Characterization and Nano-Efficacy Study of Palladium Nanoparticles against Larvae of *Anopheles stephensi* (Liston)

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Abstract*: Nanoparticles are now being used to reduce the risk of mosquito-borne diseases. Nano-palladium has been used as a catalyst and in disease control. We aim for the green synthesis of palladium nanoparticles (PdNPs) using plant extract. The synthesized nanoparticles have a wide range of applications like nano-toxicity and efficacy against vectors of diseases. The application of environmental friendly PdNPs synthesized with the extract of plant Citrus limon against mosquito larvae could provide an effective aid against mosquito-borne tropical diseases. The synthesized nanoparticles were characterized and bioassay was evaluated against 3rd instar larvae of Anopheles stephensi mosquito. Surface plasmon resonance (SPR) band was observed at 450nm in UV-Visible spectrum. Active participation of biomolecules of leaf extract was confirmed with the band analysis of FT-IR spectrum. TEM analysis has shown the formation of nanoparticles with the diameter ranged from 1.9nm – 4.8nm. Elemental analysis of particles was done by SEM and EDX analysis. Mortality in test concentrations were recorded after 24h, 48h, and 72h of exposure. A lethal Concentrations (LC_{50}) has been calculated using probit analysis. Mortality due to leaf extract was not observed after 72h in the positive control. LC_{50} for percent test concentrations containing PdNPs showed LC_{50} at 16.038%, 13.231%, and 7.215% after 24h, 48h and 72h respectively. Results showed that larvicidal effectiveness of PdNPs increases with time. This can be useful in tackling emerging insecticide resistance and mosquitoes borne diseases worldwide.

***Index Terms*: Palladium nanoparticles; Green synthesis; Efficacy; Mosquito larvicides; Characterization; Nanotoxicity**

I. INTRODUCTION

Applications of metal nanoparticles are known in the majority of scientific industries, due to their sensitivity and unique functionality owing to their nano-scale size. Palladium nanoparticles have been commercially used as nanocatalyst for various chemical processes like dehydrogenation reaction of complex nitro-aromatic compounds [1, 2], alcohol oxidation reactions [3], in the reduction of organic dyes like diazo dye [4] and Suzuki–Miyaura (SM) cross-coupling reaction to generate biaryls [5]. In biological science toxicity of palladium nanoparticles has been assessed denoting their possible use as an anti-microbial agent and larvicidal agents to control dengue vector mosquito *Aedes aegypti* [6] and one of the malaria parasite vector *Anopheles subpictus* [7]. A greener approach utilizes natural phytochemicals of leaf extracts of different plants to synthesize nanoparticles to explore the potential use of plant diversity to create a novel formulation. Leaf extract of plants like *Cinnamomum camphora* [8], *Gardenia jasminoides* [9], *Glycine max* [10], *Delonix regia* [1], *Evolvulus alsinoides* [11], *Eclipta prostrate* [12], *Melia azedarach* [6], *Catunaregum spinosa* [4], *Tinospora cordifolia* [7], *Origanum vulgare* [5], commercial coffee and tea extract [13], and an extracellular polysaccharide Xanthan gum (XG) secreted by the fermentation of the bacterium *Xanthomonas campestris* [2] have been utilized for PdNPs synthesis. Mosquitoes threatened human life since ages. Female Anopheline mosquitoes act as a blood-sucking ectoparasite which also transmit malaria parasites in a zoonotic way. According to Global Health Observatory (GHO) data, death rates due to malaria has been reduced globally by 48% still 90% of the total deaths in WHO African Region occurred due to malaria [14]. Synthetic neonicotinoid insecticides used to control mosquito vectors, found to be harmful to further environmental application due to its bioaccumulation in soil and threat to the natural biological activity of earthworm [15]. Microevolution of insecticide-resistant Aedes and Anopheles mosquito species, due to selection pressure created by continuous use of synthetic insecticides over decades has occurred [16, 17]. This has led to the necessity to develop eco-friendly and effective formulation to keep the mosquito population in check.

II. MATERIALS AND METHODS

A. Preparation of Leaf Extract

Leaves of plant *Citrus limon* were collected and identified in DEI University, Agra, India. The leaves were washed and dried in a shed at room temperature. Then the leaves were finely chopped and transferred into a conical flask containing triple deionized water to make 25% aqueous broth and subjected to heat at 75°C for 1h. The active components of the broth were filtered using whatman filter paper-1 and the filtrate was stored at the 4°C in the refrigerator.

B. Preparation of Palladium Nanoparticles (PdNPs)

Palladium (II) chloride and D-Glucose was purchased from HIMEDIA and triple deionized water used throughout the procedure.

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A flask containing 45ml of 2mM Palladium Chloride and 2ml of 0.1M D-Glucose in deionized water subjected to heat and constant stirring. To the solution 5ml of prepared leaf extract was added on cooling followed by 10µl of 1M KOH, the solution was stirred constantly until the color of the solution changes from brown to dark brown. This solution was kept in dark at room temperature for 48h. Later, settled down particles were centrifuged and sonicated for 15 min. to obtain a colloidal solution for further use.

C. Characterization of Nanoparticles

The samples were diluted 1:10 in DI water and characterization studies performed after 48h. The absorption spectra of diluted sample was recorded in a range of 300nm – 700nm using UV-Visible spectroscopy on a Hitachi U-3900 spectrophotometer. The absorbance (%transmittance) spectral bands to detect the functional groups of organic molecules present in the leaf extract and reduced PdNPs solution was obtained using Fourier transform infrared (FT-IR) spectroscopy on Bruker TENSOR 37 FTIR. The FT-IR bands were identified with the help of standard interpretation of Infrared Spectra [18]. The PdNPs solution was air dried on copper grids for transmission electron microscopy (TEM) and photomicrograph was obtained by Technai G 20 (FEI) TEM. The sample (200µl of 1:10) was heat dried in an incubator at 45°C for scanning electron microscopy (SEM) with attached energy dispersive x-rays (EDX) spectroscopy analysis, performed to detect elemental composition of the synthesized nanoparticles on SEM – Zeiss EV040 and EDX spectroscopy on PANalytical X'pert PRO.

D. Mosquito Larvicidal Bioassay

Larvae of *Anopheles stephensi* were collected from the surrounding pools of water bodies in DEI campus Agra (27°, 10' N, 78° 05' E) and identified in Department of Zoology, DEI, Agra, India. Larvae were twice washed in distilled water and nanotoxicity assessment was performed according to WHO standard manual to test mosquito larvicides [19]. The percent test concentrations were prepared to contain 1ml, 2ml, 5ml, 10ml, 12ml, and 15ml of PdNPs solution in 100ml distilled water. Three replicates prepared with each containing twenty 3rd instar larvae. The positive control was prepared to contain 10ml of prepared leaf extract in 100ml distilled water. A negative control was set up containing larvae in distilled water. The test concentrations were observed after 24h, 48h, and 72h of exposure to record the mortality.

E. Data Management and Statistical Analysis

The obtained toxicity data was tabulated in MS Excel and average mortality was calculated among three replicates. The average mortality was converted into Corrected % mortality using Abbott's formula [20]. Probit analysis was performed to calculate LC_{50} values using SPSS software [21, 22]. The values for probit equations (y), coefficient of determination (R^2), chi-squared test (χ^2), lethal concentration 50% (LC_{50}), and 95% Lower and Upper Confidence limit (LCL-UCL) were obtained for test concentrations after 24h, 48h, and 72h. Probability values for corrected % mortality were converted using NORMSIV function in MS Excel to generate transformed probit values. The obtained transformed probit values were plotted in Origin software to obtain a comparative probit graph [23].

III. RESULTS AND DISCUSSION

A. UV-Visible Spectroscopy

The color of the solution containing PdNPs turned light brown to dark brown (Fig. 1 c) after 48h. The optical properties of synthesized PdNPs solution showed significantly different absorbance spectra in compare to the leaf extract suggesting complete reduction over time. The broad peak was observed around 400nm-450nm (Fig. 1). A similar weak peak observed at 420nm for PdNPs synthesized with leaf extract of *Glycine max* and the visible change in color from light to dark brown was reported, linked with the formation of nanoparticle [10]. PdNPs synthesized with *Cinnamomum camphora* leaf extract showed similar absorption spectra [8]. An identical surface plasmon resonance (SPR) band around 425nm is visible for PdNPs synthesized with the extract of plant *Tinospora cordifolia* [7].

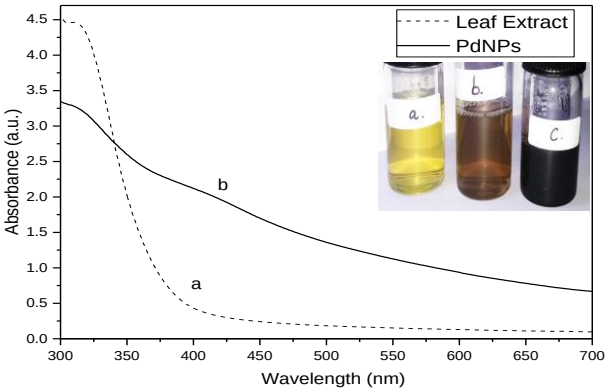

Fig. 1. UV–visible spectra of: a) Aqueous Leaf Extract of *Citrus limon*; b) Synthesized PdNPs diluted (1:10); c) Synthesized PdNPs (original sample).

B. Fourier Transform Infrared Spectroscopy

The FT-IR spectrum of *Citrus limon* leaf extract (Fig. 2a) shows a deep broad absorption band resulting due to the presence of many hydroxyl group containing compounds. The presence of transmittance band from 3200cm^{-1} to 3640cm^{-1} depicts hydrogen bonded O–H stretch of alcohols, polyols and phenols (flavonoids). Weak transmittance peak at 2100cm^{-1} depicts the presence of C≡C stretch of alkyne group, Cyanide ion, thiocyanate ion, and related ions (2200–2000cm^{-1}) and transition metal carbonyls compound groups (2100–1800cm^{-1}) The transmittance peak at 1635 cm^{-1} corresponds to NH bends of the N–H groups of secondary amines (1650–1550cm^{-1}), C=C stretch of acetylenic compound (1680–1620cm^{-1}), and amide of carbonyl compound group (1680–1630cm^{-1}). Peak in the fingerprint region of FT-IR spectrum at 684cm^{-1} is related with the C–S stretch of thiols and thio substituted compounds. The absorption bands of PdNPs solution (Fig. 2b), shows a reduced O–H band at 3300cm^{-1} which depicts that hydroxyl groups of alcohols,

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polyols and phenols have participated in the bioreduction of palladium to palladium nanoparticles. Bands at 2995cm^{-1} and 1740cm^{-1} shows C–H stretch associated with the aliphatic and aromatic structures, 2353cm^{-1} represents shifted peak for C≡C stretch of acetylenic compounds, corresponding to the exposed compounds after PdNPs formation. The band at 1616cm^{-1} is associated with C=C stretch of alkene group and can also represent N–H bend due to the presence of amines, 1355cm^{-1} and 1199cm^{-1} collectively represents skeletal vibrations due to C–C stretching in alkane groups of aliphatic and aromatic structures. The band at 1199cm^{-1} also shows C–N stretch due to presence of secondary amines. The weak band at 894cm^{-1} and 804cm^{-1} shows the C–O–O– stretch of peroxides, and at 696cm^{-1} shows the presence of C–S stretch due to thiols. [18].

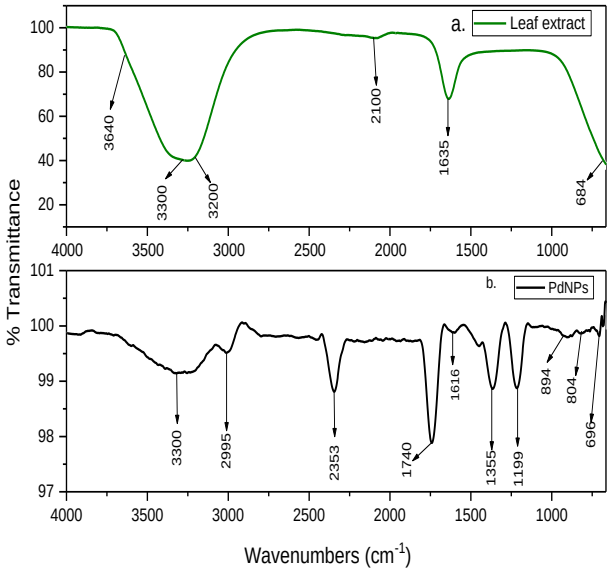

Fig. 2. FTIR - Analysis of Aqueous Leaf Extract of *Citrus limon* and Synthesized PdNPs.

C. Transmission Electron Microscopy

The spherical shaped palladium nanoparticles observed in TEM micrographs (Fig. 3). The nanoparticles appeared to be covered with the capping agents present in the extract. The agglomeration of surface capping agent is observed but no agglomerated nanoparticles within it. According to histogram generated using ImageJ software [24] (Fig. 4) to analyze the size distribution of PdNPs. The size of PdNPS ranged from 1.9nm – 4.8nm with the mean diameter of 2.3nm ± 0.04nm.

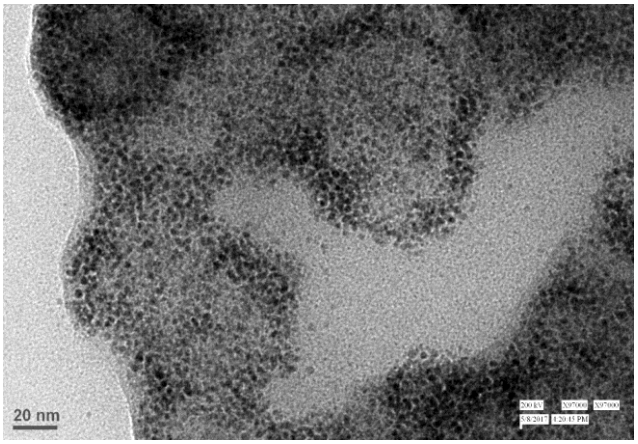

Fig. 3. TEM Micrograph of PDNPs Synthesized by Aqueous Extract of *Citrus limon*

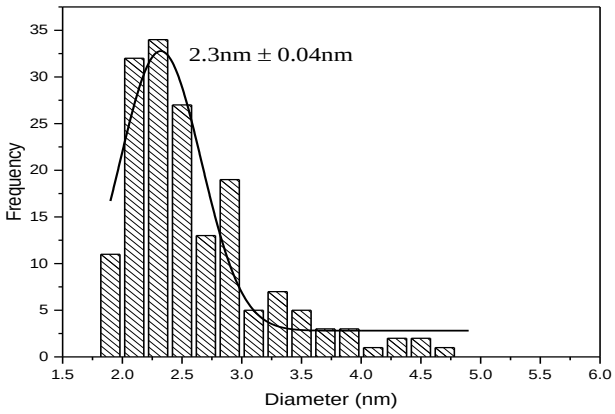

Fig. 4. Histogram of Particle Diameter (nm) Estimation of PdNPs Synthesized by Aqueous Extract of *Citrus limon*

D. EDX Spectroscopy Attached to SEM

The sterile carbon tape coated with PdNPs sample visualized in SEM micrograph as small illuminating white dots (Fig 5) used for EDX analysis (Fig. 6). The characteristic optical absorption peak at 2.99keV with high intensity of 90 counts per second (cps/eV) in L-series confirmed the synthesis and presence of PdNPs in the solution.

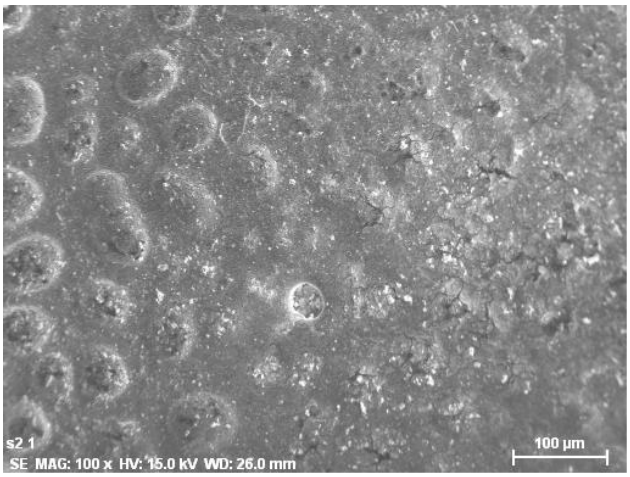

Fig. 5. SEM Micrograph of Synthesized PdNPs

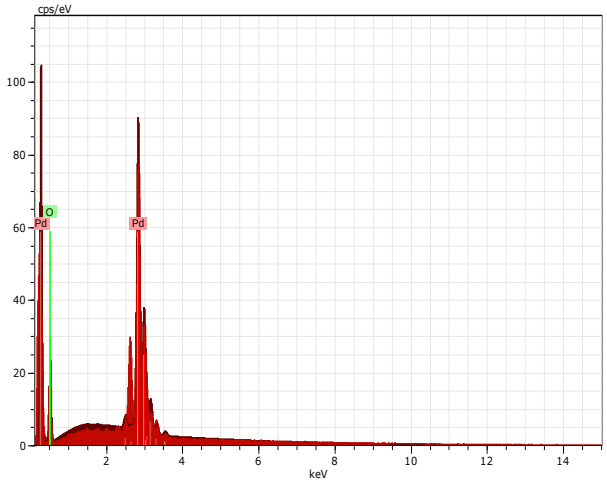

Fig. 6. XRD Analysis of Synthesized PdNPs Shown in SEM Micrograph

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E. Bioassay

The larvae were observed to survive in negative controls containing only distilled water and positive controls containing leaf extract, without the food source for more than 72h. A comparative probit regression graph is generated for bioassay result after 24h, 48h, and 72h of exposure (Fig. 6). Probit equations were generated from the data using SPSS (Table 1). LC_{50} value was estimated at 16.038% of test concentrations of PdNPs after 24h, 13.231% after 48h and 7.215% after 72h. The coefficient of determination (R^2) is 0.99 after 24h, 0.96 after 48h, and 0.92 after 72h, which indicates the reliability of data and the high fitness of regression line for the obtained data on the plot. Chi-squared (χ^2) test results (Table 1) at degrees of freedom (*df*)=3 and 0.5 level of significance showed homogeneity of data after 24h thus not reliable to calculate the confidence limit (CL) for LC_{50} after 24h. The data obtained after 48h and 72h showed heterogeneity of data and thus it is reliable to calculate the confidence limit (CL) for LC_{50}. 95% confidence limits were obtained by calculating y-intercept ± standard error (SE).

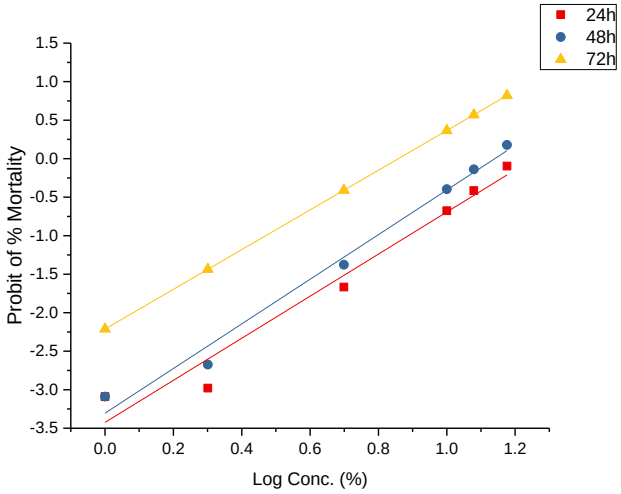

Fig. 7. Probit Transformed Graph for Probit Analysis after 24h, 48h, and 72h of PdNPs Exposure.

Table I. Results of PDNPS Bioassay

Species	*Anopheles stephensi*		
Exposure time	**24h**	**48h**	**72h**
Probit Equation (y= a + b x)	y = -3.89 + 3.22x	y = -3.44 + 3.05x	y = -2.26 + 2.62x
S.E.	0.860	0.665	0.263
LC_{50} (%) (95%LCL-UCL)	**16.038** (13.969-19.305)	**13.231** (9.537-16.853)	**7.215** (1.705-13.041)
$\chi^2_{0.5}$ $^{df=3}$	0.271	3.040	17.272
R^2	0.9989	0.9664	0.9212

Recently a comparable study has been reported for PdNPs efficacy against the 3rd instar larvae of *Culex quinquefasciatus* and *Anopheles subpictus* mosquito. The leaf extract of *Tinospora cordifolia* used in PdNPs synthesis showed larvicidal properties after 24h with LC_{50} at 49.712mg/L against *Culex quinquefasciatus* and LC_{50} at 45.920mg/L against *Anopheles subpictus*. Efficacy of PdNPs test concentration showed LC_{50} at 4.989mg/L for *Culex quinquefasciatus* and LC_{50} at 6.409mg/L for Anopheles *subpictus* [7]. PdNPs synthesized with the help of *Melia azedarach* leaf extract showed anti-larvicidal activity against *Aedes aegypti* as we have found against *Anopheles stephensi* and it has also shown anti-fungal activity against *Aspergillus niger*, *Fusarium solani*, *Nigrospora oryzae* and *Trichoderma viride*, and anti-bacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* [6]. Anti-cancer activity of PdNPs showed the reduced viability of cells in human ovarian cancer cell line culture, in comparison to our synthesis they used *Evolvulus alsinoides* leaf extract which has natural antioxidant properties [11]. PdNPs with an average size of 27±1.3 nm showed antiplasmodial activity against *Plasmodium berghei* in Swiss albino mice model with inhibitory concentration (IC50) of 10.29 mg/kg/body weight and cytotoxic anti-cancer activity against HepG2 cancer cell line, *Eclipta prostrate* leaf extract was used in the synthesis of these nanoparticles [12].

In our lab, a similar study on synthesis of silver and gold nanoparticles via microbes like *Listeria monocytogenes*, *Bacillus subtilius* and *Streptomyces anulatus* (Soni and Prakash 2015a), fungal strains like *Chrysosporium tropicum*, *Aspergillus niger*, *Fusarium oxysporum*, *Chrysosporium keratinophilum* and *Verticillium lecanii*, [25-28], leaf extract of plants like *Azadirachta indica* [29], *Ficus riliosa* [30] and bark of *Cinnamomum zeylanicum* [31] had been carried out, and the synthesized nanoparticles were tested for their toxicity against mosquito larvae, pupae and adults of *Anopheles stephensi*, *Culex quinquefasciatus*, *Aedes aegypti* and their anti-microbial and anti-fungal activity has also been accessed [30, 32]. The earlier studies indicated an effective PdNPs based formulation for mosquito control and its additional activities. However, we have successfully investigated the possibility in tropical vector species viz. *Anopheles stephensi*. . Majority of PdNPs applications can be seen in the field of catalytic converters where the harmful substances in automobile exhaust are converted into less noxious substances, therefore, their utility in medicine and biology is warranted. We have found that PdNPs can also be used for mosquito control against *Anopheles stephensi* species. This study will further facilitate the control of Malaria which can be restricted developing countries and areas of tropical climate.

IV. CONCLUSION

We have confirmed the synthesis of palladium nanoparticles with the help of leaf extract of plant *Citrus limon*. These nanoparticles were 2-4.8nm in size and were spherical in shape. We have reported the positive larvicidal effects of palladium nanoparticles against 3rd instar larvae of *Anopheles stephensi* mosquito. PdNPs have also been used in biomedical sciences, to treat diseases like cancer and malaria and its unique catalytic property can be used to degrade non-biodegradable environmentally toxic compounds to biodegradable and eco-friendly non-toxic compounds. Based on our findings we can say that the synthesized formulation is effective against mosquito larvae and can be used in larvicidal formulations to control emerging insecticide resistant mosquito population.

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