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# Lavicidal potential of *Aspergillus terreus* conidial suspension and metabolites against anopheles mosquito

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**Abstract**
*Aspergillus terreus*, a common soil saprobe produce conidia that are pathogenic to *Anopheles gambiae* (s.s) and *Anopheles Coluzzii* larvae. The bioassay was conducted according to WHO-2005 protocol with slight modification. The $LC_{50}$s of conidia was found to be $6.43\times10^6$ and $4.55\times10^4$ conidia/ml at 24 and 48-hours respectively. Mycelium extracted metabolites of *A. Terreus* also showed promising larvicidal activity against *A. gambiae* S-form and *A. colluzzii*, ethyl-acetate extract is most active compared to ethanol and diethyl-ether extracts (LC50s; 664.73, 873.14 and 1509.12µg/ml respectively), but least active compared to chemical larvicides (Temephos and Malathion). GC-MS characterization of ethyl-acetate extract showed 7 major compounds viz. 9- Octadecanamide (Z) (16.59%), Octadecanamide (14.70%), 9- Octadecanamide, N, N- diethyl (11.80%), N- Isobutyl (6Z, 8E) decadienamide (8.37%), Lupenyl acetate (6.25%), 9, 12- Octadecadienoic acid, methyl ester (5.27%) and γ- Tocopherol (3.75%), and the effect of some or all of these compounds and perhaps some of the minor compounds might be responsible for the observed larvicidal activity of the extract.

**Keywords:** *Aspergillus terreus*, *Anopheles Gambiae* (s.s), *Anopheles Coluzzii.* larvicidal potential, GC-MS characterization

## 1. Introduction
*Anopheles gambiae* is the major vector of *plasmodium* parasite in Africa, female *Anopheles* mosquitoes ingest *plasmodium* parasite during blood meal from infected individual, and upon subsequent feeding, transmit it to its next host. The transmission of malaria parasite is most prevalent in developing countries, especially in tropical and subtropical areas, which form a considerable part of sub-Sahara Africa [1]. It is estimated that; about 36% of the world population are at risk of malaria infection, of which, Africa contribute about 90% [2].
Nigeria; the most populous country in Africa, is one of the Malaria endemic areas in the word, with about 50% of its population experiencing at least one episode of malaria annually [3], in addition, children and pregnant women experience higher incidences due to low immunity [4]. Despite the substantial amount of effort made in combating malaria, the disease is still one of the major country's health burdens, due to ever-growing resistance of *Anopheles* mosquitoes to insecticides; the world's prime choice curb nuisance biting by mosquitoes and their involvement in transmission of pathogens including *plasmodium* [5], which enable them to survive or avoid exposure to insecticides, thus leading to increased survival and biting rates [6].
The use biological control agents such as entomopathogenic fungi as alternative to synthetic chemical insecticides against vector insects is currently gaining a lot of scientific interest, because of their eco-friendly and host specific nature [7, 8]. These fungi are ubiquitous, easy to isolate and cultivate under laboratory condition and mostly host specific [9]. Conidial suspension and mycelium extracted metabolites of different entomopathogenic fungi, notably *M. anisopliae*, *B. bassiana*, *A. Niger*, *A. flavus* among others have been reported to exhibit promising larvicidal activity against mosquito larvae [1, 8, 10, 11, 12, 13], in view of this, current study focuses on evaluating larvicidal efficacy of conidiophores and mycelium bioactive metabolites of A. *terreus* on *Anopheles* mosquito.

## 2. Materials and Methods

### 2.1 Isolation, identification and culture of *Aspergillus terreus*

Soil sample was collected around Biochemistry department, Bayero University, Kano. The fungus was isolated using soil suspension and selective media procedures, and was identified at microbiology laboratory complex, Bayero University Kano, using macro and micro morphological characteristics [14, 15, 16]. Fungal spores were harvested from 10-days old PDA plates in 0.05% Tween-20 (used as negative control), and the spore suspensions were standardized at $2.2\times10^6$, $2.2\times10^5$, $2.2\times10^4$ and $2.2\times10^3$ conidia/ml.

### 2.2 Extraction and formulation of metabolites

A small portion of actively growing fungal mono-culture was aseptically inoculated into 7 flasks each containing 150ml Czapeks' dox broth (3g $NaNO_3$, 0.5g $MgSO_4.7H_2O$, 1g $K_2HPO_4$, 0.5g KCl and $FeSO_4.7H_2O$) and then incubated in an orbital shaker at 130rpm and 28 °C for 7 days. After the incubation, fungal mycelium was harvested and washed several times with distilled water, and the bioactive metabolites were then extracted using three different organic solvents; Ethanol, Diethyl-ether and Ethyl-acetate for another 7-days under the same condition. Crude extracts produced were concentrated using rotary evaporator at 45 °C and then air dried at room temperature. 100mg of dried residue of each extract was dissolved in 100mg of Dimethyl-sulfoxide (DMSO) (used as negative control) and 5 different concentrations ((100, 200, 300, 400 and 500µg/ml) were formulated.

### 2.3 Collection, Maintenance and Identification of *Anopheles* larvae

*Anopheles* larvae were collected from Auyo, Jigawa State of Nigeria; and were identified using morphological and behavioral characteristic [17]. The larvae were maintained at laboratory condition (28±2 °C and 60±15% R.H) for 24-hours before conducting bioassay. After the bioassay, 30 larvae were randomly selected and subjected to specie identification using *Anopheles gambiae* specific SINE 200 primers [18].

### 2.4 Bioassay

Bioassay was conducted according to WHO-2005 protocol with slight modification.

**Set-1:** Three replicates of 5 disposable cups each containing 10 healthy fourth instars of *Anopheles* larvae were prepared, of which, 4 were exposed to each of the formulated conidia concentrations ($2.2\times10^6$, $2.2\times10^5$, $2.2\times10^4$ and $2.2\times10^3$ conidia/ml) while 1 was exposed to 0.05% Tween-20 (Negative control). The number of dead larvae was counted for two days at 24-hours interval, and the percentage mortality was reported as average of the three replicates.

**Set-2:** 5 disposable cups each containing 10 healthy fourth instars of *Anopheles* larvae was prepared for each extract, and the larvae were exposed to five concentrations (100, 200, 300, 400 and 500µg/ml) of the extracted metabolites. 3 disposable cups each containing 10 larvae were prepared as control, 2 were exposed to chemical insecticides (Temephos-156.25µg/ml and Malathion- 781.25µg/ml) as positive controls, while the remaining 1 was exposed to DMSO as negative control. The whole experimental set-up was prepared in triplicate and the number of dead larvae was counted 24-hours post exposure. The percentage mortality was reported as the average of the three replicates.

### 2.5 Extract Characterization

Ethyl-acetate extract residue was dissolved in n-Hexane and spectral analysis of the bioactive compounds was carried-out through Gas-Chromatography Mass-Spectroscopy [19], and the bioactive compounds were identified by comparing their mass spectra and retention indices with those of NIST mass spectral library.

### 2.6 Data Analysis

Bioassay data was analyzed using Statistical Package of Social Sciences version 16.0; mean percentage mortality was determined using One-way Analysis of Variance (One-way ANOVA). Probit analysis was conducted to determine the medium lethal concentration ($LC_{50}$) of fungal conidiophores and extracted bioactive metabolites. All percentage mortalities were corrected based on mortality of negative control group using Abbott's formula [20].

## 3. Results and Discussion

### 3.1 Conidial Bioassay

Table 1 shows larvicidal activity of *A. terreus* conidial suspension against *Anopheles* larvae. The results showed that *A. terreus* conidia were pathogenic against *Anopheles* larvae, the mean percentage mortality was found to increase with increase in conidial concentration and exposure time. The highest mean percentage mortality (77.8%) was recorded at 48 hours exposure time at $2.2\times10^6$ conidia/ml, while the least mortality (13.3%) was recorded at $2.2\times10^3$ conidia/ml at 24-hours exposure time. The medium lethal concentrations were found to be $6.43\times10^6$ and $4.55\times10^4$ conidia/ml at 24 and 48 hours exposure time respectively. Statistical analysis shows that *A. terreus* conidia resulted in significant mortality as compared to negative control ($F= 20.250$, $df= 3, 11$ $p= 0.000$ at 24 and $F= 24.856$, $df= 3, 8$ $p=0.000$ at 48-hours).

**Table 1:** percentage mortality of *Anopheles* larvae exposed to different concentration of *A. Terreus* conidial suspension

| Exposure time | Concentration (conidia/ml) | Percentage Mortality ± S.E | $LC_{50}$ (conidia/ml) (LCL-UCL) |
|---|---|---|---|
| 24_Hours | $2.2\times10^6$ | 46.7 ± 3.33 | $6.43\times10^6$ |
| | $2.2\times10^5$ | 26.7 ± 3.33 | $(1.74\times10^6\text{-}7.43\times10^{7)}$ |
| | $2.2\times10^4$ | 16.7 ± 3.33 | |
| | $2.2\times10^3$ | 13.3 ± 3.33 | |
| 48_Hours | $2.2\times10^6$ | 77.8 ± 6.41 | $4.55\times10^4$ |
| | $2.2\times10^5$ | 59.3 + 3.70 | $(2.18\times10^4\text{-}9.01\times10^{4)}$ |
| | $2.2\times10^4$ | 44.4 ± 0.00 | |
| | $2.2\times10^3$ | 29.6 ± 3.70 | |

Negative control group (treated with 0.05% Tween-20) recorded 0% and 10% mortality at 24-hours and 48-hours respectively, (n=10)

At the time of compiling this result, no report evaluating the larvicidal activity of *A. terreus* conidia on *Anopheles* mosquito was available; however, many scientists have reported the potency of different entomopathogenic fungal conidial suspension against larvae of different mosquito species, relating the effect of conidial concentration and exposure time on mortality of the host insect, all of which, correspond favorably to the finding of this research [11, 8, 21, 22, 23].

### 3.2 Extract Bioassay

Table 2 shows larvicidal activity of mycelium extract of *A. terreus* against *Anopheles* larvae. The extracts were found to exhibit certain larvicidal activity against *Anopheles* larvae, irrespective of the extraction solvent. The mean percentage mortality also increases with increase in extract concentration. The highest mean percentage mortality (40%) was recorded upon treatment with 500µg/ml of ethyl-acetate extract, while both ethanol and diethyl-ether extracts resulted in 23.3% mortality at 500µg/ml concentration. Statistical analysis shows that ethyl-acetate extract resulted in significant mortality compared to negative control ($F= 25.625$, $df= 4, 14$ $P= 0.000$). The $LC_{50}$ values of the extracts were determined using probit analysis (Table 2), the result showed that the $LC_{50}$ of ethyl-acetate is lowest (664.73µg/ml) which indicates higher larvicidal efficiency compared to ethanol (873.14µg/ml) and diethyl-ether (1509.12).

**Table 2:** Larvicidal efficiency of *A. terreus* extracted metabolites against Anopheles mosquito larvae

| Solvent | Concentration (µg/ml) | Percentage mortality | $LC_{50}$ (µg/ml) (LCL-UCL) |
|---|---|---|---|
| Ethanol | 100 | 0.0[a] | 873.14 |
| | 200 | 3.3[a] | (656.56-1661.42) |
| | 300 | 13.3[a] | |
| | 400 | 16.7[a] | |
| | 500 | 23.3[a] | |
| Diethyl-ether | 100 | 3.3[a] | 1509.12 |
| | 200 | 6.7[a] | (843.71-9721.77) |
| | 300 | 10.0[b] | |
| | 400 | 16.7[a] | |
| | 500 | 23.3[a] | |
| Ethyl-acetate | 100 | 3.3[a] | 664.73 |
| | 200 | 6.7[a] | (522.87-1066.22) |
| | 300 | 23.3[a] | |
| | 400 | 26.7[a] | |
| | 500 | 40.0[b] | |

*S.E= a= 3.33, b= 0.00, Negative control group (treated with DMSO) records 0% mortality. Positive control group 1 and 2 (treated with Temephos (176.25µg/ml) and Malathion (781.25µg/ml) respectively) recorded 100% mortality, (n=10).

Reports on comparative study on larvicidal efficacy of mycelium; Ethyl-acetate and Methanolic extract of *A. Terreus* against *Anopheles stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti* larvae [24] and *Beauveria bassiana* against; *Aedes aegypti* larvae [25], also shows higher larvicidal activity of ethyl-acetate extracts, which correspond favorably to the findings of this research. Based on this result, ethyl-acetate extract, which exhibited better larvicidal activity, is regarded as the most active extract, and ethyl-acetate is considered a better solvent for extraction of *A. terreus*-mycelium bioactive secondary metabolite for control of *Anopheles* mosquito larvae.

Comparison between Ethyl-acetate extracted metabolite and standard chemical insecticide; Temephos and Malathion, shows that; at 156.25µg/ml, Temephos resulted 100% mortality after 24 hours, while the extract causes only 6% mortality at the same concentration while at 781.25µg/ml, Malathion resulted in %100 mortality 24 hours post exposure time, while the extract causes approximately 57% mortality at the same concentration as shown in Figure 1.

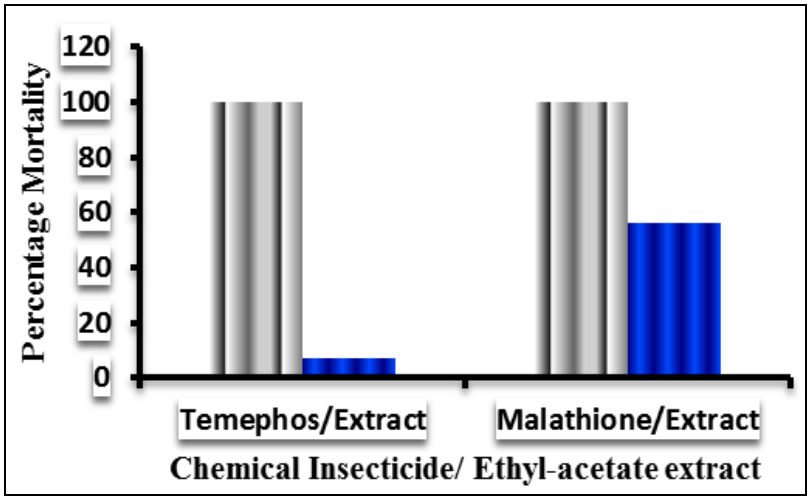


**Fig 1:** Comparison between larvicidal efficacies of Ethyl-acetate extracted metabolites of *A. terreus* and standard chemical larvicides

Bhan *et al.* [26] while evaluating the synergistic effect of *A. flavus*-ethyl-acetate mycelium extract on Temephos, reported a similar scenario, where the fungal extract was reported to have a medium lethal concentration of; 13.616, 14.347, and 10.027 ppm after 24, 48 and 72 hours, respectively, while Temephos recorded 0.0060, 0.0055 and 0.0042 ppm after 24, 48 and 72 hours, respectively. Also Vivekanandhan *et al.* [27] while evaluating synergistic effect of *Fusarium oxysporum* extract on Temephos, reported a similar scenario, where the result show a medium lethal concentration of the extract to be 109.2, 70.8 and 302.3µg/ml, while Temephos shows 50.7, 36.4 and 117.3µg/ml against larvae of *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* respectively.

### 3.3 Anopheles identification

Figure 2 shows gel electrophoreses of SINE200 PCR products. The result showed that the samples analyzed showed distinct bands corresponding to *Anopheles gambiae* S-Form and *Anopheles colluzzii*. 13% of the samples analyzed showed bands within approximately 200bp, and are grouped as *Anopheles gambiae* S-Form, while 73% are within approximately 500bp, and are grouped as *Anopheles colluzzii* [18], while the remaining failed reactions may be due to failed amplification or the larvae does not belong to *Anopheles gambiae* (Sensu Lato) group.

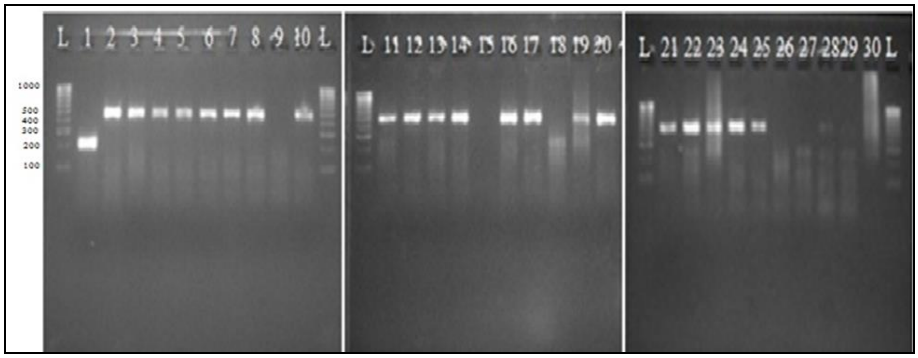


Figure 2: Agarose gel of SINE200 PCR Showing *Anopheles colluzzii* and *A. gambiae* S-form. Lane L- DNA ladder (1000bp), Lane 1, 18, 28 and 29- An. Gambiae S-form (249bp), Lane 2-8, 10, 11-14, 16, 17, 20 and 21-25- An. Colluzzi (479bp).

### 3.4 GC-MS characterization result

Table 3 showed the major compounds identified from GC-MS analysis oethyl-acetate extract. Twenty eight compounds were identified from GC-MS analysis of ethyl-acetate extract, of which, 7 most abundant compounds (Based on Area and Area percentage) are reported as shown below.

**Table 3:** The major bioactive compounds of *A. Terreus* ethyl-acetate extracted metabolites identified using GC-MS

| S/N | R. Time (Mins) | Molecular Weight (g/mol) | Compound | Formula |
|---|---|---|---|---|
| 1 | 13.608 | 281.5 | 9-Octadecenamide, (Z)- | $C_{18}H_{35}NO$ |
| 2 | 16.085 | 283.5 | Octadecanamide | $C_{18}H_{37}NO$ |
| 3 | 31.204 | 337.6 | 9-Octadecenamide, N,N-diethyl- | $C_{22}H_{43}NO$ |
| 4 | 12.288 | 223.4 | N-Isobutyl-(6Z,8E)-decadienamide | $C_{14}H_{25}NO$ |
| 5 | 26.065 | 468.4 | Lupenyl acetate | $C_{32}H_{52}O_2$ |
| 6 | 12.668 | 294.5 | 9,12-Octadecadienoic acid, methyl ester | $C_{19}H_{34}O_2$ |
| 7 | 16.760 | 416.7 | γ-Tocopherol | $C_{28}H_{48}O_2$ |

The compounds identified are relatively new compared to the compounds usually reported in the literature with larvicidal activities; this might be associated with the choice of the growth media. However, some of these compounds have been reported to exhibit certain biologically and pharmacologically important activities; Lupenyl-acetate has been reported to exhibit anti-microbial and anti-inflammatory properties [28], 9, 12-Octadecanoic acid, methyl ester is reported to exhibit antimicrobial activity [29], while isobutyl amides (N-Isobutyl-(6Z, 8E)-decadienamide) demonstrate anti-microbial and larvicidal activities [30], other identified compounds have not been reported to have biological activities. However, among the minor compound identified; 9, 12-Octadecanoic acid (Area- 127686, Area%- 0.13%) has been widely reported as important component of ethyl-acetate-mycelium extracted metabolites of *Beauveria bassiana*, exhibiting larvicidal activity against different insect larvae including mosquito [10, 25, 31].

## 4. Conclusion

*A. terreus* conidia and mycelium extracted metabolites exhibit promising larvicidal activity against *Anopheles gambiae* S-form and *Anopheles Colluzzii* larvae. Ethyl-acetate extracted metabolite is most active compared to ethanol and diethyl-ether extracts, but least active compared to standard chemical larvicides. GC-MS characterization of ethyl-acetate extract showed 7 major compounds and the effect of some or all of these compounds and perhaps some of the minor compounds might be responsible for the observed larvicidal activity of the extract.

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