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# Green and eco-friendly lemongrass palladium metallic nanoparticles for biocompatibility studies in *Danio rerio* and cytotoxicity studies in *Aedes aegypti* mosquito larvae

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**Abstract**
The LgPd NPs were synthesized biochemically, which are green NPs. The size of the NPs was around 20 nm, with an oval shape shown in the TEM of previously published data. These NPs were effectively trapped in mosquito larvae and caused the death of Aedes larvae due to ROS formation. Similarly, the NPs were treated with zebrafish embryos to check their toxicity level in higher animals, but it was observed that there was no near-death were recorded. The 10 µg/mL of LgPd NPs were enough to cause mortality in mosquito larvae, but a similar concentration in zebrafish embryos will not be lethal at all. The mechanism behind it was studied in these two species concerning the EPR effect and RES system and the generation of ROS supporting the proper development of higher animals and their effective body mechanism. This evaluation helps the biocompatible nature of prepared NPs and shows larvicidal activity against mosquito larvae. The current study explored the minor concentration, i.e. 10 µg/mL of LgPd NPs, successively killing the larvae, proving their larvicidal activity. ROS production deforms the larval structures; ROS formation was eliminated in zebrafish, and its effect was neutralized. This targeted therapy was helpful in theranostics purpose in living animals.

**Keywords:** *Danio rerio*, cytotoxicity, *Aedes aegypti*, larvicidal, EPR effect, RES, LgPd NPs

## Introduction

The zebrafish, also known as *Danio rerio*, is a well-known animal model for studying various diseases in human beings. The reason for choosing zebrafish as a model organism is the fast embryo development. The zebrafish eggs hatched in the next 48 hr, and the larvae will start to swim freely in the water [1]. Upto this developmental event, all the organelles were formed and started functioning normally. During the first few events of embryogenesis, the embryo becomes transparent so that we can study all the components of our interest nicely. Therefore, this efficient development of *Danio* provoked researchers to study all the pathways during the differentiation in a rapid duration, reducing uiredtiredxperimenting on multiple occasions [2]. Instead of a mice model, such a study will take a shorter period to execute. The zebrafish model works almost similarly to human beings. Hence, the diseases such as cancer, HIV, Alzheimer's, cardio disorders, neurodegenerative disorders, lung infections, kidney dysfunctions, and many more are ongoing research in this model to find the possible alternative and theranostics for patients [3]. The zebrafish is a bisexual organism. The female *Danio* possesses the belly for egg development with silver-coloured bands alternating with blue ones. The male *Danio* is streamlined with pale yellow-coloured bands prominent during the breeding season. It is a freshwater fish and can be cultured in a lab environment. It is easy to culture and maintained in a small space as well. Therefore most of the research in today's date is carried out in *Danio rerio* [4]. The *Aedes aegypti* is a vector mosquito for various diseases; hence, it must be controlled and killed to protect human life from its threat. The mosquito spreads viral and parasitic diseases effectively from one individual to another. Mosquitoes are present in every part of the world. Therefore it is essential to control their strength in advance to get protected from diseases [5]. The WHO says 6, 27, 000 deaths occur worldwide due to vector-borne diseases (VBDs), particularly due to malaria spread by mosquitoes [6]. In most cases, the Aedes are responsible for spreading chikungunya, yellow fever, dengue and zika virus [7]. The mosquito is an arthropod with jointed appendages and can fly as it belongs to class dipterans. The body system is not well developed; all the crucial and true organs are absent. There is an absence of a liver in the mosquito, due to which the malarial parasite cannot multiply in the mosquito; it needs a host to perform the multiplication after invading the liver. Therefore, such mosquitoes are the only vectors that carry life-threatening viruses and parasites along with them to attack host animals. There is a primitive system present in mosquitoes. Hence, they can be killed easily, but their reproduction rate is high, and in a few days of development, they are ready for reproduction and lay the eggs in the form of a raft [8]. They do not need any special treatment for their survival. They need little water resources where they can lay the eggs and produce the larvae of mosquitoes. The Aedes are the blood-sucking vectors; hence they can easily transfer the pathogens from one to another through their bite. They suck the blood, and at the same time, the pathogen gets to enter the host blood. The parasites do not play any multiplication or reproduction in the mosquito body due to the lack of real organs. Therefore the parasites or viruses in the mosquito body do not multiply due to the absence of an adequate amount of nutrient supply [9]. Once the infected mosquito invades the host's skin, the parasites enter and perform their function, including multiplication; then, various steps in the liver and finally enter into the blood and show the deadly symptoms. Therefore, it is essential to kill the mosquitoes to protect humans from such symptoms. Therefore by looking into this issue, we have come up with a solution which targets the mosquito larvae, enters their body, disrupts the cell membrane and kills the larvae before they enter the adult stage [10]. The lemongrass palladium metallic nanoparticles (LgPd NPs) are the remedy to reduce the mosquito population in their essential habitat, i.e. water. Therefore, to protect living beings, such alternative solutions must be explored. The LgPd NPs are larvicidal in nature reported previously [11] and hence, used effectively to cause lethality in the larval population of mosquitoes. The LgPd NPs are eco-friendly, and green synthesized NPs. Therefore, it does not harm the animals and is biocompatible with them. These NPs are target-oriented and kill the outside scavengers. The remaining portions of NPs were removed by the reticuloendothelial system (RES). The removal can be done via liver and kidney, called hepatobiliary and renal clearance [12]. In the case of mosquito, no such system is present that recognize the NPs and stop them from harming themselves. Therefore, NPs enter into the body of such vectors and accumulate in newly forming structures during their development. This effect is called as enhanced permeation and retention (EPR) effect. Hence, the nano size of LgPd NPs plays a vital role in entrapping them into the newly forming structure and deforming them by the cumulative action of lemongrass extract by forming reactive oxygen species (ROS) and palladium NPs. Therefore, it disrupts the structure, breaks the lipid bilayer and kills the larvae; hence, it can be called larvicidal or insecticidal [13].

## Methods

### Materials

Light microscope, previously synthesized LgPd NPs, Zebrafish received from ARI, Pune, Zebrafish eggs (embryos), Aedes larvae, Milli-Q water, Graphpad prism 5 software etc.

### *Identification of Aedes larvae*

The Aedes mosquito and larvae its identified to study their modus operandi to infect living beings. The Aedes mosquitoes are preferably grown in a water medium and develop into larvae pupa and adult mosquitoes. The larvae were readily available in surrounding water bodies or could be cultured and reared in the lab for experimentation. Hence, the mosquito larvae were cultured according to the previously published data. In brief, the aquarium water was used to culture Aedes mosquito larvae. The significant difference was only the change in mosquito species. The present mosquito larvae species were identified under a light microscope, pictured well and stored in data for further record [14].

### *Synthesis and characterization of green and eco-friendly metallic LgPd NPs*

The production and its characterization were already done in previous publications. In short, the LgPd NPs were characterized for size via TEM analysis, and it was the confirmatory study for the shape and size of the NPs prepared [11].

### *Biocompatibility studies in the zebrafish embryos*

The *Danio rerio,* i.e. zebrafish embryos, were cultured and reared in our lab for the production of embryos and to perform the biocompatibility studies in the same animal model. These fish were obtained as a gift sample from ARI, Pune. The zebrafish were kept for breeding in a 2:1 ratio of female to male respectively, overnight. There was a lid placed between the two sexes for a thorough night. In the morning, the lid was removed, allowing them to spawn freely. In a short duration of about half an hour, the spawning between male-female was completed, and after the fourplay, the female zebrafish lay the fertilized eggs. These eggs were fertilized already due to male sperm in the water [15]. The fish were removed from the breeding tank, and eggs were collected and washed with milli-Q water. Later, these eggs were kept in an E3 medium for further development and experimentation. The collected eggs were now called an embryo. Those embryos are now referred to as single-cell embryos with yolk sacs. Soon the development starts and the embryo will be differentiated and determined. To study the effect of LgPd NPs on fish embryos, the NPs solution was added to a 6-well plate containing 10 embryos in each well. The E3 medium was added 3 mL in each well. The well without NPs solution was considered as a control. 10 µg/mL to 30 µg/mL of NPs solution were added sequentially and mixed well in 6 well plates. The biocompatibility was recorded respective to the control, and a similar experiment was repeated thrice to confirm the results [16].

### *Cytotoxicity evaluation in Aedes mosquito larvae*

The Aedes larvae were cultured according to the previously published data, and later on, it was added with LgPd NPs. The incubation period was 96 hr. The experiment was performed in a Petri dish. The 10 larvae were placed in each petri dish, and accordingly, the concentration of NPs from 10 µg/mL to 30 µg/mL was added with serial dilution. The larvae were observed and recorded the data for their cytotoxicity at the respective time. This experiment was performed with slight modification to previously published data. Once the incubation time was over, the larvae were counted in each Petri plate. The plate without NPs was labelled as a control [17].

***Generation of ROS and related cytotoxicity***

The ROS was formed during the incubation period with LgPd NPs. These ROS species were known to cause lethality in mosquito larvae. Therefore, the count decreased in number as the incubation period went on. The LgPd NPs were entered through the membranous pore called the siphon tube of larvae during its respiration and trapped in its body permanently. The larvae did not have enough body toxicity removal mechanisms. Hence got affected by the ROS production of NPs, and later on, it continued, the mosquito larvae were killed automatically. The ROS were produced by the action of lemongrass plant extract which readily produces ROS in an aqueous environment with a heating effect and causes membrane damage to the insects were reported [18].

**Results and Discussions**

***Identification of Aedes larvae***

The larvae were identified in the light microscope. The main difference between the Aedes larvae and other mosquito larvae was the presence of white markings on the legs and lyre marks on the thorax along with dark colour at the 4$^{th}$ instar stage of Aedes mosquito larvae. Such characteristic features made these mosquito larvae different from the other mosquito species. The Aedes larvae were identified, and the images were successfully captured in our data. Further, the data were compared with other mosquito larvae for confirmation of the results. It was observed that the presented mosquito species belong to the Aedes mosquito species (Fig. 1. A) [19].

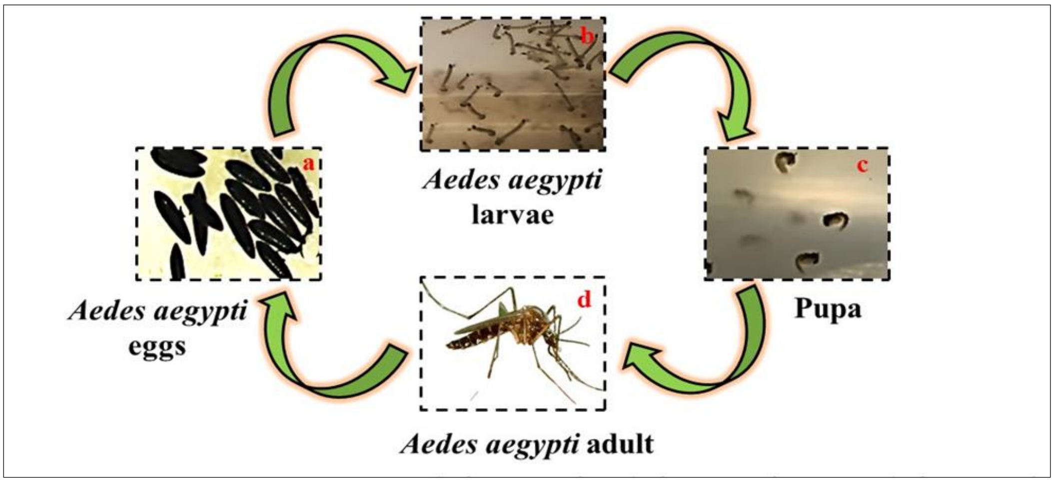


**Fig 1:** A) developmental stages of aedes aegypti a) Aedes aegypti eggs, b) aedes aegypti larvae, c) pupa d) aedes aegypti adult mosquito

***Synthesis and characterization of green and eco-friendly LgPd NPs***

The synthesis of LgPd NPs was performed with the use of previously published data. The synthesis of such NPs was eco-friendly to the living ones [11].

**Biocompatibility studies in the zebrafish embryos**

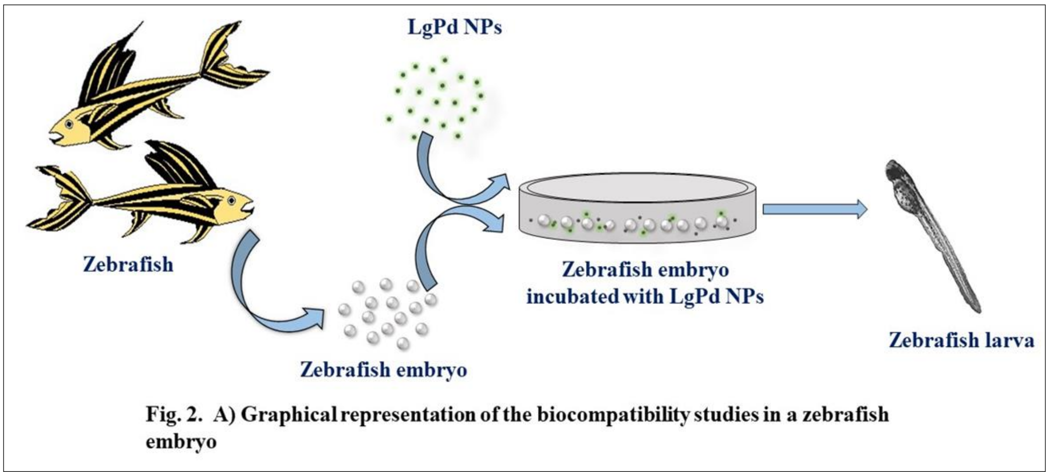


**Fig 2:** A) graphical representation of the biocompatibility in a zebrafish embryo

The biocompatibility studies were performed in the zebrafish embryo. The zebrafish eggs were kept in 6 well plates for incubation and mixed with E3 medium and LgPd NPs solution for further treatment. The results were observed nicely and predicted that the zebrafish embryo was not harmed by the normal concentration, such as 10 µg/mL of LgPd NPs. Therefore, it was proven that the LgPd NPs were not lethal for zebrafish embryos. The LgPd NPs were passively entered into the body of the zebrafish embryo by the mechanism of membrane permeability (Fig. 2. A) [20].

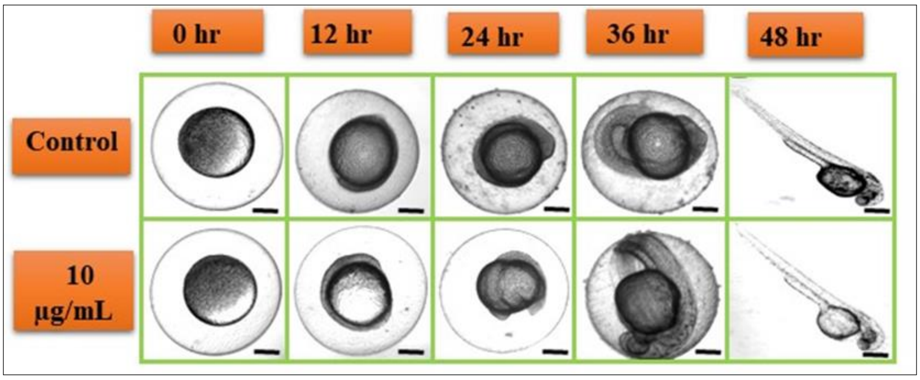


**Fig 3:** A) Biocompatibilty studies in zebrafish embryos Incubated with and without LgPd Nps upto 48hr

These NPs were biocompatible with the fish embryos. The developing embryos were persisting the organelles in their body, successfully eliminating NPs from the body by renal clearance or hepato-biliary clearance. It was reported that the highly developed organisms had suitable toxicity removal mechanisms. The NPs were injected externally, so it was exterior in their origin; hence, the body pretends them to be foreign particles and emits signals for urgent removal with a cascade of reactions. These signals were sufficient to remove such foreign particles even if they were in nanosize. The larger toxicity removal gland such as the liver played a vital function in the removal and acceptable elimination of toxic substances or foreign particles from the body. The LgPd NPs have been synthesized via the green route. Hence, such particles were already eco-friendly in nature. It was designed to automatically remove it from the body after its successful action body mechanism helps them to get eliminated themselves after their successful action [21].

In this experiment, we wanted to show that the NPs were biocompatible with the animal body and that it was not harmful to the living ones explored during the investigation. The 10 µg/mL concentration of NPs was effective and didn't show any cytotoxic effect in the zebrafish embryo (Fig. 3. A). The NPs were designed to accumulate in the living body where they found a space of nanosize, for example, in the developing arteries, or during the angiogenesis (metastasis), such particles were entered into those portions and act accordingly. The phenomenon by which such NPs accumulate the tissue was referred to as the enhanced permeation and retention effect (EPR effect). The zebrafish model was effectively studied for disease accumulation and theranostic. Therefore, the zebrafish model successfully demonstrated the EPR effect and the successive elimination of NPs after their action was achieved by the reticuloendothelial system (RES system). A similar experiment was repeated thrice and obtained identical results. The graph was plotted for the viability of embryos for an excellent understanding of the actual concentration effect of LgPd NPs (Fig. 4. A) [22].

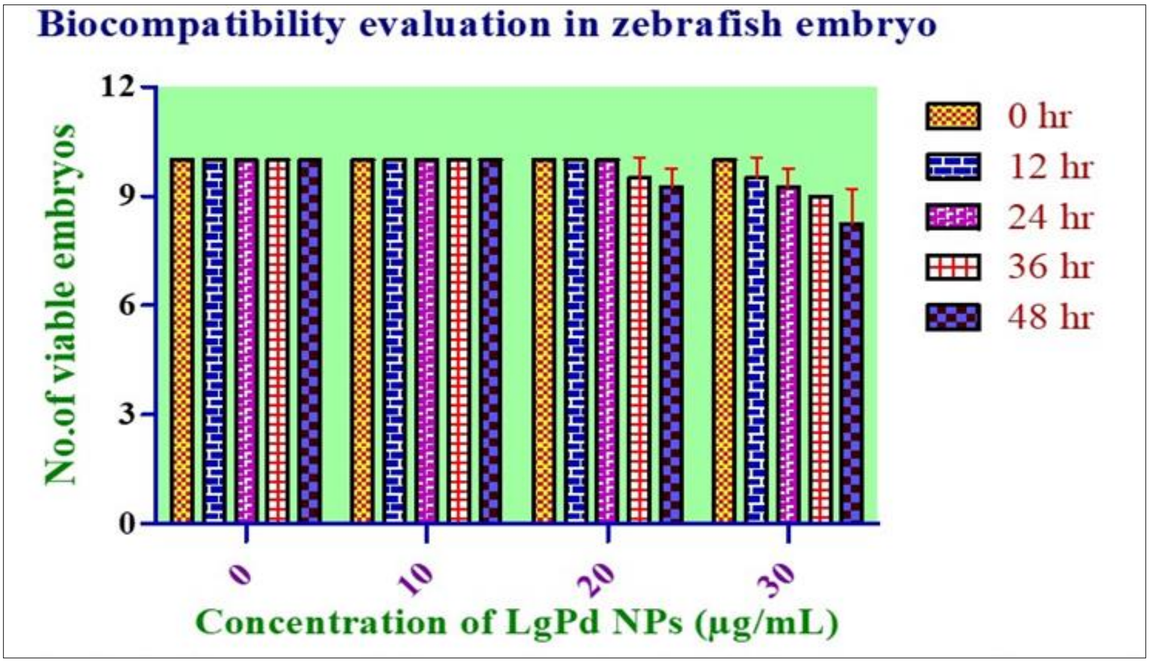


**Fig 4:** A) Biocompatibility studies in zebrafish embryos with varied concentrations of LgPd NPs

***Cytotoxicity evaluation in Aedes mosquito larvae***

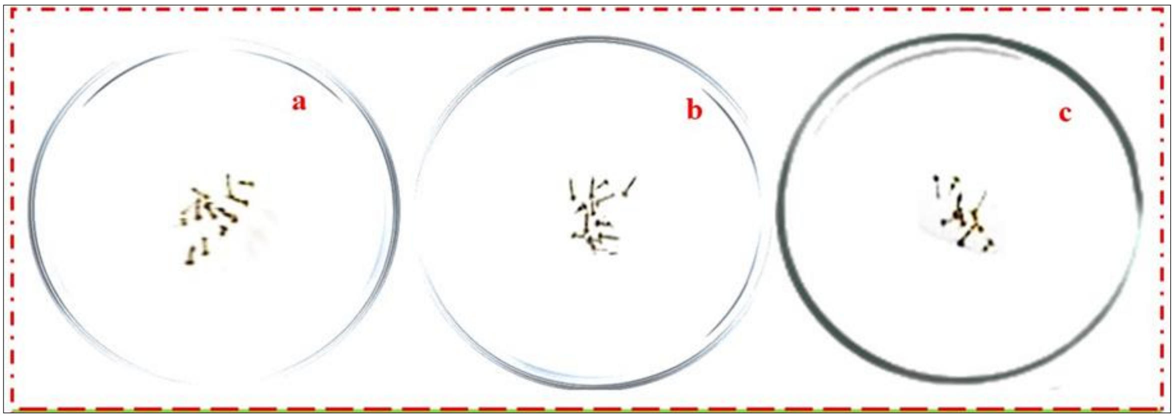


**Fig 5:** A) Aedes Aegypti mosquito larvae treated with lemon grass palladium metallic NPs a) 10 µg /mL of LgPd NPs b) 20 µg ML of LgPd Nps c) 30 µg/mL of LgPd Nps

The cytotoxicity profile of LgPd NPs on the Aedes mosquito was evaluated successfully. The concentration of 10 µg/mL of LgPd NPs was causing lethality and mortality in Aedes mosquito larvae observed in the experiments. The results were more or less familiar with previously studied and published data with *Culex pipiens.* Most mosquito larvae follow similar development patterns and the life developmental stage. The Aedes mosquito larvae follow the same way, such as egg to larvae, then converted to pupa stage and finally into adult mosquito larvae. The synthesized NPs were immensely effective in killing the larvae of Aedes reported in our study. The resulting larvae accumulated with the LgPd NPs [23].

Later on, these larvae were subjected to further forming reactive oxygen species (ROS) due to their higher concentration in the body. The larvae did not have the mechanism to remove the accumulated NPs in the body due to the loss of organelles essential for removing harmful content from the body. Therefore it results in the death of larvae. The experiment explored the best results for killing larvae and gave an alternative solution for the routinely used cures to prevent mosquito growth and spread. The final results showed that all the 10 larvae died during the experiment performed in a Petri dish with LgPd NPs (Fig. 5. A). The results were plotted with the number of dead larvae versus the concentration of NPs, and obtained results were analysed (Fig. 6. A). This experiment was performed in triplicate, and more or less similar results were found in the subsequent investigation [24].

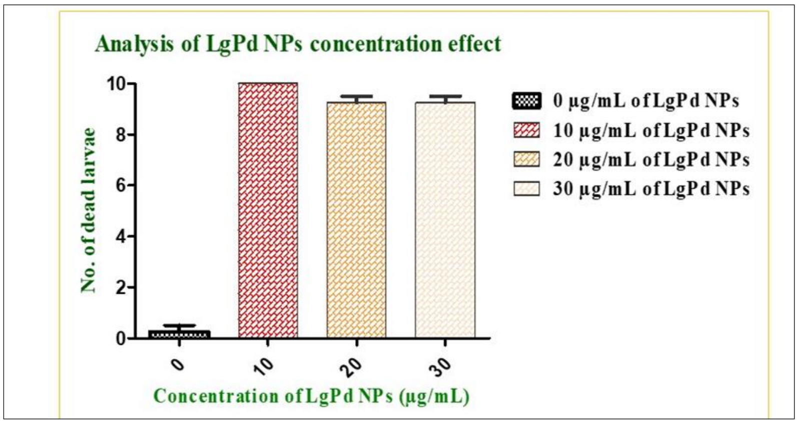


**Fig 6:** A Larvicidal Activity of LgPd NPs against Aedes Aegypti

**Generation of ROS and related cytotoxicity**

The ROS formed was due to the time action of LgPd NPs. Therefore, ROS generation is supposed to be an essential characteristic of NPs. The design of LgPd NPs made it essentially efficient in forming ROS. The lemon grass plant extract was biocompatible with living ones. But the production of ROS may facilitate the degradation of the cell membrane and finally leads to cause the death of cells, were reported in earlier research. Nanotechnology enables the active entry of the NPs into a respective target. In higher animals, the cytoplasmic organelles were responsible for removing toxicants from the body. Since creatures like mosquitoes could not survive in higher concentrations due to the loss of machinery to remove such ROS-forming species [24]. The higher animals such as zebrafish could manage the ROS species formed during the action of NPs and their body, removing such species which damaged their fate. Though this target-oriented therapy is responsible for curing diseases, the leftover content was expelled from the body. The ROS effectively killed cancer cells or abnormally growing cells as they were targeted in the case of Aedes mosquito larvae; no such elimination mechanism for

ROS was present; hence, they damaged the structure of larvae by deforming the membranous entity. The ROS is free radical species consisting of a singlet oxygen state and efficiently causes mortality in mosquito larvae. The LgPd NPs were biocompatible in zebrafish and lethal in Aedes mosquito larvae due to the formation of ROS. The larvae and the Petri dish were kept in the oven at 30 °C for around 10 minutes. The heat energy gained by the particles facilitates the production of and kills the mosquito larvae. The heat generation provokes the formation of the hydroxide free radical molecule. In the presence of superoxide dismutase, it forms hydrogen peroxide ($H_2O_2$). Later, the Fenton reaction proceeds to form the radicals. The heat reaction and lemon grass plant extract of LgPd NPs generates ROS in a cascade of response and cause the death penalty in Aedes larvae. This study showed an additional mechanism that rapidly deforms the larval structures [25].

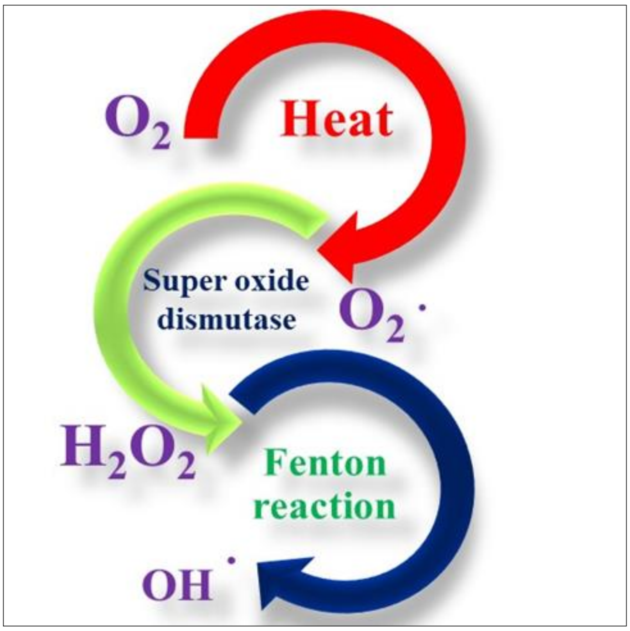


**Fig 7:** Genesis of free radical species

## Conclusions

The synthesized LgPd NPs were effective in killing the Aedes mosquito larvae. Hence, it has proven larvicidal activity. Similar NPs didn't affect zebrafish embryos; they showed their eco-friendly and non-toxic nature in higher animals. These NPs can be used in targeted therapies for the treatment of diseases. The synthesized NPs have medicinal importance as they produce ROS at the cellular level and damage larval development. The 10 µg/mL of NPs concentration was enough to cause the death of Aedes mosquito larvae. The current study opens the door for the further study of the EPR effect and RES system mechanism of higher animals and their advantages respectively. The loss of such systems in cold-blooded animal harm their survival. Therefore, the prepared NPs system is enormously helpful in preventing vectors and alternatively reducing the spread of vector-borne diseases.

## Declaration of competing interest

The author declares there is no conflict of interest to explore.

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