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The implicit remedy to eradicate *Culex Pipiens* with eco-friendly bio-integrated lemongrass palladium metallic nanoparticles

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Abstract
The mosquitoes are responsible for the vector born diseases. The different species of mosquitoes spread various diseases all over the world. The mosquitoes are of different types depending on their habitat and occurrence. The familiar illnesses spread by mosquitoes include malaria, dengue, chickungunya, WNV, and yellow fever, and recently known for its involvement in spreading the zika virus. In the mosquito's body, harmful parasites and viruses survive; hence, they serve as a carrier or vector. These parasites get protected in the gut and liver of the host; they multiply and develop fully. After mosquito bites, the parasites or viruses are ready to span the host membrane o birds, animals, primates & human beings. During the bite, the proboscis of a female mosquito spans the host's skin and sucks the blood, resulting in the exchange of body fluid. It allows the entry of antigens into the host body and multiples rapidly in the next 48 hr. As a result, the immune response generated by the host is not efficient after a few days of infection, and the host starts to develop the symptoms of the respective disease. If the patient is not diagnosed earlier, it may be life-threatening to the individual. The *Culex pipiens* species of mosquito dwell near the household areas, most probably the human locality, and spread the disease like WNV, and yellow fever; such mosquito species can be controlled by bio blended metallic nanomaterials such as LgPd NPs (lemongrass palladium metallic nanoparticles) around 20 to 30 nm in size. Due to the nanosize of LgPd NPs, it accumulates in the developing larvae of *culex* spp. and during its 4th instar stage of metamorphosis, 95 % of larval death occurs. The 10 µg/mL concentration of LgPd NPs, sufficient to cause high mortality in *Culex* spp., were observed. Also, the cumulative effect of lemongrass (*Cymbopogan citratus*) (anti-microbial, anti-parasitic & anti-viral) and palladium as a carrier moiety suppresses the growth. It shows the zero tolerance towards *Culex pipiens*.

Keywords: *Culex pipiens*, mosquitoes, larvae, host, vector-borne disease, LgPd NPs

Introduction
Mosquitoes (Diptera: Culicidae) are found worldwide. Their occurrence is almost every part of the world, such as frigid regions or a place like Antarctica. In Iceland, mosquitoes are none or are rarely present (Kraig W. nationalgeographic.com & India Today Web Desk, 2017) [1, 2]. The mosquitoes are mostly habituated in the tropical region of Asia, America and the temperate region of the world (Bellone R and Failloux AB, 2020) [3]. They adapted to survive in all environmental conditions and reproduce easily even after exposure to pesticides. A single female mosquito can lay 4000-5000 (10 broods) egg's during her entire life cycle (TERMINIX, https://www.terminix.com) [4]. The mosquito prefers to breed in shallow and stagnant water bodies. It includes the sewage water of the municipality, the water from industrial effluents, lake water and improperly stored drinking water (WHO, Publication No. 66, 1982) [5]. After spawning with the male mosquito, the female mosquito is ready to lay fertilized eggs on the surface of nearby water sources. The female mosquitoes have a good fecundity rate, and the malarial parasite infection may decrease it in the female mosquito (Vézilier J *et al.*, 2012) [6]. It lays a bundle of eggs at a time (50-500) in the form of an egg raft. These egg rafts are nothing but the egg cluster, and we can see them with our naked eyes. This egg hatched and developed into mosquito larvae within 48-72 hr after the pupa stage. Once they hatch, the stages of metamorphosis or moulting initiate, starting from 1st instar larvae to 4th instar larvae. Later on, it gets converted to pupa and finally moulted into the adult mosquito (OLABS, MeitY, CDAC, 402) [7]. There are different types of mosquito species present worldwide. Some known mosquito species include *Anopheles spp.*, *Culex spp.*, *Aedes spp.* etc. These varieties of mosquitoes are the causal vectors to spread a variety of diseases (malaria, yellow fever, dengue) and viruses such as chikungunya, WNV (West Nile Virus) and zika virus, respectively (Tandina F *et al.*, 2018) [8]. They are the bad carriers for the spread of parasites and viruses that are deadly to animals and human beings. The *Culex pipiens* occurs mostly near the human houses, and its likely targets are the birds such as poultry, pigeon, and humans (Jour TY *et al.*, 2019) [9]. The female mosquitoes have long proboscis to pierce the skin of animals rather than male mosquitoes. Therefore, most female versions are blood-sucking, and only a few survive on the nectar of flowers. The male mosquitoes predominantly survive on the nectar of flowers (National Museum of Natural

History, Cypress Hansen, 2021) [10]. Therefore, female *Culex* mosquitoes are more dangerous than males. It sucks the blood of animals and simultaneously transfers the parasite responsible for yellow fever, viral diseases like WNV in human beings, birds, horses etc., detected after certain blood tests and diagnosis. To avoid such abnormal fever cases in animals and humans, there is a need for effective tools that control the mosquito population at the ground level. There are various scientifically proven medications available today, but it still has certain limitations. These remedies cannot eradicate them. Instead, they have certain drawbacks, such as forming resistant mosquito species (Cuervo-Parra JA *et al.*, 2016) [11]. Other issues include allergies to coil smoke from mosquito repellents, lung infections due to prolonged inhalation of coil smoke, and kidney-related diseases also detected in the mouse model (Anusha C *et al.*, 2017) [12]. The bi-product left over after use and its disposal causes the problem of environmental solid waste management, and it is a little costly to use daily. For example, tools like mortein, good knight, good knight fast card, mosquito repellent, and baby mosquito repellent work in small spaces in the gaseous state. Rather, they will not provide any substantial work at ground level, where the mosquito begins its life, i.e. the water. These products are functional only in urban areas and not in villages because they do not have a solution from the basics, and there is no assurance of complete rid of mosquitoes. On the other hand, they are not budget-friendly for poor and middle-class families to use daily. Therefore, there is a need for a one-stop solution to the demand of the present condition. Hence, eco-friendly lemongrass palladium metallic nanoparticles (NPs) are designed to avoid allergic reactions and organ damage from the smoke of repellent products in animals and human beings. These NPs are biologically synthesized using plant extract, and hence, these are biocompatible with the livestock. The LgPd NPs are biocompatible, bio blended and can be used in dual therapy. The plant extract act as an anti-microbial, anti-parasitic & anti-viral and the palladium metallic moiety helps as a carrier. Apart from that, the nano size of NPs allows them to enter the newly developed larvae via siphon tube, and due to the EPR (Enhanced permeation and retention) effect, NPs accumulate in the larval structure (Yao Y *et al.*, 2020) [13]. There is no hepato-biliary and renal clearance system present in the mosquito larvae; hence, the accumulated NPs, due to their anti-microbial, anti-parasitic & anti-viral properties, cause mortality in *Culex pipiens* larvae during the moulting stage.

Experimental

The materials used to perform the study were shown in schematic 1 (S1) and used in their original received form.

Materials

Chemicals, Instrument and Software Applications

Aquarium wastewater, Glass aquarium, Glass beaker (500 mL), Fish powder worm food purchased from Amazon. in. The lemongrass plant obtained from the campus of SRTMUN, $PdCl_2$, HCl, and H_2O_2 were purchased from Sigma Aldrich, Magnetic stirrer, Probe sonicator (Ultra Autosonic India, 1800 W), UV-Vis spectrophotometer (190-1100 nm, Deeksha Analytical Pvt. Ltd. Bengaluru), TEM (Hitachi High-Tech, Europe), Microscope (Olympus MX, Japan), UV lamp (Philips™ - UV), Graphpad Prism 5 software application etc.

Culture of Culex Pipiens Larvae

This experiment was designed with minor modifications to the original one to culture mosquito larvae. The experimental setup was as follows. The aquarium wastewater was added to the empty glass aquarium, kept overnight in a dark condition, and covered using the lid with a small opening to enter female *Culex pipiens*. On the following day, the top was opened up, and the egg rafts were collected and transferred to a glass beaker (1 egg raft/beaker) (S1) (Dehghan H *et al.*, 2013) [14].

Identification of *Culex pipiens* Eggs and Larvae

The collected *Culex pipiens* eggs & the developing larvae were observed under the microscope (Fig. 1. A. a - d).

Rearing of *Culex pipiens* Larvae

The mosquito larvae started hatching from the egg raft. The powder form of fish food was added (if needed) to each beaker to feed the larvae and grow them rapidly. The complete life cycle of the development of *Culex pipiens* took almost 8 to 10 days of duration starting from egg to adult mosquito formation (Fig. 1. A. a - d).

Collection and Preparation of Lemongrass Plant Extract

The lemongrass plant was collected from the SRTM university campus, Nanded, MH, India. The collected lemongrass was washed twice with deionized water carefully. After removing dirt and solid particles from the lemongrass, it was gently soaked with a hair dryer. This experiment was designed with few modifications to achieve the original aim. Later on, the lemongrass was chopped with a fine scissor into small pieces, and around 5 g of finely chopped grass pieces were weighed. These lemongrass pieces were added to 250 mL of Milli-Q water in 500 mL of the beaker and kept boiling at 50 °C for the next 1 hr. Once the boiling was over, let the solution cool down to room temperature and filter through the sieve and whattman filter paper to remove the remaining solid debris. The solution is then kept for centrifugation at 2000 rpm for around 10 minutes. In the next step, the pellet was discarded, and the supernatant was stored at 4°C for further use. The final concentration of lemongrass extract was 20 mg/mL (Fig. 1. B) (Emmanuel A *et al.*, 2017) [15].

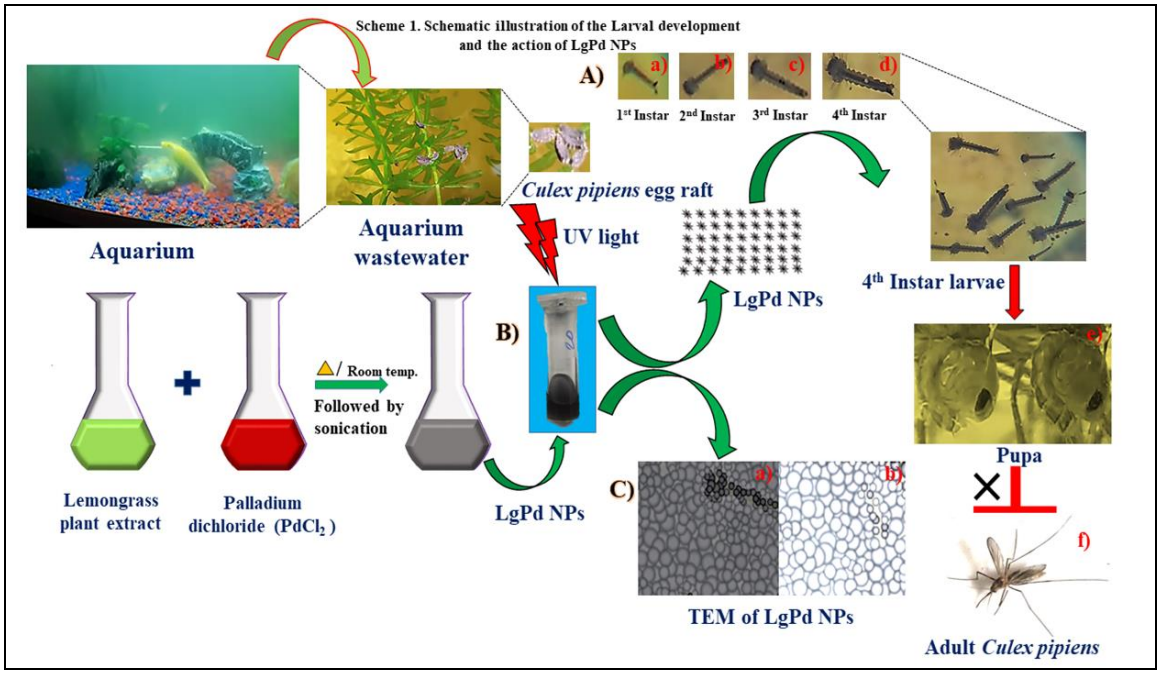

Fig 1: A) Larval developmental stages a) 1st Instar b) 2nd Instar c) 3rd Instar d) 4th Instar e) Pupa f) Adult Mosquito, B) Solubility of LgPd NPs, C) TEM of LgPd NPs a) Dark view b) Light view

Synthesis of Bio-Integrated LgPd NPs

The palladium dichloride is poorly soluble in water. It is completely dissolved in 7% of HCl & 5% of H_2O_2 solution at 60°C under stirring conditions for the next 2 hr; as a result, the pale violet coloured solution of $PdCl_2$ will form. Therefore, in the next step the 5 mg of $PdCl_{2\ was}$ weighed on the weighing balance and dissolved in 50 mL of 7% of HCl & 5% of H_2O_2 mixture together (Nguyen VNH *et al.*, 2011) [16]. This experiment of NPs synthesis was performed with light changes in the original investigation (Dan Z *et al.*, 2020) [17]. The final concentration of the solution was 0.2 mg/mL. In the next step, 5 mL of $PdCl_2$ solution was mixed with 95 mL of lemongrass plant extract, and the solution was kept for stirring overnight at room temperature. Later on, this solution was sonicated for 10 minutes to avoid agglomerates, and the prepared NPs sample was ready for further characterization (Yamilee KD *et al.*, 2021) [18]. The final concentration of the synthesized LgPd NPs was 10 µg/mL of solution (Fig. 1. B).

Characterization of Bio-Integrated LgPd NPs

All the characterization of LgPd NPs is described in (S1).

Larval Toxicity of Nanoparticles (Quantitative Analysis)

The reared larvae of *Culex pipiens* were subjected to the action of NPs. The 5 mL of LgPd NPs were added to 500 mL of a beaker containing the 4th stage instar larvae of a single egg raft and kept under incubation for the next 48 hr at room temperature (Abdul J *et al.*, 2021) [19]. The control was set without adding NPs, and during imaging, 2 mL of bleach solution was added to both the beakers (Fig. 3. A. a). In the next step, the toxicity and results were analyzed. This experiment was done with certain modifications.

Results and Discussions

Identification of *Culex pipiens* Eggs and Larvae

The collected *Culex pipiens* eggs and the developing larvae were observed under the microscope. The eggs were pale lavender, and they stuck together one by one and it formed a structure called egg raft (S1) (Centre for disease control and prevention, CDC, 2020) [20]. In each raft, the number of eggs varied from 50 to 500. In the 3rd instar stage, the larvae had two predominant black spots on its dorsal side, which is the characteristic feature of *Culex* larvae (Sallum MAM *et al.*, 2020) [21]. In the 4th instar stage, those spots started to vanish and became dull in colour (S1). After the 4th instar stage, the pupa or tumbler was formed, and it was the last moulting stage before growing into an adult mosquito similar to a fatty comma in appearance. In the final stage, the mosquitoes were developed into adults and started to leave their respective places as usual (Fig. 1. A. a - d, S1).

Characterization of Bio-Integrated LgPd NPs

The process of reduction reaction synthesized the nanomaterial. The action of lemongrass extract reduced the $PdCl_2$, and the reduced $PdCl_2$ converted to metallic ions (Pd^{++}) & finally the NPs were synthesized. The further reaction and followed step of sonication increase their stability and restrict the nanomaterial from agglomeration. Therefore, it resulted in the formation of 20-30 nm size LgPd NPs, which were characterized further. The biosynthesized LgPd NPs were observed under UV lamp for short-wavelength (254 nm) and long-wavelength

(320 nm) to determine whether they showed any inherent fluorescence properties or not (S1). During this experiment, little bleach was added to avoid the light scattering and blue coloured autofluorescence. The resulting analysis shows there was no fluorescence detected in the NPs. The LgPd NPs were then subjected to UV-Vis analysis and followed the protocol (Sallum MAM *et al.*, 2020) [21]. The aqueous solution of LgPd NPs absorbs the visible light wavelength and shows a small peak at 450 nm. This indicates the transition of π–π* bonding of electron ((Fig. 2. A. b). TEM is the important characterization step in measuring the size and shape of the nanomaterial. The NPs diluted solution was placed on the TEM grid's rough surface to determine its actual size. The size of the LgPd NPs measured with the TEM was in the range of 20-30 nm (Fig. 1. C. a, b). The particle was spherical with light black. There was no agglomeration between the particle, and serially arranged LgPd NPs were observed in the TEM analysis (Yamilee KD *et al.*, 2021) [22].

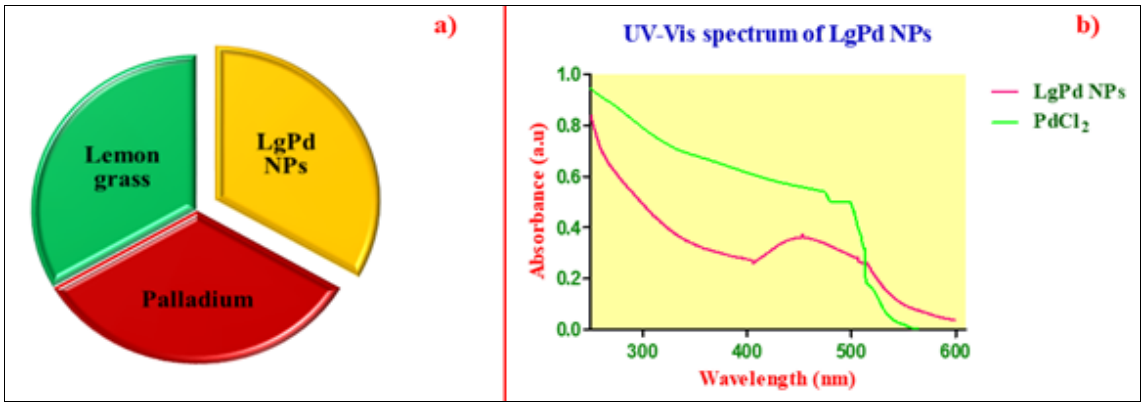

Fig 2: A) Biosyntthesis of LgPd NPs a) Graphical representation b) UV-Vis spectra of LgPd NPs

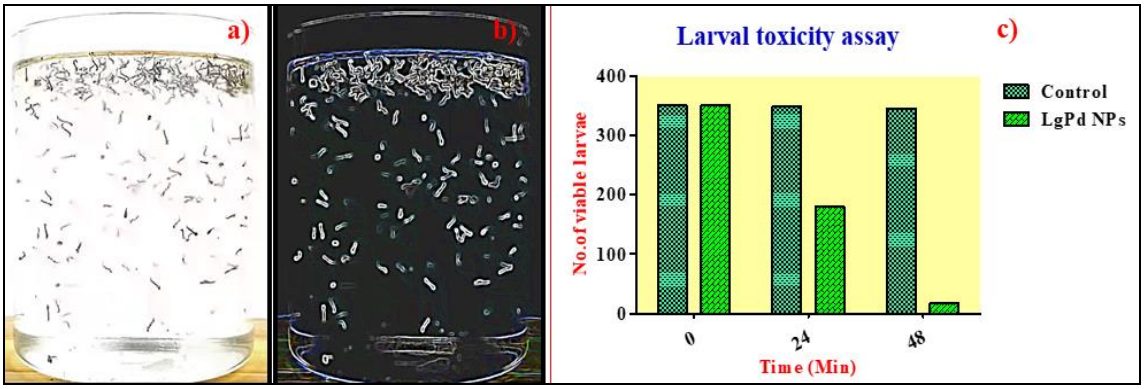

Fig 3: A) Larval toxicity assay a) Bright field image b) UV blue filter image c) Larval toxicity assay

Larval Toxicity of Nanoparticles (Quantitative Analysis)

To study the larval toxicity of LgPd NPs on *Culex pipiens*, the developed larvae were transferred into the 500 mL beaker. The transferred larvae were in the 4th instar stage of development. The reason behind choosing the 4th stage instar larvae was that the metabolic rate and the respiration process in the 4th instar stage of larvae were high compared to another stage of the development (Padmanabha M *et al.*, 2020) [23]. Due to increased respiration, the rate of siphoning of larvae to exchange gaseous or liquid material and food content from the outer environment was also raised. This results in the accumulation of LgPd NPs due to its nanosize and the EPR effect inside the developing larvae and leads to the death of the larva in the next 48 hr. These larvae could not surpass this stage and will never enter the pupa stage. The larva mortality was seen after adding LgPd NPs with naked eyes and under a UV lamp (Fig. 3. B. a). The mortal larvae were floating on the water surface and in the middle of the beaker. The floating of dead larvae is feasible from the entropy point of view. It is energetically favourable for the water molecules to retain their motion to the normal stage (Huggins DJ, 2015) [24]. Initially, the larvae were 350 in number. Out of which almost 95 % of larvae died in the 4th instar stage, and the remaining 5 % were physically impaired and converted into pupa stage, few of them died, and the resistant survived. It was observed that only 17-18 ±2 larvae surpassed the 4th instar stage with physical impairment. Most of them died again in the development's pupa stage, and only 2-3 ±1 were converted to adult mosquitoes (S1) (Fig. 3. B. b). In the case of control, all the larvae were hatched and converted to adults with zero mortality. The control beaker was already covered with a nylon net and the adult mosquitoes formed were caught and killed by the mosquito repellent spray (Fig. 3. B. a). This experiment was repeated thrice and plotted the graph with standard deviation.

Conclusion

The results showed that the LgPd NPs successfully inhibited mosquito larvae growth, even if some were resistant. Still, they become physically impaired and will not survive until the end of the adult stage. Therefore,

the 10 µg/mL concentration of NPs is enough to kill almost one egg raft of *Culex pipiens* bearing 350 eggs/raft successively. This study aims to open future directions concerning controlling the mosquito population by using biocompatible bioblended metallic NPs, which help reduce the vector born diseases.

Declaration of Competing Interest
The author declares there is no conflict of interest.

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