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ORIGINAL ARTICLE

Synergistic effect of entomopathogenic fungus *Fusarium oxysporum* extract in combination with temephos against three major mosquito vectors

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ABSTRACT

Mosquito control using chemical insecticides is facing a major challenge due to development of insecticide resistance. Improving the efficiency of existing insecticides using synergistic secondary metabolites of biological origin is increasingly being researched. Herein, we evaluated the toxicity of *Fusarium oxysporum* extract alone and in binary combinations with temephos, on larvae and pupae of *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefaciatus*. *F. oxysporum* extract was characterized using TLC, FT-IR and GC-MS. After 24 h of exposure, the binary combination of temephos + *F. oxysporum* extract (1:1 ratio) was highly toxic to larvae of *An. stephensi* (LC_{50}: 35.927 µg/ml), *Ae. aegypti* (LC_{50}: 20.763 µg/ml) and *Cx. quinquefasciatus,* (LC_{50}: 51.199 µg/ml). For pupae LC_{50} values were 38.668, 26.394, and 72.086 µg/ml, respectively. Histology studies of mosquitoes exposed to *F. oxysporum* extract showed vacuolation in epithelium, as well as in adipose, and muscle tissues of larval midgut. Overall, our results show that the synergistic combination of temephos and *F. oxysporum* extract is highly effective to control mosquito young instars.

KEYWORDS

Fusarium oxysporum; temephos; Zika virus; FT-IR; GC-MS; histopathology

Introduction

Mosquitoes (Diptera: Culicidae) spread several diseases, which include malaria, dengue, yellow fever, filariasis, Japanese encephalitis and Zika virus. *Anopheles stephensi* is a major one of the leading vectors of malaria in India and other tropical and subtropical areas of the world [1]. *Aedes aegypti* is a day-biting mosquito responsible for the transmission of chikungunya, dengue fever, Rift Valley fever, Yellow fever and Zika virus, among others [2,3]. The Southern house mosquito, *Culex quinquefasciatus* a night biting mosquito responsible for the transmission of lymphatic filariasis, Japanese encephalitis and West Nile virus [4].

Vector control programs currently employ several synthetic insecticides, which include organophosphates, carbamates and pyrethroids, for the effective control of mosquito vectors [5,6]. However, the continuous overuse of a limited number of chemical pesticides result in fast development of multiple resistance in mosquitoes [7,8]. Currently, insecticide resistance is a major problem in Integrated Vector Management of mosquitoes. Besides, synthetic chemical insecticides also cause side effect to humans, non-target species and environmental pollution [5]. Several methods for the management of insecticide resistance in mosquitoes have been explored. Among these methods using synergistic combinations of bioactive metabolites from plants and fungi along with chemical insecticides is gaining importance [9], this can be attributed to the multiple modes of action of bioactive "green" metabolites. Research on bioactive metabolites from plants and fungal species showed relevant insecticidal properties [10–15].

Entomopathogenic fungi are among the most important species considered as potential biological control agents [16]. As significant number of them are preferred as they exhibit selective toxicity, do not persist, and do not need to be ingested [17]. There are several entomopathogenic fungi which have been tested for mosquito larval control. *Metarhizium anisopliae* has excellent toxic activity against *Ae. aegypti* [18], its secondary metabolites shows larvicidal and adulticidal activity against *An. stephensi* [19]. *Fusarium oxysporum* is a pathogenic soil-borne fungus distributed worldwide. Agricultural pests are naturally infected by *F. oxysporum* [20]. *F. oxysporum* strains can infect and kill a large range of hosts with agricultural and medically importance [21]. Synergism is the joint action where one component of the mixture has the effect of increasing the potency of the other component of the mixture, such that their combined effect is greater than the sum of their individual effects, as recently elucidated for binary mixtures of green biopesticides [22,23]. Besides, some insecticides

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have the capacity to increase stress and affect insect behavior, which may lead to improved performances of entomopathogens [24].

Temephos is an organophosphate insecticide which is recommended by WHO for the control of mosquito larvae [25]. The use of larvicides is highly regulated by WHO as there is an immediate danger of biomagnification of pesticides in food chain. There have been several reports showing that *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* have developed resistance to temephos. Hence in order to increase the effectiveness of temephos, a combination with fungal secondary metabolite might be ideal. Therefore, in the present study, we evaluated the synergistic mosquitocidal activity of *F. oxysporum* with temephos on larvae and pupae of three mosquito species, namely *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* . Chemical characterization *F. oxysporum* extract using TLC, FT-IR, and GC MS was carried out. In addition, midgut histology of the three mosquito species exposed to the fungal extract was investigated.

Materials and methods

Isolation and identification of F. oxysporum

Fusarium oxysporum MG574894.1 (Supplementary Data Figure S1) fungal strain was isolated from a dead *Spodoptera litura* in cotton field, Dharmapuri, Tamilnadu, India. *F. oxysporum* was characterized and sequenced. The sequenced data was submitted in National Center of Biotechnology Information (NCBI), the data base accession number is MG574894.1. Pure fungal strains were cultured in Potato Dextrose Agar (PDA) (Hi-media, India) for 7 days in the dark (28 ± 2 °C) inside an incubator at the Molecular Entomology Laboratory, Department of Biotechnology, Periyar University (Salem, India). *F. oxysporum* was primarily identified based on morphological characteristics colony, i.e. growth, presence or absence of aerial mycelium, colony colour, and colony pigment [26]. Mainly lacto phenol cotton blue was use for primary stain and the species was photographed under light microscope (Olympus CH-20i/India) at 40X magnification, (Supplementary Data Figure S2).

Preparation of the fungal broth

F. oxysporum spores were harvested by the flooding sterile distilled water with 0.05% Tween 80 (Sigma, USA). This fungal suspension was filtered by using sterile cloth for removal of fungal hyphae, conidia clumps and media debris. The filtered fungal suspension was estimated by using heamocytometer to count the spore concentration, then adjusted to 1×10^7 conidia/ml. The fungal broth was prepared with 250 ml of potato dextrose broth (PDB) in 500 ml Erlenmeyer flasks. 1×10^7 conidia/ml was inoculated in 250 ml of broth culture, then the broth was incubated (28 ± 2 °C) and placed in an orbital shaker (Rivotech-22038A2) at 130 rpm for 7 days, (Supplementary Data Figure S1b).

Extraction and concentration of F. oxysporum metabolites

After 7 days, the fungal biomass was filtered through Whatman no. 1 filter paper. Then, the biomass was washed with sterile distilled water for 3 times for the removal of media components. Then, 25 g of fungal biomass was transferred to a 500 ml Erlenmeyer flask containing 250 ml of ethyl acetate and extracted for 7–9 days using an orbital shaker (Rivotech-22038A2) with 130 rpm at 28 ± 2 °C. After incubation the fungal filtrate was filtered through Whatman No.1 filter paper.

The fungal crude filtrate was concentrated to eliminate ethyl acetate by using a rotary evaporator at 40 ± 5 °C (Superfit, India, Model R/150/01) under reduced pressure 23–27 mm hg at 40 ± 5 °C and the residue obtained was stored at room temperature.

Larval and pupal toxicity

An stephensi, *Ae. aegypti* and *Cx. quinquefasciatus* eggs was provided from the Institute of Vector Control Zoonoses (IVCZ) Banahalli, Tamil Nadu, India. The eggs hatched in distilled water and were maintained at 28 ± 2 °C and 60 ± 10 R.H. and 12:12 (L: D) photoperiod. We provided dog biscuits and yeast (3:1 ratio) as food source for the larvae.

The *F. oxysporum* extract, temephos and binary mixtures of *F. oxysporum* extract + temephos were tested against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* 4th instar larvae and pupae, following the method by WHO [27]. For each replicate, 25 mosquito larvae or pupae were stored in 300 ml plastic cups containing 250 ml of distilled water plus the desired concentration (i.e. 100, 200, 300, 400 and 500 µg/ml) of the selected treatment, each concentration was replicated three times. The control was 25 individuals exposed to the same dose of ethyl acetate. After 24 h, the mortality (%) was calculated and corrected with control mortality using the formula by Abbott [28].

Midgut histology

Treated and control *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* larvae were separately embedded in 3% formaldehyde solution for 2 h at 4 °C. The blocks were cooled at 27 °C for 3 h and cut into 8 µm thickness, with 1.3 mm ribbons, using a microtome (Leica, Germany). Cross-sectioned larval midgut sections were stained with Ehrlich's haematoxylin and eosin, after air drying the collected sections were viewed under a light microscope (Olympus-CH20i/India), with magnification at 100X and 400X.

Table 1. Larvicidal activity of *Fusarium oxysporum* fungal extract against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* 24 h post treatment.

Mosquito species (n^a = 375)	LC_{50} (95% LCL-UCL) (µg/ml)	LC_{90} (95% LCL-UCL) (µg/ml)	LC_{99} (95% LCL-UCL) (µg/ml)	χ^2 (df = 4)
Anopheles stephensi	109.248 (26.596–160.622)	471.871 (412.356–572.163)	767.502 (646.431–988.095)	0.152
Aedes aegypti	70.789 (8.635–119.862)	358.435 (316.461–422.297)	592.940 (507.507–742.851)	4.408
Culex quinquefasciatus	320.307 (276.749–367.614)	738.217 (628.484–940.646)	1078.922 (891.394–1431.661)	0.048

Notes:n^a = total number of mosquitoes used per each species, 25 per replicate, three replicates were carried out, five concentrations were tested, LC_{50} = lethal concentration killing 50% of exposed organisms, LC_{90} = lethal concentration killing 90% of exposed organisms, LCL = 95% lower confidence limits, UCL = 95% upper confidence limits, χ^2 = chi square (not significant, $p > 0.05$); df = degrees of freedom.

Table 2. Pupicidal activity of *Fusarium oxysporum* fungal extract against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* 24 h post treatment.

Mosquito species (n^a = 375)	LC_{50} (95% LCL-UCL) (µg/ml)	LC_{90} (95% LCL-UCL) (µg/ml)	LC_{99} (95% LCL-UCL) (µg/ml)	χ^2 (df = 4)
Anopheles stephensi	247.552 (168.241–306.155)	826.426 (662.296–1216.275)	1298.358 (998.426–2024.911)	0.107 n.s.
Aedes aegypti	197.989 (123.192–247.973)	663.423 (558.218–871.396)	1042.873 (844.107–1448.418)	0.454 n.s.
Culex quinquefasciatus	330.377 (276.730–393.263)	851.210 (693.877.1192.077)	1275.661 (1004.632–1872.644)	0.072 n.s.

Note: n^a = total number of mosquitoes used per each species, 25 per replicate, three replicates were carried out, five concentrations were tested, LC_{50} = lethal concentration killing 50% of exposed organisms, LC_{90} = lethal concentration killing 90% of exposed organisms, LCL = 95% lower confidence limits, UCL = 95% upper confidence limits, χ^2 = chi square (not significant, $p > 0.05$); df = degrees of freedom.

Table 3. Larvicidal activity of temephos against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* 24 h post treatment.

Mosquito species (n^a = 375)	LC_{50} (95% LCL-UCL) (µg/ml)	LC_{90} (95% LCL-UCL) (µg/ml)	LC_{99} (95% LCL-UCL) (µg/ml)	χ^2 (df = 4)
Anopheles stephensi	50.703 (44.643–106.710)	360.454 (316.010–429.602)	616.981 (519.594–783.284)	4.561 n.s.
Aedes aegypti	48.340 (26.114–92.328)	259.645 (227.412–305.259)	431.913 (370.118–542.836)	3.136 n.s.
Culex quinquefasciatus	146.133 (56.232–200.429)	590.233 (501.650–760.274)	952.290 (776.815–1304.658)	0.719 n.s.

Notes: n^a = total number of mosquitoes used per each species, 25 per replicate, three replicates were carried out, five concentrations were tested, LC_{50} = lethal concentration killing 50% of exposed organisms, LC_{90} = lethal concentration killing 90% of exposed organisms, LCL = 95% lower confidence limits, UCL = 95% upper confidence limits, χ^2 = chi square (not significant, $p > 0.05$); df = degrees of freedom.

Table 4. Pupicidal activity of temephos against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* 24 h post treatment.

Mosquito species (n^a = 375)	LC_{50} (95% LCL-UCL) (µg/ml)	LC_{90} (95% LCL-UCL) (µg/ml)	LC_{99} (95% LCL-UCL) (µg/ml)	χ^2 (df = 4)
Anopheles stephensi	57.454 (51.961–123.004)	455.939 (393.263–567.673)	780.808 (644.885–1048.881)	0.506 n.s.
Aedes aegypti	36.439 (25.801–92.338)	429.398 (370.336–533.228)	749.762 (618.963–1010.067)	0.371 n.s.
Culex quinquefasciatus	117.271 (47.564–162.710)	440.433 (388.772–523.229)	703.893 (601.028–883.063)	4.569 n.s.

Notes: n^a = total number of mosquitoes used per each species, 25 per replicate, three replicates were carried out, five concentrations were tested, LC_{50} = lethal concentration killing 50% of exposed organisms, LC_{90} = lethal concentration killing 90% of exposed organisms, LCL = 95% lower confidence limits, UCL = 95% upper confidence limits, χ^2 = chi square (not significant, $p > 0.05$); df = degrees of freedom.

Table 5. Larvicidal activity of a binary combination of *Fusarium oxysporum* fungal extract + temephos against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* 24 h post treatment.

Mosquito species (n^a = 375)	LC_{50} (95% LCL-UCL) (µg/ml)	LC_{90} (95% LCL-UCL) (µg/ml)	LC_{99} (95% LCL-UCL) (µg/ml)	χ^2 (df = 4)
Anopheles stephensi	35.927 (25.592–52.953)	187.092 (112.472–192.010)	287.243 (230.356–583.259)	0.812 n.s.
Aedes aegypti	20.763 (12.902–42.894)	130.353 (85.576–168.794)	253.552 (202.768–419.726)	0.506 n.s.
Culex quinquefasciatus	51.199 (29.819–95.321)	225.580 (181.947–277.315)	451.226 (372.079–622.215)	4.830 n.s.

Notes: n^a = total number of mosquitoes used per each species, 25 per replicate, three replicates were carried out, five concentrations were tested, LC_{50} = lethal concentration killing 50% of exposed organisms, LC_{90} = lethal concentration killing 90% of exposed organisms, LCL = 95% lower confidence limits, UCL = 95% upper confidence limits, χ^2 = chi square (not significant, $p > 0.05$); df = degrees of freedom.

Table 6. Pupicidal activity of a binary combination of *Fusarium oxysporum* fungal extract + temephos against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* 24 h post treatment.

Mosquito species (n^a = 375)	LC_{50} (95% LCL-UCL) (µg/ml)	LC_{90} (95% LCL-UCL) (µg/ml)	LC_{99} (95% LCL-UCL) (µg/ml)	χ^2 (df = 4)
Anopheles stephensi	38.668 (34.401–52.921)	195.048 (153.728–241.172)	385.588 (317.664–536.697)	1.668 n.s.
Aedes aegypti	26.394 (22.173–42.153)	92.331 (76.932–115.284)	246.274 (221.394–283.249)	0.771 n.s.
Culex quinquefasciatus	72.086 (60.932–98.302)	227.304 (190.058–275.098)	422.610 (353.125–563.736)	3.011 n.s.

Notes: n^a = total number of mosquitoes used per each species, 25 per replicate, three replicates were carried out, five concentrations were tested, LC_{50} = lethal concentration killing 50% of exposed organisms, LC_{90} = lethal concentration killing 90% of exposed organisms, LCL = 95% lower confidence limits, UCL = 95% upper confidence limits, χ^2 = chi square (not significant, $p > 0.05$); df = degrees of freedom.

Thin layer chromatography (TLC)

F. oxysporum crude metabolites were separated by using thin layer chromatography (TLC) with silica gel 60 size mesh coating on 20 mm × 20 mm glass slide. The chloroform : methanol solvent system was used as mobile phase, with different mobile phase solvent ratios (i.e. 10:0; 9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9; 0:10), at 26 ± 2 °C and 40% R.H.).

Fourier transformed infrared (FT-IR) spectroscopy

F. oxysporum extract was dried, and powder was subjected to FT-IR spectroscopy. Characterization involved FT-IR analysis of the dried powder of ethyl acetate extract by scanning it in the range 500– 4000 cm^{-1} at a resolution of 4 cm^{-1}. These measurements were carried out on a Bruker Optics (Germany) Tensor 27 model in the diffuse reflectance mode operating at a resolution value of 0.4 cm^{-1} in KBr pellets. The pellets were later subjected to FT-IR spectroscopy measurements.

GC-MS analysis

Clarus 680 was used in the analysis employing a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethyl polysiloxane, 30 m × 0.25 mm ID × 250 µm df and the components were separated using helium as carrier gas, at a constant flow of 1 ml/min. The injector temperature was set at 260 °C during the chromatographic run. 1 µl of extract sample was injected into the instrument, the oven temperature was as follows: 60 °C (2 min), followed by 300 °C at the rate of 10 °C min^{-1} and then 300 °C, where it was held for 6 min. The mass detector conditions were: transfer line temperature 240 °C, ion source temperature 240 °C, and ionization mode electron impact at 70 eV, a scan

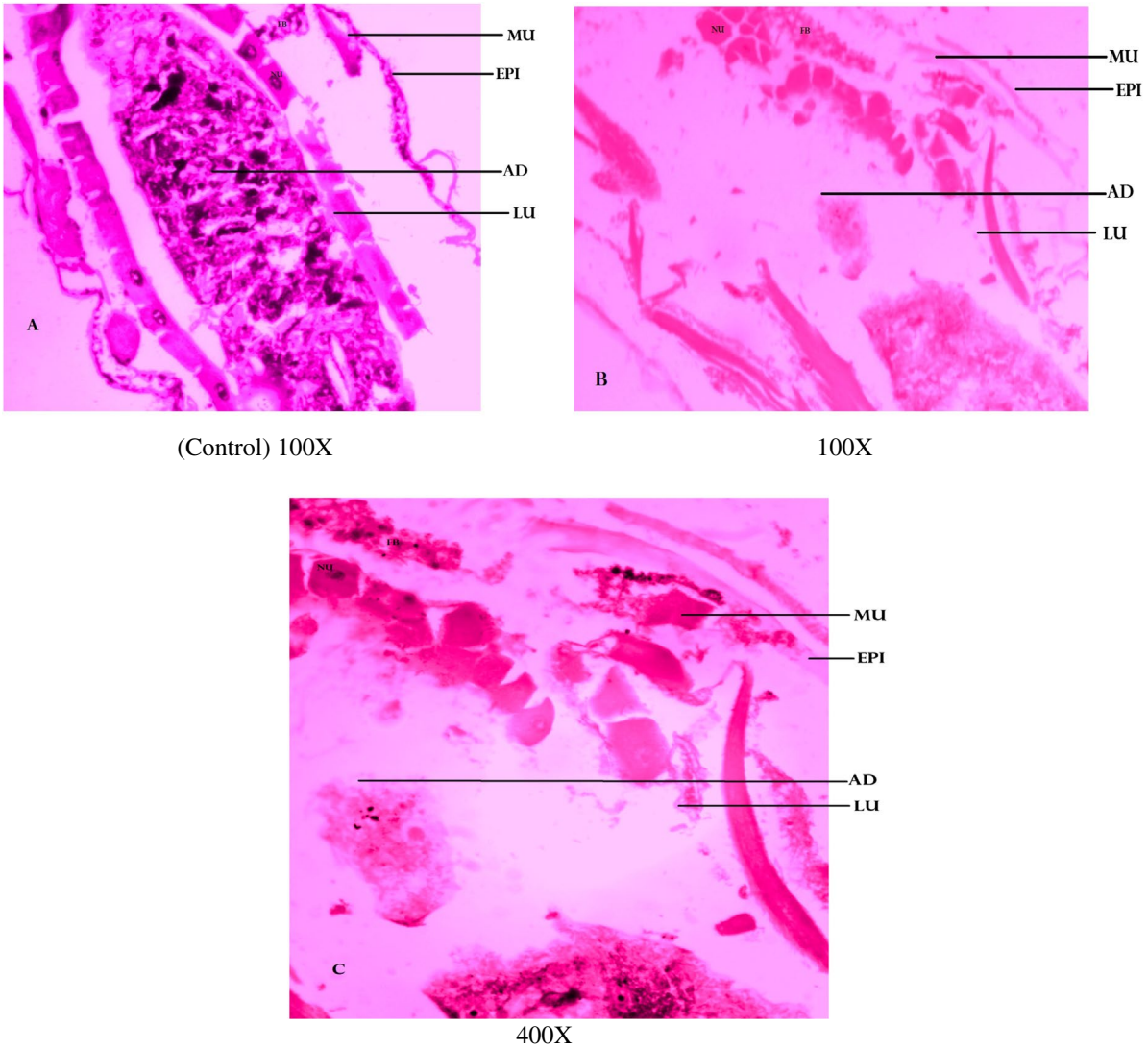

Figure 1. Cross sections of 4th instar larvae of *Aedes aegypti* treated or untreated with the *Fusarium oxysporum* extract. (A) Control was compared with (B) 100X and (C) 400X treated larval tissues, showing vacuolated gut epithelium (epi), gut lumen (lu), adipose tissue (ad) muscles (mu) nucleus (nu) and fat body (fb).

Notes: Larval mid-gut section was stained with Ehrlich's haematoxylin, stained mid-gut tissues were viewed and photographed under light microscope at 100X and 400X magnification.

time 0.2 s and scan interval of 0.1 s. Considering fragments from 40 to 600 Da, the spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

Statistical analysis

Ae. aegypti, *An. stephensi* and *Cx. quinquefasciatus* larval and pupal mortality data were subjected to analysis of variance (ANOVA) of arcsine square root transformed mortality percentages). The lethal concentrations required to kill 50, 90 and 99% (LC_{50}, LC_{90}, and LC_{90}) of larvae and pupae 24 h post-treatment were calculated by probit analysis with a reliability interval of 95% using the SPSS 16 software.

Results

Larval and pupal toxicity

Larvicidal activity of *F. oxysporum* extract was investigated on *An. stephensi*, *Ae. aegypti* and *Cx. quinquefaciatus*. 24 h post-treatment, LC_{50} values achieved by the *F. oxysporum* extract were 70.789 µg/ml for *Ae. aegypti* larvae, while *An. stephensi* and *Cx. quinquefasciatus* larvae was relatively more tolerant to the fungal extract (LC_{50} 109.248 and 320.307 µg/ml, respectively), LC_{90} and LC_{99} are detailed in Table 1. LC_{50} values against pupae were 247.552, 197.989 and 330.377 µg/ml, respectively. LC_{90} and LC_{99} are shown in Table 2.

Temephos was investigated alone against larvae of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* were 50.703, 48.340 and 146.133 µg/ml, respectively (Table 3). LC_{50} values against pupae were 57.454, 36.439 and 117.271 µg/ml, respectively (Table 4).

24 h after exposure to binary combination of temephos and *F. oxysporum* extract (1:1 ratio) resulted in lower LC_{50} values on *An. stephensi* (35.927 µg/ml), *Ae. aegypti* (20.763 µg/ml) and *Cx. quinquefasciatus* (51.199 µg/ml) (Table 5), if compared to the products tested alone. Furthermore, LC_{50} values on pupae were 38.668, 26.394 and 72.086 µg/ml, respectively (Table 6).

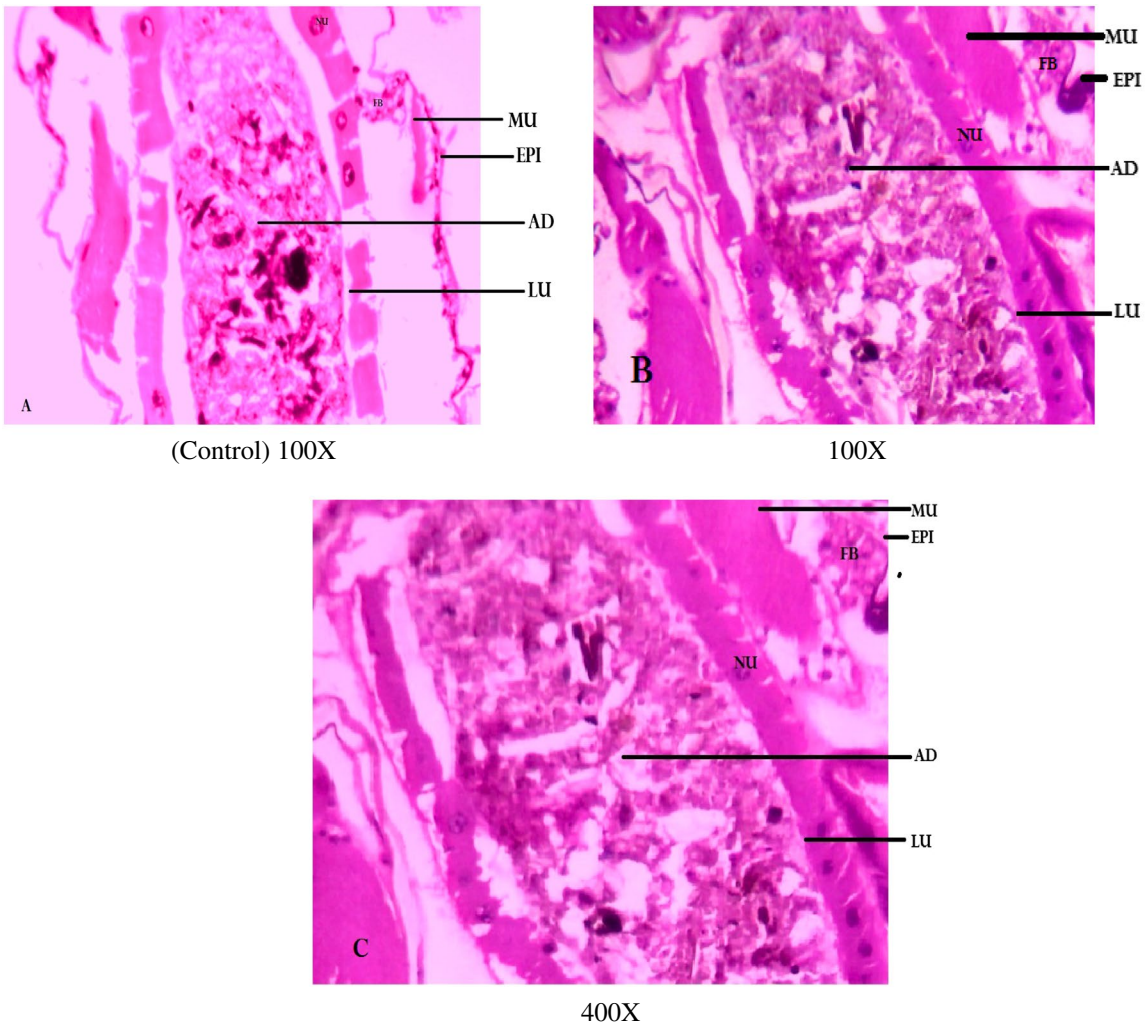

Figure 2. Cross sections of 4th instar larvae of *Anopheles stephensi* treated or untreated with *Fusarium oxysporum* extract. (A) Control was compared with (B) 100X and (C) 400X treated larval tissues, showing vacuolated gut epithelium (epi), gut lumen (lu), adipose tissue (ad) muscles (mu) nucleus (nu) and fat body (fb).

Notes: Larval mid-gut section was stained with Ehrlich's haematoxylin, stained mid-gut tissues were viewed and photographed under light microscope at 100X and 400X magnification.

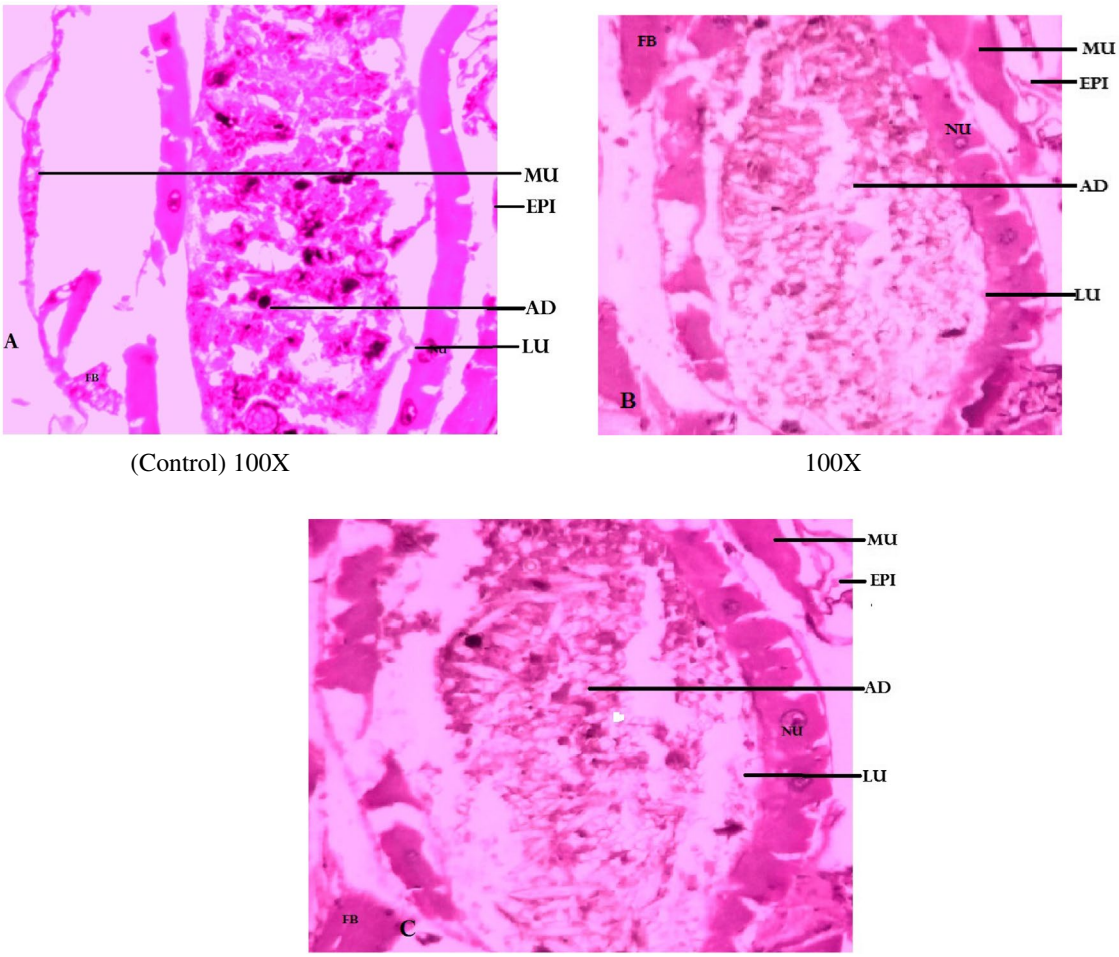

Figure 3. Cross sections of 4th instar larvae of *Culex quinquefasciatus* treated and untreated *Fusarium oxysporum* extract. (A) Control was compared with (B) 100X and (C) 400X treated larval tissues, showing vacuolated gut epithelium (epi), gut lumen (lu), adipose tissue (ad) muscles (mu) nucleus (nu) and fat body (fb).
Notes: Larval mid-gut section was stained with Ehrlich's haematoxylin, stained mid-gut tissues were viewed and photographed under light microscope at 100X and 400X magnification.

Histology studies on mosquito larvae

Ae. aegypti larvae were highly affected by the exposure to the fungal extract alone. Cross sections of fourth instar larvae of *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus* treated with *F. oxysporum* extract highlighted the damage done by the extract in midgut tissues. *Ae. aegypti* larval midgut showed the highest damage with distinct vacuolation in midgut epithelial cells, adipose tissue and muscles; similar damage to a lesser degree was observed in *Cx. quinquefasciatus* and *An. stephensi* larvae. These changes were not detected in control larvae at 100X magnification (Figure 1–3).

Chemical characterization of the F. oxysporum extract

In TLC assays, the crude ethyl acetate extract of *F. oxysporum* showed a spot with R_f value of 0.5555 (Figure 4); the distance travelled by solute was 2.5 cm, while the distance travelled by the solvent was 4.5 cm.

GC-MS of *F. oxysporum* extract showed the presence of various compounds (Figure 5), which include carbonic acid, bis (1-methylethyl) ester (9.806%), 2-propanol, 1-(1-methylethoxy)- (10.977%), 4-heptanol, 2-methyl- (19.955%), and 4 h-1-benzopyran-4-one, 5,6,7,8-tetrahydro-3-hydroxy-2-methyl- (21.451%) (Table 7).

The FT-IR spectrum showed main peaks at 3390.86, 3253.91, 2954.95, 1707.00 and 1257.57 cm^{-1} (Figure 6); the broad peak at 3390.86 cm^{-1} was assigned to wagging vibration of N–H stretching, while the broad peak at 3253.91 cm^{-1} was assigned to N–H stretching, and the sharp peak 1707.00 cm^{-1} outlined C=C bending (Table 8).

Discussion

The effective control of mosquito vectors with synthetic insecticides is a major concern for human health and the environment [7]. This study shows the efficacy of *F. oxysporum* extract conjugated with temephos as a potential mosquitocidal combination against three major mosquito vectors. Fungal metabolites known to

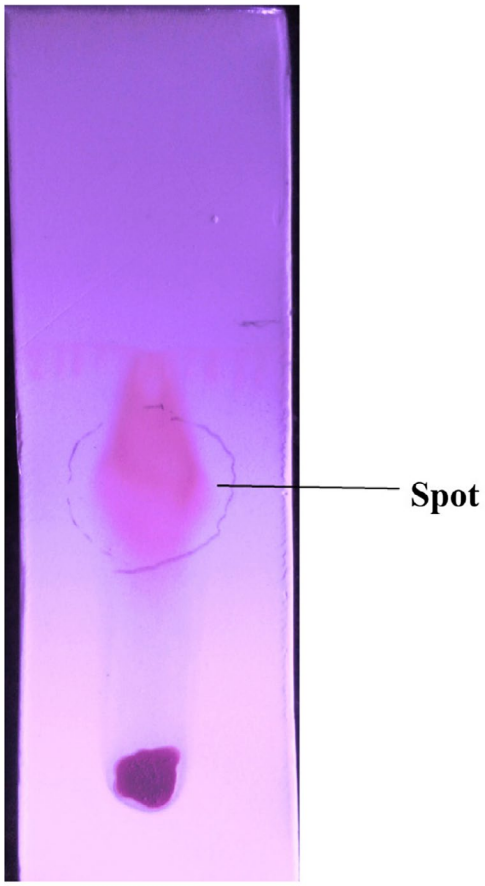

Figure 4. Thin layer chromatography (TLC) of the *Fusarium oxysporum* extract showing a major central spot.
Notes: The chloroform : methanol solvent system was used as mobile phase, with different mobile phase solvent ratios (i.e. 10:0; 9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9; 0:10), at 26 ± 2 ºC and 40% R.H.).

possess insecticidal activity [29–32]. *Streptomyces* and *Actinobacteria* secondary metabolites show remarkable insecticidal activity against *An. stephensi* [10,33], while the ethyl acetate extracts of marine *Actinobacteria*, and *Streptomyces* are highly toxic to larvae of *An. stephensi* and *Cx. tritaeniorhynchus* 24 h post-treatment [34].

Combinations of plant extracts with chemical insecticides have been shown to increase insecticidal activity in several instances [35,25]. Similar synergistic effects have been detected also testing binary mixtures of mosquitocidal essential oils [22,23] and selected compounds [36]. In our study, the extract of *F. oxysporum* in binary combination with temephos showed a synergistic action against larvae and pupae of all the tested mosquito species, when compared with either temephos alone or with the fungal extract alone.

Our findings on the use of a binary combination of chemical insecticide and fungal secondary metabolites support earlier studies. Indeed, the combined use of *Beauveria bassiana* and *Metarhizium anisopliae* metabolites with permethrin has been found to be very effective to kill mosquito larvae [37]. Increased toxicity of *M. anisopliae* metabolites in combination with neem oil against *Anopheles gambiae* and *C. quinquefasciatus* adults has been also reported [38]. Concering tests on other insect species, *B. bassiana* secondary metabolites combined with synthetic insecticides abamectin, triflumuron and carbaryl showed boosted insecticidal activity against the Colorado potato beetle [39]. Furthermore, the combination of chemical insecticide imidacloprid with *B. bassiana* and *M. anisopliae* enhanced the toxic effect and reduce survival *Diaprepes abbreviatus* larvae [40]. Combinations of imidacloprid with secondary metabolites from *M. anisopliae* show remarkable toxicity against German cockroaches [41]. *Lecanicillium muscarium* metabolites in combination with either imidacloprid, buprofezin, diflubenzuron, and nicotine, were found to be effective for controlling adults of *Bemisia tabaci* [42].

Similiarly, *B. bassiana* and *M. anisopliae* conidia and their secondary metabolites in combination with sub-lethal doses of imidacloprid were effective against adults of *Attasexdens rubropilosa* [24]. Combination of *M. anisopliae* secondary metabolites with synthetic pesticides organophosphate and diflubenzuron achieved a joint toxic action against larvae of *Anomala cuprea* [43].

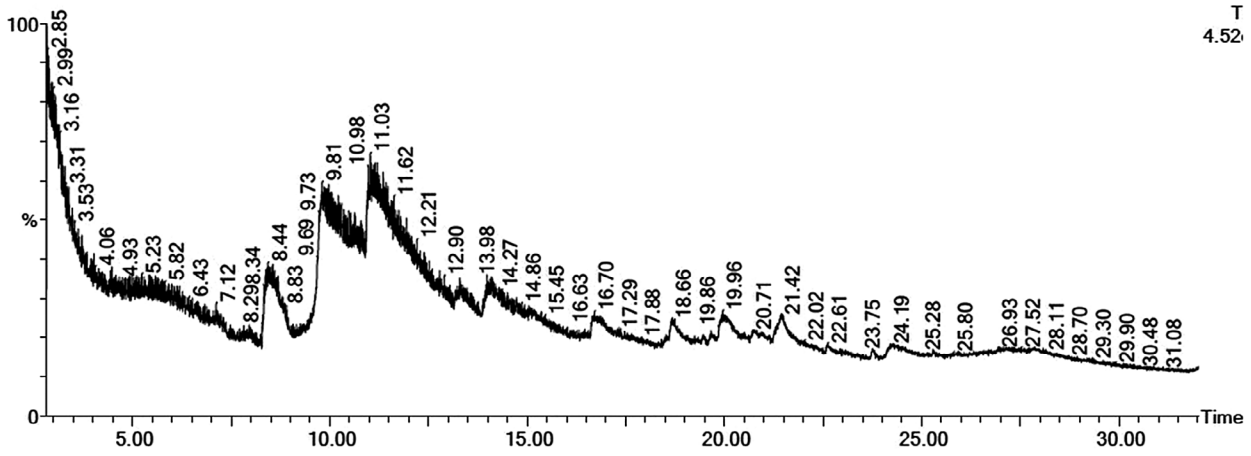

Figure 5. GC-MS analysis of the *Fusarium oxysporum* extract.
Notes: Oven initial temp 60 °C for 2 min, ramp 10 °C min^{-1} to 300 °C, hold 6 min, inject auto = 250 °C, volume = 1 µl, split = 10:1, carrier gas = He, solvent delay = 2.00 min, transfer temp = 240 °C, source temp = 240 °C, scan 50 to 600 Da, column 300 m~250 µm.

Table 7. Major compounds identified in the ethyl acetate extract of *Fusarium oxysporum*.

No.	R.T.	Compound name	Molecular Weight	Formula	Area (%)	Bioactivity	References
1	9.806	Carbonic acid, bis (1- methylethyl) ester	146	C7H14O3	3.375	Pesticide	[46]
2	10.977	2-Propanol, 1-(1-Methylethoxy)-	118	C6H14O2	1.893	Unknown	–
3	11.027	2-(2-Hydroxyethoxy)Ethyl Acetate	148	C6H12O4	1.699	Unknown	–
4	11.202	Diisopropyl Ether	148	C6H12O4	2.069	Unknown	–
5	18.740	Benzenemethanol, Alpha.-(Trichloromethyl)-, Acetate	266	C10H9O2Cl3	1.748	Unknown	–
6	19.955	4-Heptanol, 2-Methyl	130	C8H18O	3.745	Unknown	–
7	21.451	4-1-Benzopyran-4-One, 5,6,7,8-Tetrahydro-3-Hydroxy-2-Methyl	180	C10H12O3	4.004	Antimicrobial and larvicidal activity	[47]

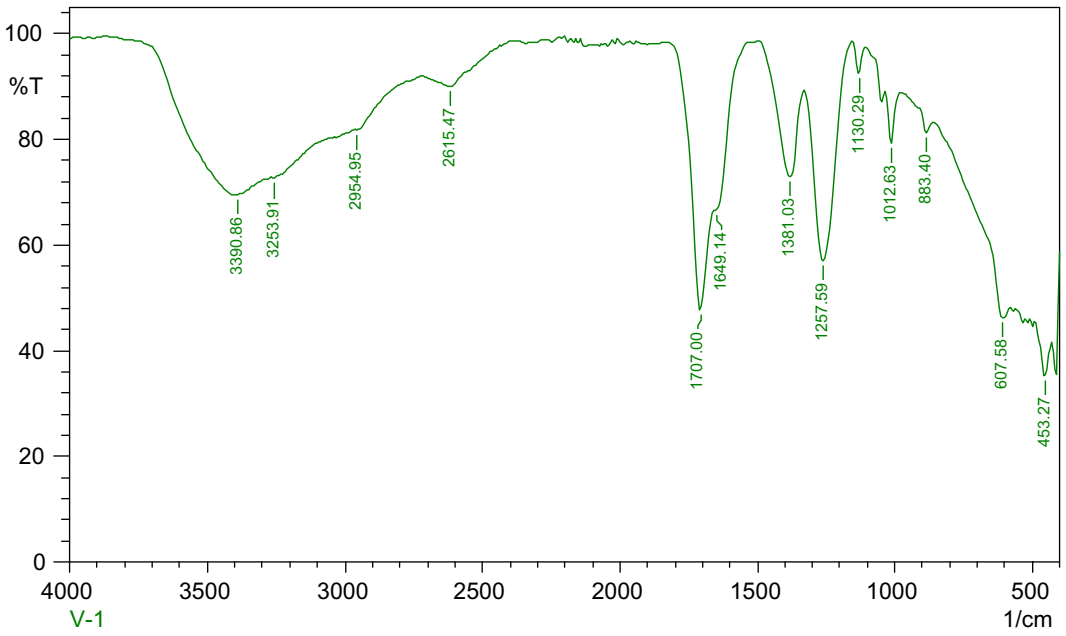

Figure 6. FT-IR spectrum of the *Fusarium oxysporum* extract.

A major advantage of using binary mixtures with green metabolites as insecticides is the reduction of the amount of chemical insecticide used, reducing environmental pollution and the negative impact on non-target organisms. Second, and most importantly, natural product metabolites exert their toxicity through multiple mechanisms of action, thus reducing the possibility of resistance development in target mosquitoes [44]. In this binary combination of chemical insecticide and fungal extract, the two products exert toxicity in mosquitoes through different mechanisms of actions; the fungal extract induces mechanical stress on mosquito young instars and also contains toxic molecules detailed above, while the chemical insecticide affects the central nervous system, through inhibition of cholinesterase [21]. GC-MS data shows the presence of carbonic acid, bis (1-methylethyl) ester, 2-propanol, 1-(1-methylethoxy)-, 4-heptanol, 2-methyl-, and 4 h-1-benzopyran-4-one, 5,6,7,8-tetrahydro-3-hydroxy-2-methyl- as main compounds. Notably, it has been earlier elucidated that carbonic acid, bis (1-methylethyl) ester, 4-heptanol and 1–4-benzopyram-4-one-5, 6, 7, 8-tetrahydro-3-hydroxy-2-methyl possess insecticidal activity against mosquitoes [45], allowing us to argue that these three constituents can be actively involved in the observed synergistic effect highlighted in the present work.

Table 8. FT-IR spectroscopy of *Fusarium oxysporum* mycelial extract.

Wave number (cm^{-1})	Peak assignment	Visible intensity	Functional group
3390.86	N–H stretching	Broad peak	Amine group
3253.91	N–H stretching	Broad peak	Aliphatic group
2954.95	C–H bending	Broad peak	Alkane group
2615.47	C–H stretching	Broad peak	Ether group
1707.00	C=C bending	Medium	Alkane group
1649.14	C=O Stretching	Medium	Ether group
1381.03	C–H bending	Sharp	Alkane group
1257.59	C–O Stretching	Sharp	Alkane, Ether group
1130.29	S=O Stretching	Sharp	Sulfone group
1012.63	C–O Stretching	Sharp	Alkane
883.40	C=C bending	Medium	Alkane group
607.58	C–Br stretching	Medium	Ether group

Overall, our result shed light on the use of a cheap fungal extract from fungus easy to culture in combination with temephos, to achieve synergistic effects in larvicidal and pupicidal treatments for the control of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*. Therefore, we suggest that the selection of appropriate combinations of currently marketed insecticides and pathogenic fungal

extracts offers the prospect for effective mosquito control, reducing costs and resistance problems derived from the continuous overuse of conventional insecticides.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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