75cm^{-1} indicate the presence of aromatic rings in *Aspergillus carbonarius.*. The band between1456.30-1454.38cm^{-1}assigned to H-C-H bend indicate the methyl compounds. The peak at 1410.01-1400.37 cm^{-1} assigned to -NO_2 bend indicates the nitro compounds. The band between 1373.36-1305.85cm^{-1} assigned to N=O bend indicates the nitro compounds. The C-O stretch from 1244.13-1236.11 cm^{-1} assigned to C-O stretch confirm diethyl ester compounds in *Penicillium verrucosum, Penicillium chrysogenum, Aspergillus carbonarius, Aspergillus aculeatus* while the peak at 1244.13cm^{-1} assigned to N=O bend indicates the nitro compounds in *Talaromyces albobiverticillius* metabolite. The C-O stretch from 1151.54-1039.67cm^{-1} assigned to C-O stretch confirms diethyl ester compounds. The peak at 929.72cm^{-1} given to C-C indicates skeletal vibration in *Aspergillus carbonarius* and *Aspergillusaculeatus*. The peaks at 898.68-696.33cm-1 indicate the presence of aromatic rings. The band between 669.33-611.45cm^{-1} indicate the presence of alcohol, -OH out-of-plane bend. The band between 559.38-459.07cm^{-1} assigned to C-I stretch indicate the presence alkyl halides compounds.

FTIR analysis of the metabolite of *Penicillium verrucosum*(PV-m),*Aspergillusaculeatus1* (AA-mI), *Aspergillus aculeatus II*(AA-m2), *Aspergillus carbonarius* (AC-m) and *Talaromyces albobiverticillius* (TA-m) revealed the presence of various chemical constituents. They showed different peaks, indicating transitions between vibration levels of different molecules. Similarly, the presence of different functional groups of compounds was identified with a variation in the peaks ratio (Kalaiselvi *et al.,* 2012). The FTIR spectral analyses showed important absorption bands at various wave number (cm^{-1}) indicating the presence of chemical structures. So, the FTIR spectroscopy revealed the absorption peaks of different functional groups of the chemical compounds present in the metabolite of the soil fungi metabolite. The FTIR spectrum was used to locate the functional groups of the active components present in the soil fungi metabolite on the basis of their peak values in the range of IR radiation. FTIR analysis results revealed the presence of alcohol, alkanes, alkenes, aromatics, carboxylic acids, phenol, amines, amides, nitromethane, nitrotoluene and halides in the metabolite soil fungi which may provide an insight in its use as a biopesticide for larvae. FTIR spectrum has shown to be an effective instrument for differentiating, classifying and discriminating closely related compounds (Ramamurthy *et al.,* 2007; Ragupath *et al.,* 2011; Zavoi *et al.,* 2011, *Csernatoni et al.,* 2013).

3.3. Larvicidal activities of fungal metabolites

The larvicidal potentials of the fungal metabolites of *Penicillium verrucosum* (PV-m), *Aspergillus aculeatus 1* (AA-mI), *Aspergillus aculeatus II* (AA-m2), *Aspergillus carbonarius* (AC-m) and *Talaromyces albobiverticillius* (TA-m) showed excellent but varying activities against the mosquito larvae. The lethal dose (LD_{50}) of the metabolite at time interval between 10sec to 4minute was within the range of 0.05-0.3g/ml (Table 7). The LD_{50} of the metabolite were observed to be 0.12g/ml, 0.05g/ml, 0.2g/ml, 0.34g/ml and 0.18g/ml for PV-m, AA-mI, AA-m2, AC-m and TA-m respectively. The larvicidal activity was seen to be within the range of 16-180% with the metabolite of *Talaromyces albobiverticillius* being the highest at concentration of 0.18g/ml at 60secs as recorded in Table 7 below. This was followed by the metabolite of *Aspergillus aculeatus II* (AA-m2) having a larvicidal activity of 170% at 50secs, the result of which similar to earlier reported work (Rana *et al.,* 2008). Therefore, it can be concluded that metabolite

of fungi isolated from soil habitats can kill larvae of mosquito and can be harnessed for bio-control of mosquitoes which are biological vectors of malaria parasites. These potential fungal isolates or their extracellular metabolites can further be used in integrated pest management programs.

Table 7: *Larvicidal activities of fungal metabolites*

Fungal Isolate	Fungal Metabolite	LD_{50}Conc (g/ml)	% Larvicidal activity	Time (secs.)
Penicillium verrucosum	PV-m	0.12	16	2400
Aspergillus aculeatus 1	AA-mI	0.05	16	1250
Aspergillus aculeatus II	AA-m2	0.20	170	50
Aspergillu scarbonarius	AC-m	0.34	20	50
Talaromyces albobiverticillius	TA-m	0.18	180	60

4. CONCLUSION

It has been established that the secondary metabolites of soil fungi contain compounds with potential use in curbing the menace of malaria by eliminating the vector at larva stage. Being a biologically produced metabolite, it is believed to be eco-friendly therefore pose no harm to the environment while it protects human life by eradicating mosquito larva which in turn control and reduce malaria infection among the populace hence ensuring the safety of human life.

Acknowledgements

The authors are indebted to the Tertiary Education Trust Fund (TETFUND) for providing them with materials to carry out this research. The authors are also appreciative to the management of LAUTECH, Ogbomoso for the provision of Laboratory space. Thank you.

Conflict of Interest

There is no conflict of interest among the authors.

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