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Chemical constituents of thermal stress induced *Ganoderma applantum* (Per.) secondary metabolites on larvae of *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus* and histopathological effects in mosquito larvae

C. Logeswaran, P. Vivekanandhan, M.S. Shivakumar*

Molecular Entomology Laboratory, Department of Biotechnology, Periyar University, Salem, 636 011, Tamil Nadu, India

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ABSTRACT

Entomopathogenic fungus contains variety of secondary metabolites, which are known to have pharmacological and insecticidal properties. Stress plays an important role in production of toxic secondary metabolites. Thermal stress plays major role in production of toxic compounds. In the present study the toxicity of thermal temperatures induced *G. applantum* secondary metabolites were tested for larvicidal activities on *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus*. Our results show that after 24 h post treatment, stress induced *G. applantum* ethyl acetate extract produce strong larvicidal activity in *An. stephensi* (LC_{50}: 53.035 mg/l),*Cx. quinquefasciatus* (LC_{50}: 85.618 mg/l), and *Ae. aegypti* (LC_{50}: 90.018 mg/l). *G. applantum* secondary metabolites were characterized using TLC and FT-IR. FT-IR shows two major functional groups which are, OH stretch and carboxylic groups. GC-MS analysis shows presence of two major compounds namely, Benzyl Chloroformates and hexyl nitro cyclohexene. Histopathological study shows that *G.applantum* extract, produce vacuolation in epithelial cells of midgut, far body and muscle tissues in mosquito larvae. Our overall findings suggest that thermal stress induced *G. applantum* culture, can produce virulent metabolites are toxic to mosquito larvae. These secondary metabolites can be used for effective, green pesticides for future mosquito control programs.

1. Introduction

Mosquitoes are responsible for transmission of major human disease namely, malaria, filariasis, dengue, yellow fever, Japanese encephalitis and zika (Naqqash et al., 2016; Benelli and Duggan, 2018). Mosquito borne disease are responsible for deaths around two million people every year (Cullen and Arguin, 2014; Jiang and Mulla, 2009). Currently 3500 mosquito species have been identified in three genera namely; *Anopheles, Aedes*, and *Culex* which are economically important to human health (Bensansky and Collins, 1992; Hill and Crampton, 1994; Reiter, 2007).

Several synthetic chemical insecticides have been developed worldwide to reduce the prevalence of mosquito vectors about twelve insecticides from four classes of insecticide have been recommended for indoor residual spraying (IRS) while only pyrethroids (Cypermethrin and Permethrin) have been approved for treating bed nets (Nauen, 2007; WHO, 2016). Chemical pesticides shows remarkable toxicity on insect pests but repeated use of same or different types of chemicals cause environmental contamination and mosquitoes will get insecticide resistance capacity (Kajla et al., 2016; Khater, 2012).

The major source of biological control agents are plants, aquatic organisms, invertebrate predators, nematodes, protozoa, fungi and bacteria (Vivekanandhan et al., 2018a,b,c,d,e,f). The biological agents and their secondary metabolites/essential oils are very effective and alternative of synthetic chemicals in vector control programme. In recent years, biopesticides having attributes of being environmentally safe (Chandler et al., 2010), and which are highly selective have been seen as an alternative to chemical insecticides (Hussain et al., 2010). Several environmental stress factors, namely temperature, drought, alkalinity, salinity and UV radiation are harmful to the bacteria, fungus and plant, among them temperatures plays an important role in primary and secondary metabolites synthesis in organisms (Griesser et al., 2015; Christie et al., 1994; Yang et al., 2007). Different physical and chemical tools are used for enhancement of toxic secondary metabolite production in culture media (Szabó et al., 2003; Nowak et al., 2010).

Entomopathogenic fungi are the important microbial biological control agents against medical and agricultural pests (Meyling and Eilenberg, 2007). Several entomopathogenic fungi like as *Verticillium lecanii* (Soni and Prakash, 2012), *Fusarium oxysporum* (Vivekanandhan et al., 2018b), *Metarhizium anisopliae* (Scholte et al., 2007) and

* Corresponding author.
E-mail address: skentomology@gmail.com (M.S. Shivakumar).

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Beauveria bassiana have been shown to have biological control potential on pests (Vivekanandhan et al., 2018c). Fungal derived secondary metabolites are also used for controlling insect pests. Mushrooms belong to subdivision of Basidomycota. It consists of more than 14,000 species, these traditionally used for pharmaceutical purpose food and as antioxidants. Mushrooms are known to produce variety of secondary metabolites (Damrongpan and Urat, 2015, 2015). *Ganoderma applantum* (Ganodermataceae) is a wood-decay fungus widely distributed worldwide and typically found on surface of the stem part of the tree (Luo and Di, 2015). Several Ganoderma species are used in Chinese herbal medicine. *G. applantum* and *G. lucidum* are used in the prevention and treatment of various chronic diseases in China (Luo and Di, 2015). *G.applantum* secondary metabolites contain triterpenes, steroids and polysaccharides as major constituents (Baby et al., 2015). Ganoderma species derived secondary metabolites have many biological activities for example antimicrobial activities (Osińska-Jaroszuk et al., 2014), anti-inflammation activities (Joseph et al., 2011) insecticidal activities (Olayemi, 2013), antioxidants, anticancer, prebiotic, immunomodulating, cardiovascular, and anti-fibrotic. In our present study we investigated the toxic and sublethal effects of secondary metabolites from *G.applantum* extract on mosquito larvae and compared it with thermal stress induced secondary metabolites on *Aedes aegypti, Anopheles stephensi* and *Culex quinquefaciatus* larvae. Further secondary metabolites were characterized using GC-MS and FT-IR to find out the functional groups and their chemical composition.

2. Materials and methods

2.1. Fungal culture

Ganoderma applantum was collected from Yercaud hills (latitude 11.775251°N and longitude 78.2092576°E) and Kolli hills, Tamil Nadu, India (latitude 11.2485°N and longitude 78.3387°E). *G.applantum* was cleaned with running tap water and fungal biomass was collected in sterile polythene bags for further experiments.

2.2. Morphological confirmation of G.applantum

Fungal spore preparation was done on glass slide and adds two drops of lactophenol cotton blue stain with fungal conidia was added properly mixed. Finally observed under the light microscope at 40X magnification (Figs. 1 and 2) (Vivekanandhan et al., 2018c).

2.3. Preparation of G.applantum extracts and preparation of the fungal broth

G. applantum fungus was shade dried for 7–10 days in dark dry place at room temperature (28°C ± 3°C). Dried fungal biomass powder (300 g) was extracted with 350 ml of ethyl acetate for cooling extraction up to 25 days at (28 ± 2°C). After 25 days upper organic phase was filtered through Whatman no.1 filter paper and then crude extract

Fig. 1. *Ganoderma applantum* (per.) natural appearance.

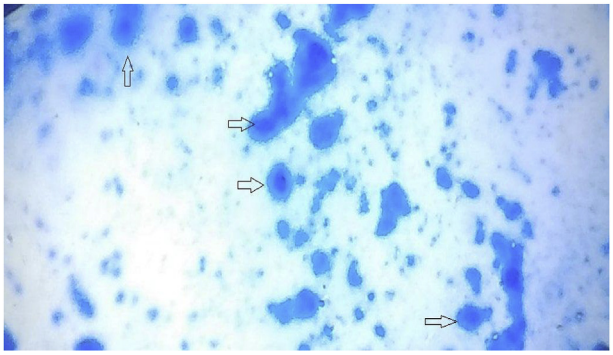


Fig. 2. The arrow indicated *G. applantum* conidia spores at 40X magnification under light microscopes.

were concentrated using rotary vacuum evaporator (Superfit/India) at (45-60°C) then the fungal crude extract were stored at (4°C) for further experiment.

G. applantum spores were harvested by the flooding sterile distilled water with 0.05% Tween 80 (Sigma, USA). Filtered fungal suspension was estimated by using heamocytometer to count the spore concentration, and then adjusted to the spore to 1×10^7 conidia/ml. The five 250 ml of fungal broth (potato dextrose broth, PDB) was prepared in 500 ml Erlenmeyer flasks then 1×10^7 conidia/ml was inoculated in five 250 ml of broth culture medium, then the inoculated broth culture was incubated in five different temperature in, 20° C, 25°C, 30°C, 35°C and 40°C through pH 7.

2.4. Extraction and concentration of G. applantum metabolites

After 15 days, fungal broth was centrifuged at 10,000 rpm for 15 min. After the centrifugation the settled pellet was discarded, then the supernatant was transferred to separating funnel with added equal volume of the ethyl acetate then mixed fungal culture filtrate with ethyl acetate for 7days. After the seven days the upper organic phase was separated and filtered through Whatman no.1 filter paper then extracted secondary metabolites was stored at (4°C) condition for further experiments.

2.5. Mosquito culture

Larvae of *An. stephensi, Ae. aegypti* and *Cx. quinquefasciatus* were procured from, Institute of Vector Control Zoonoses (IVCZ) Banahalli, Tamil Nadu, India. The larvae were maintained in plastic trays containing tap water in laboratory conditions at (27 ± 2°C) and relative humidity of 75–85%, 14:10 (Light and Dark). Powdered dog biscuit, yeast powder and millets powder was used as larvae food in the ratio of 3:3:1 (Vivekanandhan et al., 2018b).

2.6. Larval toxicity

G. applantum extract were tested against 4th instar larvae of *An. stephensi, Ae. aegypti* and *Cx. quinquefasciatus*, as per World Health Organization (World Health Organization (WHO), 2016; Vivekanandhan et al., 2018a). For each replicate, 25 mosquito larvae were taken in 300 ml plastic cups containing 250 ml of distilled water and fungal extract in thermal stress induced temperatures like, (20°C, 25°C, 30°C, 35°C and 40°C). Desired concentration (i.e. 50 mg/L, 200 mg/L, 300 mg/L, 400 mg/L and 500 mg/L) of the selected fungal secondary metabolites and each concentration were replicated three times. For control 25 individual exposed to DMSO. After 24 h, the mortality (%) was calculated and corrected with control mortality using the Abbott's formula (Abbott, 1925).

2.7. Thin layer chromatography (TLC)

G. applantum secondary metabolites compounds were separated by using thin layer chromatography (TLC) with silica gel 60 size meshes coating on 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% relative humidity (RH).

2.8. Fourier transformed infrared (FT-IR) spectroscopy

G. applantum 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.

2.9. Gas chromatography mass spectroscopy (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 mm 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, on source temperature 240 °C, and ionization mode electron impact at 70 eV, scan time 0.2 s and scan interval of 0.1s. 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.

2.10. Histopathological study

24 h post treated and control *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus* larvae were fixed with diluted formaldehyde for 2 h at 5°C. Wax blocks were cooled at 25°C up to 5 h and cooled wax were cut into 8 µm thin 1.1 mm ribbons with a microtome. Larval gut section was stained with haematoxylin and eosin stain (Vivekanandhan et al., 2018b). After air drying sections was viewed under a light microscope (Olympus-CH20, Mumbai, India) at a magnification of 400X.

2.11. Statistical analysis

Mosquitoes larval mortality data were subjected to analysis of variance (ANOVA) of arcsine square root transformed mortality percentages. The lethal concentrations required to kill 50 and 90% (LC_{50} 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-16.0 software.

3. Results and discussion

Currently repeated use chemical insecticides cause many problems to environment; non-target invertebrates and domesticated animals (Upadhyay and Jauhari, 2016). Considerable amount of attention to focused on identifying potential mosquitocidal secondary metabolites from bacteria and fungus from natural sources for mosquito control. Several studies have shown that entomopathogenic fungus cause high mortality in *Culex* (Vyas et al., 2007) and *Anopheles* species (Scholte et al., 2004).

Table 1
Larvicidal activity of field collected *G. applantum* fungal extract against fourth instar larvae of *An.stephensi, Ae.aegypti and Cx. quinquefasciatus*.

Mosquito species (na-375)	concentration (mg/l)	LC_{50}(LCL-UCL) (mg/l)	LC_{90}(LCL-UCL) (mg/l)	χ^2 (Df = 12)
An.stephensi	50 200 300 400 500	70.49 (58.10-127.10)	234.00 (132.30-1866.42)	9.24 n.s
Ae.aegypti	50 200 300 400 500	90.01 (2.25-154.35)	430.98 (267.66-6325.92)	7.02 n.s
Cx. quinquefasciatus	50 200 300 400 500	85.61 (50.19-158.09)	311.59 (174.511-366.11)	13.21 n.s

Note: na = total number of mosquito used per each species 25per replicates, three replicates were carried out, five concentration were tested, LC_{50} = lethal concentration killing 50% of exposed organisms, $LC_{90=}$ = lethal concentration killing 90% of exposed organisms, LCL = 95% LCL = 95 confidence limits, UCL = 95% upper confidence limits, χ^2 = chi square (not significant, p > 0,05): df = degrees of freedom.

Table 2
Larvicidal activities of temperature 20 °C and pH-7 condition maintained *G. applantum* ethyl acetate extract against fourth instar larvae of *An.stephensi, Ae, aegypti* and *Cx.quinquefasciatus*.

Mosquito species (na-375)	concentration (mg/l)	LC_{50}(LCL-UCL) (mg/l)	LC_{90}(LCL-UCL) (mg/l)	χ^2 (Df = 12)
An.stephensi	50 200 300 400 500	337.31 (252.77-334.95)	5908.52 (2005.53-163869.44)	0.63 n.s
Ae.aegypti	50 200 300 400 500	483.63 (411.71-1410.07)	8858.49 (2676.95-338154.94)	0.34 n.s
Cx. quinquefasciatus	50 200 300 400 500	439.598 (329.24-801.74)	6427.90 (2206.72-1418467.65)	0.33 n.s

Note: na = total number of mosquito used per each species 25per replicates, three replicates were carried out, five concentration were tested, LC_{50} = lethal concentration killing 50% of exposed organisms, $LC_{90=}$ = lethal concentration killing 90% of exposed organisms, LCL = 95% LCL = 95 confidence limits, UCL = 95% upper confidence limits, χ^2 = chi square (not significant, p > 0,05): df = degrees of freedom.

3.1. Morphological confirmation

Fungal microscopic identification was carried out based on the morphological features like conidia structures, colors, conidial shape and pigment production (Fig. 2). The fungus was identified as *G. applantum* followed by the methods of (Ragavendran et al., 2017; Vivekanandhan et al., 2018a, b).

3.2. Larval bioassay

Larvicidal activity of *G. applantum* biomass ethyl acetate extracts was investigated for larvicidal activity on *Ae. aegypti, An. stephensi* and

Table 3
Larvicidal activities of *G. applantum* ethyl acetate extracts against fourth Instar larvae *An.stephensi, Ae, aegypti and Cx.quniqufaciatus* in temperature 25°C and pH-7.

Mosquito species (na-375)	Concentration (mg/l)	LC_{50}(LCL-UCL) (mg/l)	LC_{90}(LCL-UCL) (mg/l)	χ^2 (Df = 12)
An.stephensi	50 200 300 400 500	263.09 (185.71-374.19)	5138.06 (1785.34 -148312.39)	2.04 n.s
Ae.aegypti	50 200 300 400 500	396.46 (313.308-5870.610)	3713.45 (1682.206-24490.068)	0.30 n.s
Cx. quinquefasciatus	50 200 300 400 500	325.09 (238.99-524.85)	6685.93 (2096.33-306951.32)	1.48 n.s

Note: na = total number of mosquito used per each species 25per replicates, three replicates were carried out, five concentration were tested, LC_{50} = lethal concentration killing 50% of exposed organisms, $LC_{90=}$ = lethal concentration killing 90% of exposed organisms, LCL = 95% LCL = 95 confidence limits, UCL = 95% upper confidence limits, χ^2 = chi square (not significant, p > 0,05): df = degrees of freedom.

Table 4
Larvicidal activities of *G. applantum* ethyl acetate extracts against fourth instar larvae *A.stephensi, Ae.aegypti and Cx.quniqufaciatus* in temperature 30° C and pH 7.

Mosquito species (na-375)	Concentration (mg/l)	LC_{50}(LCL-UCL) (mg/l)	LC_{90}(LCL-UCL) (mg/l)	χ^2 (Df = 12)
An.stephensi	50 200 300 400 500	144.38 (89.71-187.09)	1350.01 (800.47-4401.29)	2.52 n.s
Ae.aegypti	50 200 300 400 500	205.99 (106.51-296.30)	7129.48 (1934.46-1836466. 31)	1.19 n.s
Cx. quinquefasciatus	50 200 300 400 500	174.69 (105.39-230.29)	2569.75 (1178. 71-22117.27)	1.77 n.s

Note: na = total number of mosquito used per each species 25per replicates, three replicates were carried out, five concentration were tested, LC_{50} = lethal concentration killing 50% of exposed organisms, $LC_{90=}$ = lethal concentration killing 90% of exposed organisms, LCL = 95% LCL = 95 confidence limits, UCL = 95% upper confidence limits, χ^2 = chi square (not significant, p > 0,05): df = degrees of freedom.

Cx. quinquefasciatus. Our results clearly show that thermal stress induced *G. applantum* extract has toxicity on major mosquito species, with LC_{50} values of 53.035 mg/l in *An.stephensi*, 90.018 mg/l in *Ae.aegypti* and 85.618 mg/l in *Cx. quinquefasciatus*. Thermal stress (35°C) induced *G.applantum* secondary metabolites show strong larvicidal activity (Tables 1–6). Similar results were reported in other fungus having potential larvicidal activity against an *An. stephensi* (Saurav et al., 2013). Ethyl acetate extract from marine Actinobacteria and Streptomyces sp. shows high mortality in *An. stephensi* and *Cx. tritaeniorhynchus* (Dhanasekaran et al., 2014). *Culicinomyces clavisporus* extracellular culture filtrates show remarkable larvicidal activity against *Cx.*

Table 5
Larvicidal activities of *G. applantum* bio mass extract against fourth instar larvae of *An.stephensi, Ae.aegypti and Cx.quniqufaciatus* in temperature 35° C and pH-7.

Mosquito species (na-375)	Concentration (mg/l)	LC_{50}(LCL-UCL) (mg/l)	LC_{90}(LCL-UCL) (mg/l)	χ^2 (Df = 12)
An.stephensi	50 200 300 400 500	53.03 (25.15-73.37)	131.78 (106.25-161.43)	2.13 n.s
Ae.aegypti	50 200 300 400 500	87.886 (479.805-1764.164)	345.98 (208.06-30017-75)	10.38 n.s
Cx. quinquefasciatus	50 200 300 400 500	69.90 (347.35-676.49)	228.50 (123.32-2097.79)	9.02 n.s

Note: na = total number of mosquito used per each species 25per replicates, three replicates were carried out, five concentration were tested, LC_{50} = lethal concentration killing 50% of exposed organisms, $LC_{90=}$ = lethal concentration killing 90% of exposed organisms, LCL = 95% LCL = 95 confidence limits, UCL = 95% upper confidence limits, χ^2 = chi square (not significant, p > 0,05): df = degrees of freedom.

Table 6
Larvicidal activities of *G. applantum* ethyl acetate extracts against fourth instar larvae of *An.stephensi, Ae.aegypti and Cx.quinquefasciatus* in temperature 40°C and pH-7.

Mosquito Species (na-375)	Concentration (mg/l)	LC_{50}(LCL-UCL) (mg/l)	LC_{90}(LCL-UCL) (mg/l)	χ^2 (Df = 12)
An.stephensi	50 200 300 400 500	469.16 (367.36-1118.16)	8422.44 (2552.08-339871.50)	1.13 n.s
Ae.aegypti	50 200 300 400 500	495.46 (479.80-1764-16)	8419.08 (2713.38-210111-23)	.832 n.s
Cx. quinquefasciatus	50 200 300 400 500	441.65 (347.35-676.49)	3787.48 (1737.85-23129.73)	4.84 n.s

Note: na = total number of mosquito used per each species 25per replicates, three replicates were carried out, five concentration were tested, LC_{50} = lethal concentration killing 50% of exposed organisms, $LC_{90=}$ = lethal concentration killing 90% of exposed organisms, LCL = 95% LCL = 95 confidence limits, UCL = 95% upper confidence limits, χ^2 = chi square (not significant, p > 0,05): df = degrees of freedom.

quinquefasciatus, Ae.aegypti, and *An. stephensi* (Singh and Prakash, 2012).

Thermal stress induced *G. applantum* ethyl acetate extracts produced higher larval toxicity against fourth instar larvae of *An. stephensi, Cx. quinquefasciatus*, and *Ae.aegypti* (Table 5). This results shows that temperature stress may be involved in production of metabolites which are more toxic.

3.3. Histopathology study

Cross sections of fourth instar larvae of *Ae.aegypti, An. stephensi* and *Cx. quinquefasciatus* treated with *Ganoderma applantum* extract

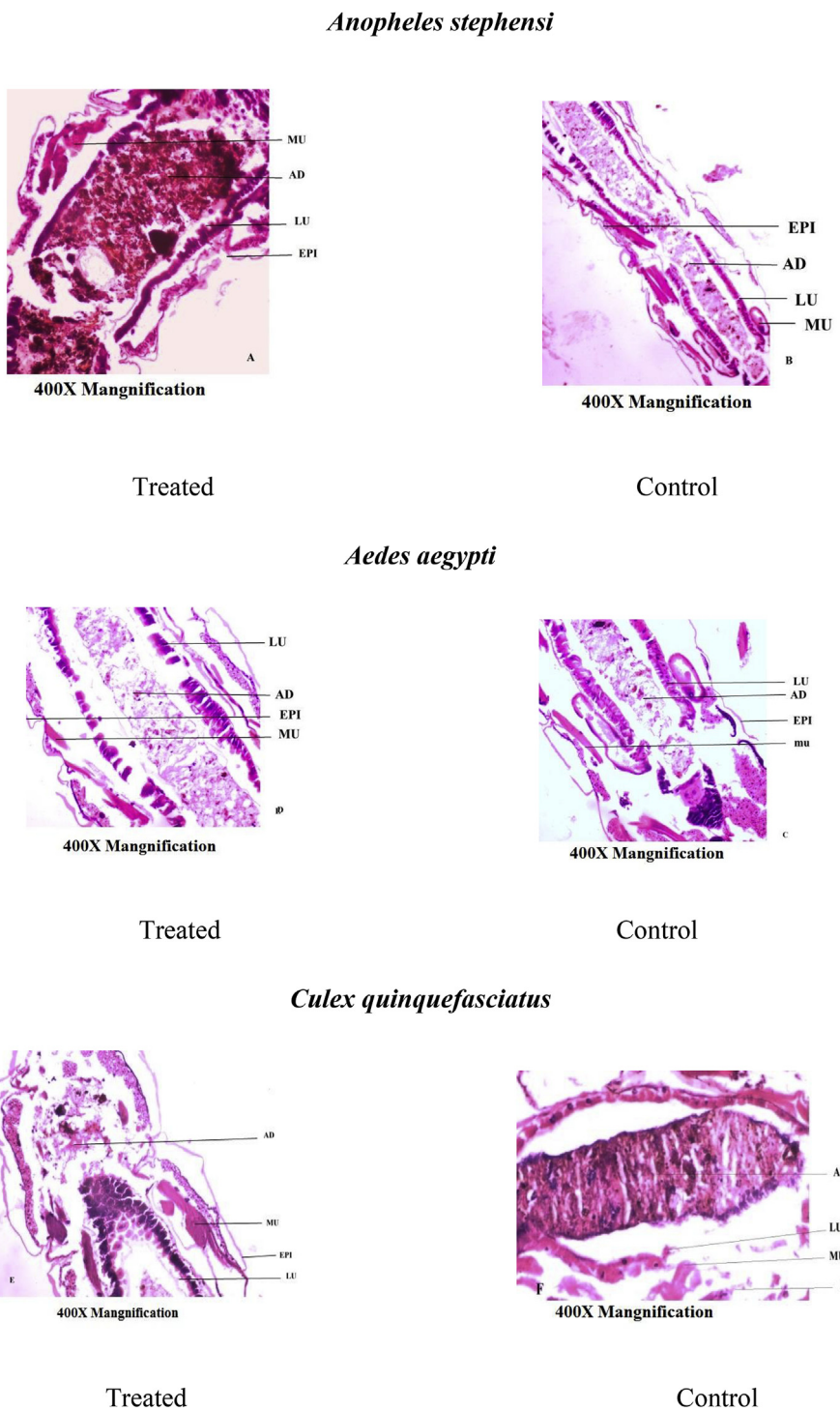


Fig. 3. Cross section of *An. stephensi, Ae. aegypti* and *Cx. quinquefaciatus* larvae. Treated and untreated with *G. applantum* secondary metabolites (**A**) is treated and (**B**) is control. Gut lumen (lu), gut epithelial lumen (epi) adipose tissue (ad), muscle (mu).larvicidal mid gut section was stained with haematoxylin and eosin and stained midgut tissue viewed and photographed under light microscope at 400X magnification.

highlighted the damage to midgut tissues. *An. stephensi* 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 *Ae.aegypti* larvae. These changes were not detected in control larvae at 400X magnification (Fig. 3).

The major components in the extracts namely hexyl nitro cyclohexene, and Benzene teracholoro dimethyl are, inferred to be the principal metabolites which may be involved in insecticidal activity. *G. applantum* metabolites can affect the larval nervous system and damage larval gut tissues producing vacuolation in epithelium cells of midgut, muscle and adipose tissues are damaged by the secondary metabolites.

3.4. Gas chromatography-mass spectrometry (GC-MS)

Gas Chromatography-Mass Spectrometry results obtained from the *Ganoderma applantum* ethyl-acetate crude extract shows (Fig. 4 and Table 7), the presence of twelve compounds namely, Benzyl

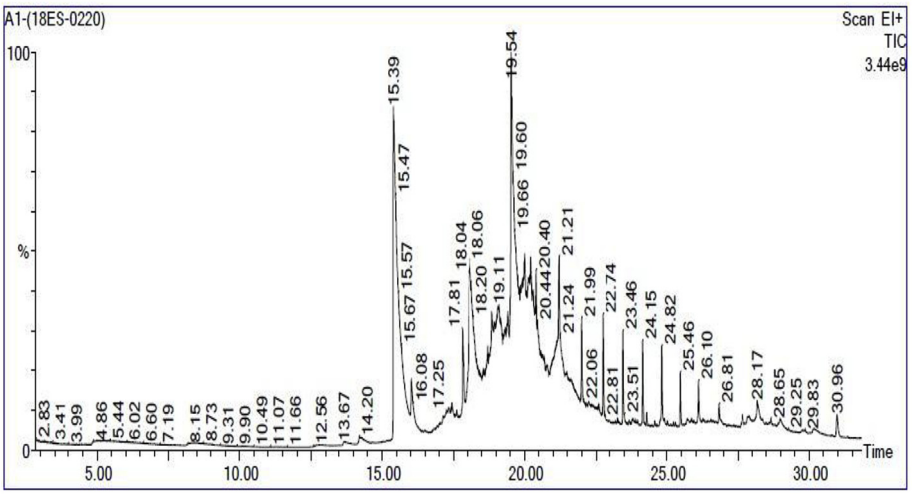
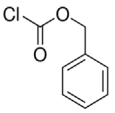
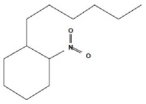
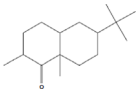
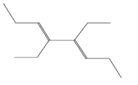


Fig. 4. GC-MS analysis of *G.applantum* ethyl acetate secondary metabolites.

Table 7
G. applantum secondary metabolites analysis of GC-MS for chemical constituents' identification GC-MS analysis of *G.applantum* ethyl-acetate crude extracts for chemical component in dimeric carbon.

S.No	Peak value	Retention time	Molecular weight	Formula	Compound name	Compound structure
1	15.42	15.394	170.59	$C_8H_7O_2C_1$	Benzyl Chloroformates	
2	16.03	16.019	254	$C_{17}H_{36}$	Heptadecane	
3	17.82	17.810	308	$C_{22}H_{44}$	Docosane	
4	18.04	18.060	312	$C_{20}H_{40}O_2$	Eicosanoic Acid	
5	18.06	18.850	213	$C_{12}H_{23}O_2N$	Hexyl nitro cyclohexane	
6	19.11	19.105	284	$C_{18}H_{36}O_2$	Tetradecanoic acid, trimethyl-, methyl ester	
7	19.54	19.415	152	$C_{10}H_{16}O$	Trans-chrysanthemal	
8	19.60	19.535	278	$C_{20}H_{38}$	Eicosadiene	
9	19.66	20.00	236	$C_{16}H_{28}O$	Naphthalenone, (dimethylethyl) octahydro dimethyl	
10	20.20	20.211	166	$C_{12}H_{22}$	Octadiene, diethyl	

Chloroformates (15.42), Heptadecane (16.03), Eicosanoic acid (18.07), Hexyl nitro cyclohexene (19.55), Tetradecanoic acid and trimethyl methyl ester (20.00), Transchrysnthemal (21.20), Naphthalenone (dimethyl) octahydro dimethyl (21.99), Octadiene, dimethyl (22.74), Docosane dibromr (23.46), Heptacosane (24.82). Minor compounds Tritraconate (25.46), Dihydro ergo sterol (28.19), Phenol (dimethyl ethyl) (30.96), (Fig. 4 and Table 7).

GC–MS analysis shows presence of twelve compounds; namely, Benzyl Chloroformates (15.42), Eicosanoic acid (18.07), Hexyl nitro cyclohexene (19.55), Tetradecanoic acid, trimethyl, methyl ester (20.00), Transchrysnthemal (21.20), Eicosadiene, naphthalenone (1, 1 dimethyl) octahydro dimethyl (21.99), Octadiene 4, 5 dimethyl (22.74), Docosane dibromr (23.46), Heptacosane (24.82) and three minor compound are, Tritraconate (25.46), dihydro ergo sterol, (28.19), Phenol, bis (dimethyl ethyl) (30.96) (Fig. 4 and Table 7).

The major components in the extracts namely hexyl nitro cyclohexene, and Benzene teracholoro dimethyl are, inferred to be the principal metabolites which are may be involved in insecticidal activity. *G. applantum* metabolites can affect the larval nervous system and also damage to the larval gut tissues for produce vacuolation in epithelium cells of midgut, muscle and adipose tissues are damaged by the secondary metabolites. Similarly Vivekanandhan et al. (2018c, d) reported that *Fusarium oxysporum*, *Beauveria bassiana* secondary metabolites highly toxic to *An.stephensi*, *Ae.aegypti* and *Cx.quinquefasciatus* mosquito larvae.

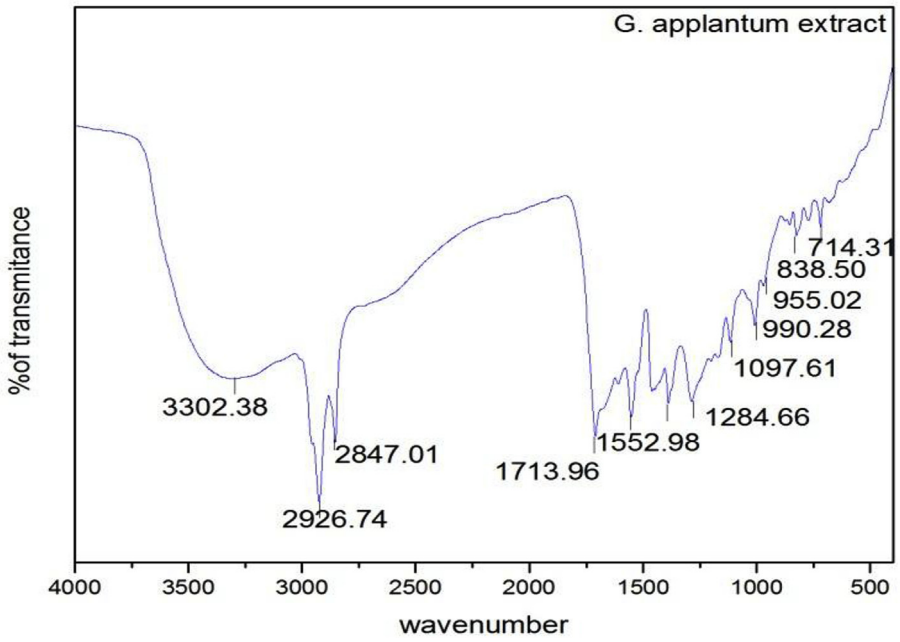


Fig. 5. FT-IR analysis of *G. applantum* secondary metabolites.

Table 8
FT-IR analysis of *G. applantum* ethyl acetate crude extract.

S·NO	Vibration	Stretch	Functional Group
1	3292.16	Dimer OH	Carboxylic acid
2	2955.00	-CH3	Alkanes
3	2924.62	CH stretch	Alkanes
4	2854.08	CH stretch	Alkanes
5	1709.76	Dimer OH	Carboxylic acid
6	1608.42	C=C stretch	Alkanes
7	1551.09	C=O stretch	Carboxylic acid
8	1460.53	Ar C-C stretch	Aromatics
9	1387.86	S-O sulfate ester	Misc
10	1285.56	C-F stretch	Alkylhalides
11	1200.19	C-H out of plane	Aromatics
12	1170.73	C-H weg (-CH_2X)	Alkylhalides
13	1115.58	C-H out of plane	Alkanes
14	1007.07	C-H weg (-CH_2X)	Alkylhalides
15	969.53	= C-H out of plane	Alkanes
16	854.73	C-H out of plane	Aromatics
17	771.90	C-H weg (-CH_2X)	Alkylhalides
18	718.71	= C-H stretch	Alkanes
19	682.68	= C-H out of plane	Alkanes

G. applantum 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.

3.5. Fourier-transform infrared spectroscopy (FT-IR)

FT-IR measurements were carried out to identify the possible biomolecule responsible for near capping and efficient stabilization of the *Ganoderma applantum* crude extract. The peaks value in the region of IR radiation in FT-IR analysis confirmed the presence of alkanes, alkylhalides, aromatics, carboxylic acid (Fig. 5 and Table 8).

FT-IR spectrum results of bioactive extracts from *G. applantum* reflect prominent peaks indicating the presence of O–H dimer stretching, corresponded to 3292.16, $=CH_3$ corresponded to the 2955.00, C-H stretching, is corresponded to the 2924.62, O-H dimer, corresponded to the 1709.76, C=C stretch, corresponded to the 1608.42, C=O stretch, corresponded to the 1551.09, C-C stretch, assigned to 1460.53, S–O sulfate ester stretching misc assigned to the 1387.86, C-F stretching corresponded to the 1285.56, C-H out of plane correspondent to the 1200.19, C-H weg (-CH2X stretching assigned to the 1170.73, C-H out of plane correspondent to the 1115.58, C-H weg (-CH2X stretching, corresponded to the 1007.07, C-H out of plane assigned to 969.53, C-H out of plane corresponded to the 854.73, C-H weg (-CH2X stretching assigned to the 771.90, C-H stretching assigned to the 718.71, and C-H out of plane assigned to the 682.68 groups (Figure: 5 and Table: 8), Carboxylic acid, Alkanes, Carboxylic acid, Aromatics, Alkylhalides which are similar to earlier studies (Vivekanandhan et al., 2018e; Ragavendran et al., 2017).

4. Conclusion

Present study shows that thermal stress induced ethyl acetate extracts of *G.applantum* show remarkable toxicity to mosquito larvae. The virulence of secondary metabolites can be increased by maintaining fungus at 35°C. FT-IR analysis shows the presence of two major functional groups of dimmer OH stretch and carboxylic group and -CH3 stretch of alkanes groups. GC-MS analyses have shown the presence of 2 major chemical constituents namely, Benzyl Chloroformates and Hexyl nitro cyclohexene may be involved in larvicidal activity. Histopathology study shows that, *G.applantum* ethyl acetate extract was produce vacuolation in epithelial cells of midgut, far body and muscle tissues in mosquito larvae. Based on the above finding, we suggest that metabolites produced by *G.applantum* can be used in the control of larval mosquito population.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101253.

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