**In Vitro Antimicrobial and Antioxidant Activity of Biogenically Synthesized Palladium and Platinum Nanoparticles Using *Botryococcus braunii***

*Botryococcus braunii* Kullanarak Biyojenik Olarak Sentezlenmiş Paladyum ve Platin Nanopartiküllerin *In Vitro* Antimikrobiyal ve Antioksidan Aktiviteleri

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**ABSTRACT**

**Objectives:** The spread of infectious diseases and the increase in drug resistance among microbes has forced researchers to synthesize biologically active nanoparticles. Ecofriendly procedures for the synthesis of nanoparticles are improving day by day in the field of nanobiotechnology. In the present study we used extract of the green alga *Botryococcus braunii* for the synthesis of palladium and platinum nanoparticles and evaluated their antimicrobial and antioxidant activity.

**Materials and Methods:** Green alga was collected from Udaisagar Lake, Udaipur (Rajasthan, India) and isolated by serial dilution method and grown on Chu-13 nutrient medium. The characterization of alga synthesized palladium and platinum nanoparticles was carried out using X-ray diffraction and scanning electron spectroscopy. The zone of inhibition was measured by agar well plate method and minimum inhibitory concentration was determined by agar dilution assay for antimicrobial activity. The antioxidant activity of the nanoparticles was also studied by 1,1-diphenyl-2-picrylhydrazyl method.

**Results:** Stable palladium and platinum nanoparticles were successfully produced using green alga. The XRD pattern revealed the crystalline nature and scanning electron micrographs showed the morphology of biogenically synthesized metal nanoparticles. Fourier transform infrared measurements showed all functional groups having control over stabilization and reduction of the nanoparticles. The green synthesized nanoparticles exhibited antimicrobial activity against gram-positive and gram-negative bacterial strains, antifungal activity against a fungus, and antioxidant activity.

**Conclusion:** The biogenic synthesis of metal nanoparticles can be a promising process for the production of other transition metal nanoparticles and new nanocatalysts will revolutionize the synthesis of organic heterocycles.

**Key words:** Palladium, platinum, nanoparticles, antimicrobial, antioxidant, biogenic

**ÖZ**

Amaç: Bulaşıcı hastalıkların yayılması ve mikroplar arasında gözlenen ilaç direncindeki artış, araştırmacılar biyolojik olarak aktif nanopartikülleri sentezlemeye zorladı. Nanopartiküllerin sentezine yönelik çevre dostu prosedürler, nanobiyoteknoloji alanında her geçen gün gelişmekteidir. Bu çalışmada palladyum ve platin nanopartiküllerin sentezlenmis bir alg olan *Botryococcus braunii* ekstraktını kullanarak sentezlenmesi ve bu nanopartiküllerin in vitro antimikrobiyal ve antioksidan aktiviteleri değerlendirildi.

**Gereç ve Yöntemler:** Udaipur'dan (Rajasthan, Hindistan) yeşil alga toplandı ve seri dilüsyon yöntemiyle izole edildi. Nanopartiküllerin sentezlenmesi, kristal yapılış gösteren, X-ışını kırımında, XRD analizleri ve tarama elektron mikroskopu underi, nanopartiküllerin morfolojisini gösterdi. Antimikrobiyal ve antioksidan aktivite değerlendirildi.

**Bulgular:** Kararlı nanopartiküller sentezlenip, in vitro antimikrobiyal ve antioksidan aktiviteleri gösterdiler. FOURIER DÖNÜŞÜM 2-PICRYLHYDRAZIL Yöntemi ile değerlendirildi.

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INTRODUCTION
The green synthesis of metal nanoparticles has three qualifying characteristics: as an environmentally safe solvent system, particle-stabilizing capping agents, and ecofriendly reducing agents. Biological synthesis using algae is one of the green approaches for the synthesis of d-block metal nanoparticles. Algae are eukaryotic, photoautotrophic, aquatic, and oxygenic organisms. Algae have more information in their genetic material to encode various reducing stabilizing agents that mediate the biogenic synthesis of metal nanoparticles. Algae acquire energy from sunlight through photosynthesis and convert inorganic carbon into organic material for their growth. Since algae are sustainable bioresources, they can be used largely in the greener synthesis of metal nanoparticles. Biogenic synthesis is the alternate route for synthesizing biocompatible metal nanoparticles to other synthesis processes such as chemical and physical. It is the newest possible way of linking nanotechnology and biotechnology in the developing field of nanobiotechnology.

Transition metal nanoparticles are regarded as important because of their biocompatibility, greener approach, ecofriendly adaptable nature, and photosynthesizing properties. Many metal nanoparticles like Cu, Ag, Pt, Au, and Pd were used in different fields such as catalysts, labeling biological substances, optoelectronics, photothermal therapy, and biological activities against microbes. In particular, the biogenic synthesis of palladium and platinum nanoparticles has attracted the attention of researchers because it is cost effective, sustainable, and ecofriendly. Palladium and platinum nanoparticles are broadly used as heterogeneous and homogeneous catalysts, drug carriers, and drugs; in many medical diagnoses without damaging the DNA structure and in cancer treatment; as nanocatalysts in environment remediation scavenging dyes from the textile industry; in Suzuki coupling reactions; and they have demonstrated antimicrobial activities and been assessed in other disciplines of biological sciences. There is a parallel increase in the use of methods for estimating the efficiency of such nanoparticles as antioxidants. One such method that is currently popular is based upon the use of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH).

Our aim in the present contribution was to synthesize and characterize palladium and platinum nanoparticles from aqueous extract of the green alga Botryococcus braunii and to evaluate their antimicrobial potential against bacterial and fungal species and antioxidant efficacy. Our study can be considered the first report on the synthesis of palladium and platinum nanoparticles using this green alga. The methods used are elucidated and the synthesized palladium and platinum nanoparticles were characterized using different techniques including ultraviolet (UV) visible spectroscopy, fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy, and X-ray diffraction.

MATERIALS AND METHODS
Chemicals and test strains
B. braunii was collected from Udaisagar Lake, Udaipur (Rajasthan, India). The reagents agar-agar, palladium acetate \( \text{Pd (OAc)}_2 \), and hexachloroplatinic acid \( \text{H}_2\text{PtCl}_6 \) were of analytical grade and were purchased from Sigma Aldrich. Bacterial strains like \textit{Pseudomonas aeruginosa} (MTCC 441), \textit{Escherichia coli} (MTCC 442), \textit{Klebsiella pneumoniae} (MTCC 109), and \textit{Staphylococcus aureus} (MTCC 96) and a fungal strain, \textit{Fusarium oxysporum} (MTCC 2087) were purchased from Microbial Type Collection, Chandigarh (India).

Isolation and culturing of \textit{B. braunii}
This green alga was isolated by serial dilution and grown on Chu-13 nutrient medium solidified by 1.5% agar-agar. The composition of the Chu-13 medium was CaNO\(_3\) (0.300 g/L), MgSO\(_4\).7H\(_2\)O (0.025 g/L), CaCl\(_2\).2H\(_2\)O (0.027 g/L), K\(_2\)HPO\(_4\) (0.010 g/L), ferric citrate (0.0035 g/L), citric acid (0.0035 g/L), Na\(_2\)CO\(_3\) (0.02 g/L), Na\(_2\)SiO\(_3\).5H\(_2\)O (0.044 g/L), and some micronutrients. Algal colonies appearing after 3 weeks of incubation were isolated and inoculated into liquid medium. For growth experiments algal species was grown for algal biomass in an incubator at 27±1 °C, 1.2±0.2 klux light intensity, and 16:8 h light:dark cycle in nutrient medium. After standardization of optimal culture conditions in Chu-13 medium the best results of growth of green alga were found.

Preparation of algal extract
The grown algal biomass was centrifuged, shade dried, and 5 g of algal biomass was taken in a 250 mL Erlenmeyer flask along with 100 mL of distilled water. Then the mixture was autoclaved for 15 min and filtered through Whatman No. 1 filter paper. The filtered extract was centrifuged and the supernatant was used as reducing agent for preparing metal nanoparticles. The prepared algal extract was kept at 4 °C in a refrigerator for further experimental use.

Synthesis of palladium and platinum nanoparticles
The nanoparticles were synthesized using the following processes.
Palladium nanoparticles: 20 mL of algal extract was mixed with 80 mL of 1 mM Pd (OAc)2 aqueous solution in a 250 mL Erlenmeyer flask at pH 6-7 and put on a magnetic stirrer at 60 °C for 3 h. Simultaneously, a positive control of Pd (OAc)2 aqueous solution and algal extract and a negative control containing only Pd (OAc)2 aqueous solution were maintained under the same conditions. The progress of the process was regularly monitored by observing color change. In the positive control the initial pale yellow solution turned dark brown, indicating formation of palladium nanoparticles, but in the negative control no change in color was observed. After the formation of palladium nanoparticles, the solution was centrifuged for 30 min and the obtained nanoparticles were washed with deionized water to remove impurities. This process of centrifugation and washing was carried out three times to achieve better separation of nanoparticles. The obtained palladium nanoparticles were oven dried at 70 °C.17

Platinum nanoparticles: 90 mL of 1 mM aqueous solution of H2PtCl6 was added with 10 mL of algal extract to a 250 mL Erlenmeyer flask at pH 6-7. The mixture was put on a magnetic stirrer at 95 °C for 4 h. Simultaneously, a positive control of H2PtCl6 aqueous solution and algal extract and a negative control containing only H2PtCl6 aqueous solution were maintained under the same conditions. In the positive control the initial light yellow solution turned brown and finally black, consistent with the formation of platinum nanoparticles, but in the negative control no change was observed. The synthesized platinum nanoparticles were separated from the mixture by centrifugation for 30 min and then washed with deionized water. This process of centrifugation and washing was repeated three times and finally the obtained platinum nanoparticles were oven dried at 58 °C for 4 h.11

Characterization of metal nanoparticles
FTIR analyses were carried out on a Perkin-Elmer instrument in the range of 4000-450 cm⁻¹ using dried powders of the metal nanoparticles. Samples for analysis were prepared under ambient conditions and mixed with KBr. X-ray diffraction measurements were carried out on a Philips Xpert Pro XRD system (DV 1650) for determining the size of the synthesized metal nanoparticles. Images were obtained with the help of a scanning electron microscope (SEM) (Model-FEI Quanta 200) for analyzing the morphology of the nanoparticles.

Evaluation of antimicrobial activity
Test microorganisms
The antimicrobial activity of the platinum and palladium nanoparticles was studied against two Gram-negative bacterial strains Pseudomonas aeruginosa (MTCC 441) and Escherichia coli (MTCC 442), two Gram-positive bacterial strains Klebsiella pneumoniae (MTCC 109) and Staphylococcus aureus (MTCC 96) and a fungal strain Fusarium oxysporum (MTCC 2087). The antibacterial and antifungal potential of the nanoparticles was assessed in terms of zone of inhibition of microbial growth by agar well plate method and minimum inhibitory concentration was determined by agar dilution assay.

Agar well plate method
Bacterial cultures were maintained in petri plates containing nutrient agar medium at 37 °C. The medium was prepared containing 10 g of beef extract, 2 g of yeast extract, 5 g of peptone, 5 g of NaCl, and 15 g of agar in 1 L of distilled water. The fungus F. oxysporum was maintained on potato dextrose agar at 25 °C. The nutrient agar and potato dextrose agar were autoclaved at 121 °C at 15 psi for 15 min and poured onto sterile petri plates to a uniform depth of approximately 4 mm. Once the medium solidified the culture was spread onto the petri plates with the help of an L-spreaders. With a sterilized 5 mm cork borer, wells were introduced into the agar and 20 μL of both platinum and palladium was added to the wells. Untreated algal extract and salt of platinum and palladium were used as negative control. The plates were incubated at 37 °C and 25 °C overnight as per requirements. The experiments were carried out in triplicate. The antimicrobial activity was evaluated by measuring the size of the clear zone around each well.28

Agar dilution method
The minimum inhibitory concentration (MIC) of these nanoparticles was determined by agar dilution technique where stocks of 50 mg/mL of the synthesized nanoparticles were resuspended in 10% dimethyl sulfoxide to produce two-fold dilutions in the range of 25-30 mg/mL and so on. Each dilution of nanoparticles was put into the melted agar. The agar was poured into petri plates and allowed to solidify. After this, bacteria prepared to a standard concentration were added as a spot to each plate, with 10⁴ colony forming units per spot. These dilution plates were then incubated at 37 °C with a control plate having no antimicrobial agent. After incubation the growth of the microbial isolates on the agar plate was assessed. The lowest concentration of nanoparticles that prevents microbial growth was considered to be the MIC value of those nanoparticles against that microorganism.18,19

DPHP radical scavenging activity
The free radical scavenging activity of all the extracts was evaluated by DPHP according to the method previously reported by Blois20 in 1958. Briefly, a 0.1 mM solution of DPHP in ethyl alcohol was prepared and 1 mL of this solution was added to 3 mL of the solution of all extracts in methanol at different concentrations (5, 10, 15, 20, and 25 μg/mL). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 541 nm using a UV-Vis spectrophotometer. Ascorbic acid was used as the reference. Lower absorbance values of the reaction mixture indicate higher free radical scavenging activity. The capability of scavenging the DPHP radical was calculated using the following formula:

DPHP scavenging effect (% inhibition) = ((A0-A1)/A0)×100, where A0 is the absorbance of the control reaction and A1 is the absorbance in the presence of all of the extract samples and reference. All the tests were performed in triplicate and the results were averaged.
RESULTS AND DISCUSSION

In the present study, we synthesized palladium and platinum nanoparticles by the use of extract of the green alga *B. braunii*. Algal extract appears to be a potential source of reducing and stabilizing agent without using any chemical as reducing agent. The complete process of formation of metal nanoparticles was initially confirmed by visual observation as shown in Figure 1. In Figure 1a the change in color from pale yellow to dark brown and in Figure 1b from light yellow to black of the reaction mixture provide a convenient sign to indicate the formation of palladium and platinum nanoparticles, respectively.\(^\text{15,16}\)

**Fourier transform infrared spectroscopy**

The FTIR spectrum of experimental samples revealed two types of vibrations, stretching and bending, in the wavelength range of 4000-450 cm\(^{-1}\). The FTIR spectrum measurements were demonstrated to identify the major functional groups present in *B. braunii* to examine their possible involvement in the synthesis of palladium and platinum nanoparticles. Different peaks were seen at 3435.88, 2923.49, 2852.33, 1637.82, 1559.61, 1414.42, 1384.79, 1069.01, 1056.17, 837.53, 781.32, 714.25, 695.06, 657, 618.16, and 532.74 cm\(^{-1}\) in the FTIR spectrum of algal extract of *B. braunii*. The peak at 3435.88 was due to N-H and O-H stretching vibrations,\(^\text{21,22}\) while the 2923.49 and 2852.33 cm\(^{-1}\) bands arose due to asymmetrical C-H stretching vibrations of -CH\(_2\) and -CH\(_3\).\(^\text{22}\) The 1637.82 cm\(^{-1}\) peak is characteristic of N-H bending vibrations in amide of protein as a capping agent.\(^\text{23,24}\) The peak at 1559.61 cm\(^{-1}\) showed the presence of a carboxyl group and the weak band at 1414.42 and 618.16 cm\(^{-1}\) was due to COO\(^{-}\) in amino acid residue of protein.\(^\text{25}\) The peak observed around 1384.79 cm\(^{-1}\) can be assigned to C-N stretching vibrations of amine. C-H bending vibrations by carbohydrates (glucose residue by C-OH bond) showed a peak at 1037.17 cm\(^{-1}\).\(^\text{26}\) The 873.53, 781.32, 695.06, and 532.74 cm\(^{-1}\) bands were demonstrated due to O-C=O bending vibrations of CO\(_3\)\(^2-\) and -CH\(_2\)\(_3\)\(_2\) and N-H wagging of amine and alkyl halide, respectively. The results of the present study have shown that hydroxyl groups have a strong ability to interact with nanoparticles. The main peaks existing in the spectrum of the algae are also present in the spectrum of the palladium and platinum nanoparticles synthesized with lower intensities and slight shift. Therefore, it may be evidenced that proteins, polysaccharides, amides, and long chain fatty acids are the biomolecules responsible for bioreduction and act as capping and stabilizing agents.\(^\text{27,28}\)

**Scanning electron microscopy**

The shape and size of the biogenically synthesized nanoparticles were elucidated with the help of SEM (Figures 2a and 2b). SEM showed that cubical, spherical, and truncated triangular palladium and platinum nanoparticles were synthesized.\(^\text{29,30}\) The size distribution histogram shows that the average size of synthesized nanoparticles was 4.89 nm and 86.96 nm for...
palladium and platinum nanoparticles, respectively. From the SEM images the number of nanoparticles (total 50 particles for each sample) was counted by ImageJ software. The following equation was used for calculating statistical properties of nanoparticles named as number average diameter ($D_n$), weight-average diameter ($D_w$), and polydispersity index (PDI).

$$D_n = \frac{\sum d_i}{n}$$

$$D_w = \left(\frac{\sum (d_i)^4}{\sum (d_i)^3}\right)^{\frac{1}{4}}$$

$$\text{PDI} = \frac{D_w}{D_n}$$

Here $d_i$ is the diameter of microspheres and $n$ represents the number of nanoparticles.

The PDI values of 0.198 for platinum nanoparticles and 0.862 for palladium nanoparticles were calculated and these values showed uniform size of synthesized nanoparticles. The PDI values were used as an indicator for the size distribution of the synthesized nanoparticles.

**X-ray diffraction**

The synthesized metal nanoparticles were further evidenced by XRD measurements. The XRD analysis of green synthesized palladium nanoparticles in Figure 3a showed major diffraction peaks at $2\theta$ of 40.1°, 46.6°, and 68.0°, corresponding to (111), (200), and (220) planes of the face-centered cubic structure of palladium nanoparticles (JCPDS no. 05-0681). The crystallite size of palladium nanoparticles was calculated from the (111) plane of face-centered cubic (fcc) palladium using the Scherrer equation. The crystallite size of the synthesized palladium nanoparticles was calculated to be around 5 nm.

Furthermore, in Figure 3b the diffraction lines at about $2\theta$ of 38.10, 46.60, 64.70, and 77.40 matched the (111), (200), (220), and (311) planes of the fcc crystal lattice of platinum (JCPDS No. 88-2343). The crystallite size of platinum nanoparticles was calculated from the (111) plane of fcc using the Scherrer equation. The crystallite size of the synthesized platinum nanoparticles was found to be 87 nm.

**Antimicrobial activity of synthesized palladium and platinum nanoparticles**

Revived bacterial strains were maintained on nutrient agar medium as shown in Figure 4 and the fungal strain was maintained on potato dextrose agar as also shown.

**Assay of biological activity**

The biological activity of the algal extract and synthesized nanoparticles was tested against both bacteria (Gram-positive and Gram-negative) and a fungus using agar well diffusion. Figure 5 shows the different zones of inhibition formed by synthesized platinum and palladium nanoparticles, antibiotics, algal extract, and salts of platinum and palladium against the test strains. The well filled with algal extract did not show any zone of inhibition but the nanoparticles synthesized from that algal culture show both antibacterial and antifungal activity with a zone of inhibition ranging from 7 to 16 mm (Table 1, Figure 6). PtNps and PdNps at 400 µg/mL concentration showed the maximum zone of inhibition against the test strains.

**Determination of minimum inhibitory concentration**

The MIC required to inhibit the growth of microbes is less in the case of platinum as compared with palladium (Figure 7). These synthesized nanoparticles show the least activity towards the fungus tested, *F. oxysporum*. The positive control drugs used...
against both gram positive and gram negative bacteria were chloramphenicol and ampicillin. Nystatin and griseofulvin were used as the positive control drugs for *F. oxysporum*. The antibiotic ampicillin does not show any activity against *P. aeruginosa* as compared to PtNps and PdNps, which show significant activity. The antimicrobial activity of nanoparticles was considered to be good if its MIC was less than 100 µg/mL, moderate if MIC was from 100 to 500 µg/mL, and poor over 500 µg/mL (Table 2).

**Antioxidant activity**

The antioxidant potential of the green synthesized palladium and platinum nanoparticles was evaluated by quantifying the DPPH free radical scavenging activity (Figure 8, Table 3). In the presence of nanoparticles, the color of the DPPH solution gradually changed from purple to pale yellow with time. The percentage scavenging of DPPH increased linearly with an increase in nanoparticle concentration from 1 to 20 µg/mL and reached 82.43% within 30 min at 20 µg/mL in the case of palladium and 78.14% at 25 µg/mL in the case of platinum. However, the positive control ascorbic acid showed 94.0% scavenging activity at a concentration of 50 µg/mL. The negative control wells loaded with algal extract did not show any color change from purple.

**CONCLUSION**

In the present work, a successful, rapid combustion method is demonstrated for the synthesis of stabilized nanoscale palladium and platinum particles for the first time with the use of these nanoparticles as potential candidates for their antimicrobial and antioxidant activities.
of extract of the green alga *B. braunii* as a reducing stabilizing and capping agent. The biogenically synthesized nanoparticles were characterized by different techniques including FTIR spectroscopy, scanning electron microscopy, and X-ray diffraction. The FTIR spectrum confirms the interaction of algal biomolecules and the formation of palladium and platinum nanoparticles. From the SEM images and XRD patterns, the prepared nanoparticles exhibited cubical, spherical, and truncated triangular shape with 4.89 nm and 86.96 nm palladium and platinum nanoparticles, respectively. The green synthesized nanoparticles exhibited antimicrobial activity against Gram positive and Gram negative bacterial strains, antifungal activity against a fungus, and antioxidant activity. This conversion of metal ions into metal nanoparticles will one day replace the other methods of synthesis of nanoparticles and could possibly be used for large-scale synthesis of technologically important applications.

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| Table 3. Comparison of DPPH scavenging activity of palladium and platinum nanoparticles |
|-----------------------------------------------|----------------------------------|
| Concentration of palladium/platinum nanoparticles (µg/mL) | % Scavenging activity of palladium nanoparticles | % Scavenging activity of platinum nanoparticles |
| 1 | 13.22 | 19.37 |
| 5 | 18.44 | 27.51 |
| 10 | 23.78 | 37.66 |
| 15 | 57.96 | 58.57 |
| 20 | 82.43 | 69.93 |
| 25 | 82.27 | 78.14 |

DPPH: 1,1-diphenyl-2-picrylhydrazyl
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