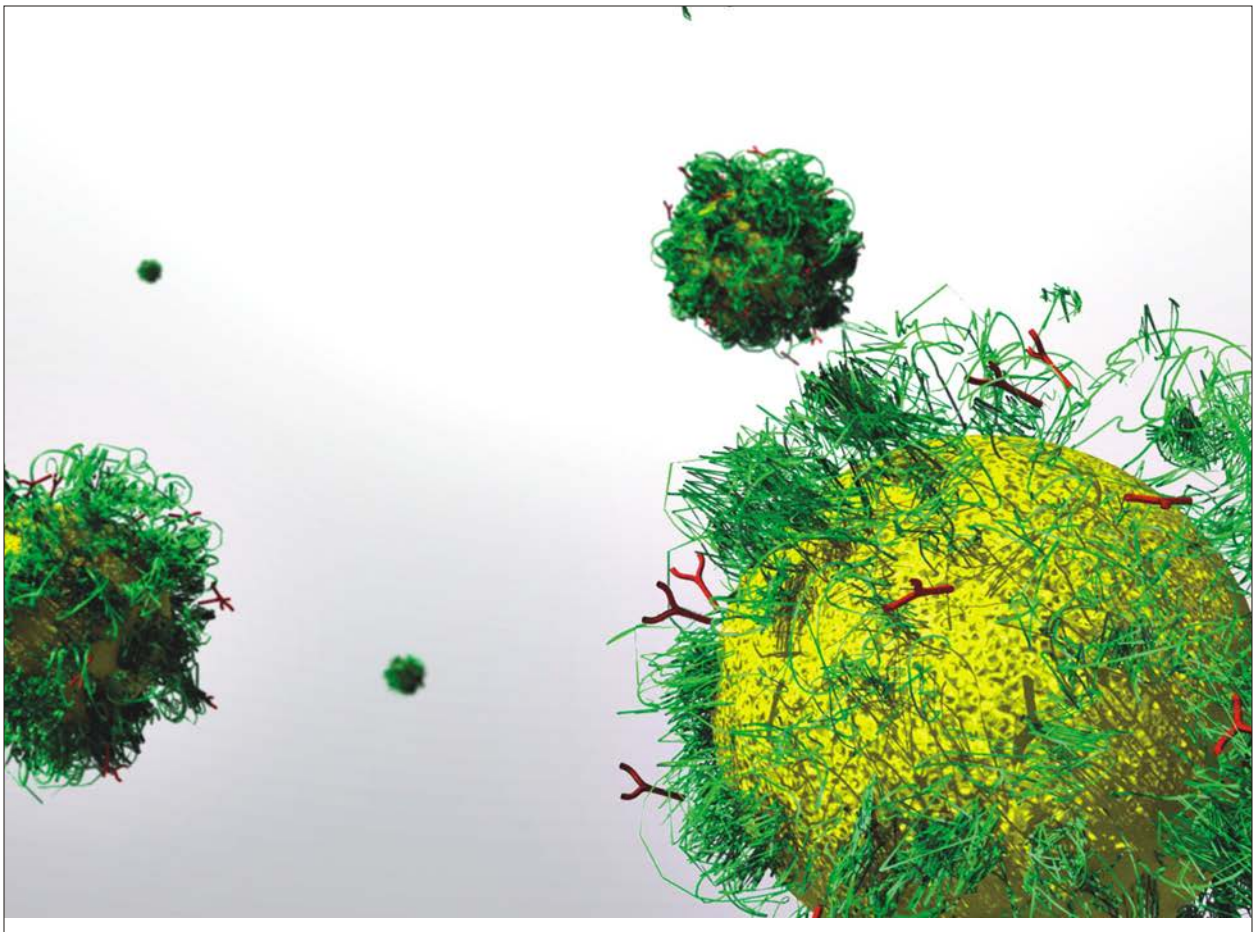

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Evaluation of Larvicidal Efficacy of Extract of the Fungus *Ganoderma lucidum*, for the Control of the Filarial Vector Mosquito, *Culex pipiens pipiens* (Diptera: Culicidae)

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ABSTRACT

The global need for alternative sustainable eco-friendly insecticides, for vector control of debilitating mosquito-borne diseases informed this study. However, insecticides of higher-plant origins are vulnerable to resistance, thus, necessitating the widening of the scope of search to include lower-plant sources, especially, those the bioactive fungal metabolites. To this end, methanolic extract of the fungus *Ganoderma lucidum* was bio-assayed against 4th instar larval stage of the filarial vector mosquito, *Culex pipiens pipiens*, following standard protocols of phytochemical extraction and testing the susceptibility of mosquito larvae to insecticides. The larvae were exposed to a series of extract concentrations ranging from 0.50-5.00 mg L^{-1} and monitored for behavioural aberrations and mortality during the subsequent 24 h post-exposure. The results indicated significant ($p<0.05$) larvicidal activities of the extract against the mosquito species and larval mortality responded proportionally to increasing extract concentration and duration of exposure. Post 24 h exposure larval mortality ranged from 0.75±0.25% in 0.5 mg L^{-1} extract concentration to 100.00±0.00% in 5.00 mg L^{-1}. Linear regression analysis confirmed dependent relationships between percentage larval mortality and increasing concentration of extract, on one hand and duration of exposure on the other, with linearity (R-square) of 92.57 and 76.06, respectively. The LC_{50} and LC_{90} values of the extract against tested larvae were estimated as 2.26 and 4.25 mg L^{-1}, respectively. These results suggest that *G. lucidum* possess bio-active phytochemicals that stand the plant species in good stead as a viable source of resistant-insulated larvicidal lead-agents, for vector control of mosquito-borne diseases.

Key words: Bioassay, duration of exposure, larval mortality, lethal concentration, methanolic extract, phytochemicals

INTRODUCTION

Human lymphatic filariasis, transmitted primarily by mosquitoes of the Genus *Culex*, threatens the health of more than a billion people in 83 countries of the world, especially in the Tropics (WHO, 2004, 2000). Filariasis affects an estimated 128 million people, incapacitating about 40 million of them world-wide and is particularly distributed in urban areas (Michael and Bundy, 1997; WHO, 2011). Such urban settlements are characterized by the proliferation of slums and development of dry season irrigation farming that, provide year-round suitable larval breeding habitats for mosquito vectors (Curtis and Faechem, 1981). This development has resulted in relatively high *Culex*-vectorial capacity and hence, endemicity of lymphatic filariasis in the areas of distribution of these vector mosquitoes (Jayasekera *et al*., 1991). Of particular epidemiological

importance are two *Culex* species namely, *Cx. pipiens pipiens* and *Cx. quinquefasciatus* that have evolved to take advantage of certain anthropogenic lapses in human peri-domestic environments. Today, these mosquitoes constitute the most incriminated vectors of filariasis, as well as, the foremost nuisance mosquitoes world-wide (Sunish *et al.*, 2007; Linthicum, 2012).

The burdens of filariasis have remained high over the decades because the two strategies available for control, i.e., parasite control (through chemotherapy) and vector control (through larviciding and adulticiding agents) have not been effective. Chemotherapy has failed for reasons including, poor efficacy and high costs of available drugs, development of parasite drug-resistance and non-availability of an effective vaccine (Burkot *et al.*, 2006). On the other hand, vector control has not yielded much positive results because the mosquito vector control interventions in the areas of distribution of filariasis are targeted at vulnerable links in the behaviour and ecological adaptability of Anopheline vectors of malaria and such are quite different from those of culicine mosquitoes. For example, the main-thrust of mosquito vector control, ever in the areas of distribution of filariasis, has been wide spread deployment of insecticide-treated bed nets and elimination or treatment of larval breeding habitats. These strategies have been effective against Anopheline vectors of malaria that are primarily endophagic (Russell *et al.*, 2013), bite late at night (Majambere *et al.*, 2013) and breed conventionally in small temporary rain pools (Olayemi and Ande, 2008). *Culex* mosquitoes, on the other hand, are dominantly exophagic and feed essentially during pre-bed time (thus, avoiding contact with insecticide-treated nets) and breed preferentially in habitats that may be difficult to eliminate such as swamps and irrigation channels (Muturi *et al.*, 2007). Therefore, effective control program for filariasis must incorporate larviciding interventions. Though, many synthetic mosquito larvicides such as Organophosphates, Organochlorines, Carbamates, etc., have been developed with proven efficacy (Zaim and Jambulingam, 2007; WHO, 2006); such successes could not be sustained as a result of associated challenges, ranging from environmental toxicity to vector resistance (Casida and Quistad, 2000; Devine and Furlong, 2007). This development has led to global searches for eco-friendly cost-effective insecticides (Zaim and Guillet, 2002; Awad, 2003).

Since, pre-historic times, plants have remained the primary source of bioactive natural compounds for treating human ailments and controlling insect pests (Sukumar *et al.*, 1991; Shaalan *et al.*, 2005). However, the search for eco-friendly insecticides from floral sources, have been dominated by higher plants, especially, the spermatophytes (Komalamisra *et al.*, 2005; Das *et al.*, 2007; Rajasekaran and Duraikannan, 2012). Yet hundreds of highly potent human and veterinary drugs, as well as, insecticides have been developed from lower plants, especially, fungi; as a result of their rich diversity of bioactive phytochemicals (Zhu *et al.*, 2011; Kulkarni, 2013; Wink, 1993). A group of fungi that have found important application in medicine are species of the *Genus Ganoderma* (Lin *et al.*, 1993; 1995; Yoon *et al.*, 1994; El-Mekkawy *et al.*, 1998; Eo *et al.*, 2000; Zhang and Lin, 2004; Sanodiya *et al.*, 2009). *Ganoderma lucidum*, in particular, is highly prized among folk remedies, due to its chemotherapeutic efficacy (Liu *et al.*, 2005) thus, resulting in an intense search for pharmacological compounds from this fungus (Wasser and Weis, 1999; Cheung *et al.*, 2000). Unfortunately, however, this search has not included those for insecticidal, especially, mosquito larvicidal lead-agents from *Ganoderma lucidum*. This is in spite of literature reports of the occurrence of derivatives of important bio-active phyto-chemicals such as saponins, tannins, alkaloids, flavonoids, etc., in species of the *Genus Ganoderma* (Boh *et al.*, 2007; Zhou *et al.*, 2007; Adelanwa *et al.*, 2010). These phyto-chemicals have demonstrated high mosquitocidal activities (Shaalan *et al.*, 2005; Deore and Khadabadi, 2009; Chaieb, 2010).

Therefore, in order to explore the fungal Genus, *Ganoderma,* as a potential source of larvicidal lead-agent for vector control of mosquito-borne disease, this study was carried out to evaluate the larvicidal activities of the crude extract of *G. lucidum* against the filarial vector mosquito species, *Cx. p. pipiens.*

MATERIALS AND METHODS

Source and collection of *Ganoderma lucidum*: Mature, field-growing, specimens of *G. lucidum* were collected from tree stumps, around the Temporary Campus of Federal University of Technology, Minna (Long. 6° 33'E and Lat. 9° 37'N), in Niger State, Nigeria. The collected specimens were identified on site and the species identity was authenticated by a Mycologist. Thereafter, a voucher specimen (FUTMN/BSH/04b-023) was deposited in the Herbarium of the Department of Biological Sciences, Federal University of Technology, Minna, Nigeria.

Preparation of plant extract: The freshly collected *G. lucidum* material was washed gently with tap water, to remove associated debris, as some of the specimens were encountered growing on tree stumps close to the soil. The plant materials were then shade-dried for five weeks at room temperature (29.00±1.00°C). The dried material was pulverized using a grinding machine. Extraction of the plant material followed standard procedures (Bagavan and Rahuman, 2011). 200 g of the pulverized material was exhaustively extracted with 200 mL methanol in a Soxhlet apparatus, operated at a temperature of 60°C, for 8 h. The extract was filtered using Whatmann filter paper No. 1. The filtrate was concentrated by drying in a rotary evaporator under reduced pressure, at 45°C for 3 h. The crude extract obtained was stored in an air-tight vial at 4°C, until needed for bio-assay.

Source of mosquitoes: The early fourth instar larvae of *Cx. p. pipiens* mosquito used for bio-assay were obtained from a Colony maintained in the laboratory of the Department of Biological Sciences, Federal University of Technology, Minna, Nigeria. The mosquito Colony was maintained following standard protocols (Olayemi and Ande, 2008).

Preparation of stock solution and test extract-concentrations: The stock solution was prepared by dissolving 1 g of the crude extract in 10 mL of methanol. Preparation of test extract-concentrations followed WHO-recommended procedures (WHO, 2005). The 100 mL of distilled water was put in a series of plastic bowls (200 mL) and the necessary quantity of stock solution, to produce a desired test extract concentration, was added to the distilled water in each bowl. The test extract-concentrations ranged from 0.2-5.00 mg L^{-1}. A control experiment was set-up, containing only methanol and distilled water in the plastic bowl. Each test extract-concentration treatment and control was in four replicates.

Larvicidal bio-assay: The bio-assay followed standard WHO protocols for testing the susceptibility of mosquito larvae to insecticides (WHO, 2005). A batch of 25 healthy early 4^{th} instar larvae of the mosquito species were introduced into each bowl of the test extract-treatments and Control experiment. The larvae were monitored for behavioural activities and mortality at specific intervals, ranging from 15 min to 24 h, post-exposure to the extract. The larvae were observed continuously for the first 3 h post-exposure to extract, for aberrations in physical activities relative to the Control. The larvae were not fed throughout the period of exposure to extract. The bioassay

tests were carried out at laboratory ambient conditions of 29±1.00°C, 72±2.00 relative humidity and 12:12 light: Darkness photoperiod. The whole experiment was repeated within 48 h of the termination of the first exercise.

Data analysis: Data obtained from the experiment were pooled and Mean±SE calculated, along with corrections for larvae mortality, using Abbot's formula (Abbott, 1925). The mean values obtained were subjected to standard statistical analysis using SPSS (Version 16.0). Mortality data, as influenced by concentration of extract and duration of exposure, were subjected to ANOVA and the statistical significance of differences between treatments was determined by Tukey's test at $p<0.05$. Probit analysis was carried out to determine the LC_{50} and LC_{90} of the extract at 95% confident limits.

RESULTS

Continuous visual monitoring of the bio-assay experiments, during the first 3 h post-exposure of the larvae to *G. lucidum* extract, revealed considerable erratic swimming behaviour of the test mosquitoes relative to the Control group. Such abnormal behaviour include, sluggishness, dis-oriented (i.e., Zig-zag) vertical movement in the water column, prolonged stay at the bottom of water in the bowls, spasmodic twitching while at rest, etc.

The results of larvicidal activity of methonalic extract of *G. lucidum* against 4th instar larval stage of *Cx. p. pipiens* are presented in Table 1. Generally, the extract elicited significant ($p<0.05$) mortality in the larvae and such mortality was both extract-concentration and exposure-time dependent. Larval mortality, post-24 h exposure ranged from 18.25±3.42% in 0.5 mg L^{-1} extract-concentration to 100.00±0.00% in 5.0 mg L^{-1}. Again, with respect to increasing toxicity of extract with duration of exposure, the least and highest larval mortality ranges were recorded in 0.50 mg L^{-1} (0.75±0.25-18.25±3.42%) and 5.00 mg L^{-1} (1.75±0.48-100.00±0.00%), respectively. During the first 12 h post-exposure of larvae to extract, mortality was not significantly different ($p>0.05$) for extract concentrations of 0.50-2.50 mg L^{-1}, unlike the results at the 18 and 24th h post-exposure. For extract concentrations of 3.00-5.00 mg L^{-1}, except during the first 15 min

Table 1: Percentage larval mortality elicited by methanolic-extract of *Ganoderma lucidum*, against *Culex pipiens pipiens* mosquitoes, in relation to increasing extract-concentration and duration of exposure

Concentration of extract	Duration of exposure to extract (h)								
(mg L^{-1})	0.25	0.50	1	2	3	6	12	18	24
0.00 (Control)	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	1.690±0.80^{a}
0.50	0.75±0.25^{b}	1.75±0.25^{b}	2.75±0.25^{b}	3.75±0.25^{b}	4.75±0.25^{b}	7.25±0.90^{b}	9.75±1.54^{b}	13.00±2.55^{b}	18.25±3.42^{b}
1.00	1.25±0.25^{b}	2.50±0.50^{b}	3.75±0.95^{b}	5.00±1.00^{b}	6.25±1.25^{b}	9.50±1.50^{b}	11.75±2.13^{b}	19.00±3.44^{c}	28.75±4.98^{c}
1.50	1.50±0.29^{b}	3.00±0.58^{b}	4.50±0.86^{b}	6.00±1.15^{b}	7.50±1.43^{ab}	9.00±1.22^{b}	12.50±2.36^{b}	17.00±3.40^{bc}	27.50±4.68^{c}
2.00	1.50±0.29^{b}	3.00±0.58^{b}	4.50±0.86^{b}	6.00±1.15^{b}	9.00±1.56^{c}	12.00±1.97^{b}	17.00±2.35^{c}	25.25±2.83^{c}	38.00±3.68^{d}
2.50	3.00±0.48^{c}	6.00±0.81^{c}	9.00±1.22^{c}	12.00±1.63^{c}	18.50±2.29^{d}	25.50±3.30^{c}	34.00±4.25^{d}	36.25±5.35^{d}	53.95±7.24^{e}
3.00	1.25±0.25^{b}	3.75±0.54^{b}	9.00±1.17^{c}	15.25V2.48^{c}	23.75±4.65^{d}	34.25±6.50^{d}	50.25±7.08^{e}	70.00±7.71^{e}	87.00±8.73^{f}
3.50	1.00±0.41^{b}	5.75±0.66^{c}	12.0±1.410^{c}	20.50±2.28^{d}	33.75±2.90^{e}	50.25±3.40^{e}	69.25±3.81^{f}	82.50±4.42^{g}	90.00±4.90^{f}
4.00	2.75±0.25^{c}	8.50±0.73^{c}	19.00±2.17^{d}	31.00±3.85^{e}	49.75±4.48^{f}	70.00±4.73^{f}	90.05±5.02^{g}	89.75±5.49^{h}	93.25±5.99^{fg}
4.50	1.50±0.29^{b}	8.75±0.77^{c}	19.75±1.17^{d}	31.75±2.25^{e}	47.25±2.90^{f}	68.25±3.30^{f}	91.50±3.55^{g}	94.00±3.84^{h}	98.75±0.61^{g}
5.00	1.75±0.48^{a}	8.00±0.96^{c}	17.25±1.44^{d}	30.50±2.07^{e}	47.75±2.55^{f}	69.50±3.03^{f}	71.25±3.03^{f}	94.75±3.32^{h}	100.00±0.00^{g}

*Values followed by same alphabets in a column are not significantly different at p = 0.05

Table 2: Linear regression analysis of larvicidal activities of the extract of *Ganoderma lucidum* against *Culex pipiens pipiens* mosquitoes, post 24 h exposure

Model	R	R^2	Significane
Extract concentration	96.23	92.57	0.002
Duration of exposure	86.81	76.06	0.008

Table 3: LC_{50} and LC_{90} (mg L^{-1}), of larvicidal activity of methanolic extract of *Ganoderma lucidum* against *Culex pipiens pipiens* mosquito larvae

LC_{50} (95% confidence limit)	LC_{90} (95% confidence limit)	Regression equation	χ^2 (df = 5)
2.26 (1.87-2.64)	4.25 (3.68-4.88)	Y = 3.54+0.65X	4.34

post-exposure of larvae, significant differences in mortality were observed among the treatments during the post exposure intervals at which umber of dead larvae were counted. On the whole, the dependent of larval mortality on duration of post-exposure to extract was more pronounced for extract concentrations of 3.00-5.00 mg L^{-1}.

The linear regression analysis confirmed dependent relationship between percentage larval mortality and increasing concentration of extract on one hand and duration of exposure on the other; with linearity (R-square values) of 92.57 and 76.06, respectively (Table 2). The LC_{50} and LC_{90} of the extract against *Cx. p. pipiens* larvae were 2.26 and 4.25 mg L^{-1}, respectively (Table 3).

DISCUSSION

Behavioural observations made on physical activities revealed erratic swimming movements and spasmodic twitching while at rest, among the larvae exposed to the extract compared with the Control group. These irregular behaviour are characteristic of neural and/or muscular mal-functioning and probably indicate the target sites of the phyto-chemicals inherent in the extract, as well as, the kind of physiological effects caused by them.

The results of this bio-assay showed that the extract of *G. lucidum* had significant larvicidal effects on the mosquitoes. This effects may be due to the presence of bioactive phytochemical compounds such as steroids, alkaloids, glycoproteins, phenols, saponins, etc., in the extract of *G. lucidum* (Sosan *et al*., 2001; Wiesman and Chapagain, 2006; Boh *et al*., 2007; Rafael *et al*., 2008; Wu *et al*., 2001). These phytochemicals have been found to possess high mosquito larvicidal efficacy (Shaalan *et al*., 2005; Deore and Khadabadi, 2009; Chaieb, 2010). Ordinarily, these secondary metabolites serve defensive purposes against parasites and pathogens that attack the host plants. This metabolite activity, probably, explains the toxicity *Ganoderma lucidum* extract to larvae of *Cx. p. pipiens*. In addition to the phytochemicals mentioned as inherent in extracts of *G. lucidum*, the most bio-active compounds in the species have been identified to be polysaccharides and triterpenes (Boh *et al*., 2007; Zhou *et al*., 2007). Though, reports on insecticidal activities of these two physiologically active *G. lucidum* phyto-chemicals are rare in literature, the anti cancer-tumor chemotherapeutic efficacy, for which the species is highly valued, have been severally attributed to the presence and activities of these compounds in the species extract (Min *et al*., 2000). Recently, triterpenes obtained from *Ganoderma* species, for example, were found to induce cytotoxic activity against cancer tumor-cells (Min *et al*., 2000; Wu *et al*., 2012), through leakage of cytochrome c from mitochondria (Green and Kroemer, 1998). According to Zafra-polo *et al*. (1996), both insecticidal and anti-tumor processes engage physiologically similar mechanisms of cell action, through the blockage of cell oxygen transport system. Therefore, the relatively high larvicidal activity exhibited by *G. lucidum* extract against *Cx. p. pipiens* mosquito

in this study, may be attributable largely to the polysaccharides and triterpenes characteristic of the extracts of *G. lucidum*. This finding, probably, sets *G. lucidum* aside from other plants that have been investigated for mosquito larvicidal potentials, as the extracts of such plants induced larval mortality through the activities of commonly available phytochemicals including, flavonoids, saponins, phenols, etc.

The results of this study indicate that percentage mortality increased significantly with a rise in the concentration of extract and duration of exposure. The observations agree with those of previous studies that bio-assayed the extracts of different species of plants against mosquito larvae (Bagavan and Rahuman, 2011; Haldar *et al.*, 2011; Sheeja *et al.*, 2012). While the proportional relationship between larval mortality and concentration of extract may be due to sudden uptake of toxic phytochemicals, above the tolerance limits of the larvae; the delayed lethal effects of duration of exposure on mortality, is probably related to gradual time-wise bio-accumulation of toxins above the threshold necessary for the manifestation of signs of poisoning. On their parts, Zebitz (1986) and Kihampa *et al.* (2009) opined that increased mosquito larval mortality due to extended exposure to a particular concentration of plant extracts may be due to disruption of endocrinological processes that, regulate ecdysis and metamorphosis in mosquito larvae.

The LC_{50} and LC_{90} values of the extract of *G. lucidum* against larvae of *Cx. p. pipiens* obtained in this study (i.e., 2.26 and 4.25 mg L^{-1}, respectively) are relatively low, compared with similar values generally reported for bio-assay of plant extracts against mosquito larvae, with a range of LC_{50} of 3.50-288.00 mg L^{-1} and LC_{90} of 15.77-1, 991.22 mg L^{-1} (Latha *et al.*, 1999; Komalamisra *et al.*, 2005; Krishnappa *et al.*, 2012; Ravi *et al.*, 2012). Variations in lethal concentration values of plant extracts against mosquito larvae have been attributed to genetic and phytochemical derivative differences among plant species; parts of plant extracted for bio-assay; type of solvent used for extraction; developmental life-stage of mosquito bio-assayed, etc. (Komalamisra *et al.*, 2005; Krishnappa *et al.*, 2012) Thus, the relatively high efficacy of the extract of *G. lucidum* stands it in good-stead, as a viable potent source of phyto-chemical lead-agent, for the development of a sustainable eco-friendly larvicide against mosquito vectors in general and *Cx. p. pipiens* species in particular.

CONCLUSION

The findings of this study revealed that *G. lucidum* holds great potentials as source of phytochemical lead-agents for the development of alternative sustainable eco-friendly larvicides for vector control of mosquito-borne diseases. This potential stems from the fact that, the extract of the plant species elicited significant larval mortality against *Cx. p. pipiens,* presumably with multi-target mechanisms; induced by medicinally-potent but insecticidally-naïve phytochemical metabolites namely, polysaccharide, tripertenes. Such insecticidally-potent but unrecognized phytochemicals in the physiological pool of insects, should yield larvicides that may not be vulnerable to resistance by mosquito vector species, as is presently the case with insecticides developed from the more physiologically-familiar phytochemicals. These physiological advantage and relatively low LC_{50} and LC_{90} of the extract of *G. lucidum* against *Cx. p. pipiens* larvae, as revealed by the results of this study, stand the plant species in good stead as source of the insecticide of choice of the near future. However, further studies are germane for guided fractional bioassay of the active principles of extracts of *G. lucidum* against different mosquito vector species, as well as, to elucidate the mechanisms of action and degree of specificity of the active principle fractions of the extracts, in order to fast-track this realization.

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