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Anti-Inflammatory and Anti-Oxidant Activity of Pterocarpus Santalinus Mediated Carbon Nanoparticles Synthesized Using Heat-Induced Method

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This study aims to assess the anti-oxidant and anti-inflammatory activity of carbon nanoparticles synthesized from red sandalwood extract.Pterocarpus santalinus-mediated carbon NPs are assessed for antioxidant and anti-inflammatory activity using hydrogen peroxide, DPPH, FRAP, egg albumin, bovine serum, and membrane stabilization assays. CNPs are taken in different concentrations of 10, 20, 30, 40, and 50 µg/ml for the assays.P.santalinus bark extract mediated CNPs exhibited antioxidant activity ranging from 63.28 to 91.22% in DPPH assay, 47.3 to 84.9% in H_2O_2 assay, 65.96 to 87.31% in FRAP assay, 66.59 to 87.61% in ABTS assay and 67.45 to 85.24% in nitric oxide assay in the tested concentration ranging from 10 to 50 µg/ml. The highest percentage of inhibition exhibited CNPs in BSA assay is 83%, in egg albumin denaturation assay - 78%, and in membrane stabilization assay - 86% for anti-inflammatory activity. At the concentration of 100µg/mL, CNPs exhibited a zone of inhibition of 9 mm against all tested pathogens - Pseudomonas sp, E.coli, E.faecalis, and S.aureus in agar well diffusion method. In all tested concentrations, CNPs did not exhibit cytotoxic activity and the % of live nauplii seen at the highest tested concentration of 80µg/mL is 100% after two days of incubation.Results show that synthesized CNPs did not exhibit cytotoxic activity. CNPs exhibited efficient antioxidant, anti-inflammatory, and antimicrobial activity. Further research needs to be conducted on CNPs as they exhibit efficient biomedical activities and can be incorporated into therapeutics in the future.

Keywords: antioxidant, anti-inflammatory, carbon nanoparticles, microwave-assisted synthesis, P.santalinus.

1. Introduction

The Greek word nanos, which means "a dwarf," is the source of the English word nano. One billionth, or 10^9, of a unit is denoted by the prefix nano. Nanomaterials, whose diameters vary from 1 to 100 nm, are produced, designed, and used in the field of nanotechnology. In his 1959 speech "There's Plenty of Room at the Bottom," Nobel Prize winner Richard Feynman introduced the idea of nanotechnology(1). Distinct properties of nanoparticles compared to their bulk counterparts are - it has extraordinary mobility, significant reactivity, and excellent electrical, chemical, optical, magnetic, and mechanical properties (2). Based on the dimension, nanomaterials are classified as zero dimension(0-D)-all dimensions of nanomaterials are in the nanoscale range;nanoparticles come under this classification,one dimension(1-D)-two dimensions are within the nanoscale range and one dimension exceeds the range,two dimension(2-D)- two dimensions exceed nanoscale range,and three-dimensional nanomaterials (3-D)- all dimensions exceed nanoscale range. Based on composition, nanoparticles can be classified as organic, inorganic-metal, metal oxides NPs and carbon-based NPs - CNTs, graphene, CNFs, fullerene, and carbon black (3).Nanoparticles are classified based on morphology - spatial organization- agglomeration- weak bond formation between NPs and aggregation - stable bond formation between NPs. Based on shape-spherical, needle, rod, cube, cube and octahedron. NPs can be crystalline or amorphous. Based on origin, nanomaterials are classified as incidental- a derivative of a chemical process or found incidentally, engineered- manufactured with defined properties for applications and naturally produced - synthesized from plants, animals and microorganisms(4). Applications of nanoparticles are environmental remediation - photodegradation, photocatalysis, water splitting, targeted drug delivery, contrast agent in imaging, theragnostic, MRI, tissue repairing, gas sensors, agriculture - pesticide, fertilizer, optical and electrochemical sensing.

Nanoparticles can be synthesized using top-down and bottom-up approaches. In the top-down approach, as the nanoparticle's dimension is high, mechanical methods are needed to decrease the size of nanoparticles. In contrast to the top-down method, the bottom-up method starts at the atomic level by assembling molecules (5). Green synthesis techniques are gradually supplanting chemical and physical methods as these methods utilize noxious chemicals, complex equipment, and excessive energy consumption. These methods utilize ultraviolet radiation, thermal decomposition, high pressure and temperature, organic solvents, and costly reagents. Green synthesis avoids use of noxious and toxic reagents, lesser energy consumption, and eco-friendly materials are used as capping agents (6). Green synthesis is cost-effective, stable NPs are formed, lesser contamination rates with large-scale production, biocompatible, and environmentally friendly. Green synthesis utilizes plant parts- stem, flower, fruit, seed, peel, bark, and root - and has phytochemicals, bacteria, algae, fungi, and yeast - which act as reducing and stabilizing agents. Synthesis using microorganisms has disadvantages of culture contamination, control of NP size is limited and a lengthy process (7).

Carbon is a distinct element that has different hybridization states - sp, sp2 and sp3 due to its valence electrons. Because of its hybridization states, carbon may create a broad variety of structures and different allotropes. Because of their strong thermal, mechanical, and electrical conductivity, carbon nanoparticles are employed in water treatment, electronic device development, excellent reinforcing materials, and electronics because of their effective electrical and optical qualities (8). Carbon exists in zero-dimensional fullerene and graphene

quantum dots (GQDs) as allotropes. Carbon nanofiber (CNF), graphene nanoribbons (GNR), carbon nanotubes (both single and multiwalled CNTs), and carbon nanohorns (CNH) are examples of one-dimensional allotropes. The two-dimensional allotrope is graphene. The three-dimensional allotrope is diamond and graphite (9).

Pterocarpus santalinus is categorized under rosewood mahogany and has butterfly-shaped red sandalwood flowers. Cedrol, a sesquiterpene molecule, contributes significantly to the flavor of sandalwood and wood. It can be used as a disinfectant since it inhibits human lung cancer cells. Because squalene prevents the body from absorbing cholesterol, cholesterol levels are reduced (10). Pterocarpus santalinus is referenced in Ayurvedic literature and utilized in traditional medicine. Because of its red heartwood, it is also known as raktchandan, red sandalwood, and red sanders. It is a member of the Fabaceae family and genus Pterocarpus. It is used for commercial purposes like handicrafts, furniture, and sculpture, it is sold illegally and over-exploited. It is reclassified as a near-threatened species after being classified as an endangered species (11). P.santalinus is a deciduous tree with a round,thick crown and a circumference of 90-160 cm. It has therapeutic activity, used to treat helminthiasis, hemorrhage, diabetes, fever, eye disease, and wounds, and controls vomiting. Heartwood of P.santalinus has anti-inflammatory, anti-hemorrhoidal, antipyretic, aphrodisiac, antidysentery, anthelminthic and diaphoretic properties.There is the presence of main chemical components like tannins, flavonoids, carbohydrates, glyceride, saponins, anthocyanins, triterpenoids, glycosides, steroids, phenols, ketones, fatty acids, ethers, polysaccharides and alcohols (12). CNPs biosynthesized using Pterocarpus santalinus plant extract is tested for antioxidant activity using hydrogen peroxide, DPPH and FRAP assay. Anti-inflammatory activity is evaluated using egg albumin and BSA denaturation assay.

2. Materials and Methods:

Synthesis of CNPs:

2.5g of powdered red sandalwood is taken in a conical flask and then add 100 ml of distilled water. Keep for boiling on the heating mantle and filter the extract using a muslin cloth. Filtered plant extract is kept for boiling to reduce it to 2 ml. Pour the extract into a petri dish and keep in the microwave oven. The burnt powder is scraped out and stored for further use.

Antioxidant activity:

Hydrogen peroxide assay:

Add 1 ml of hydrogen peroxide in each test tube. Varying concentrations of P.santalinusextract mediated CNPs and standard ascorbic acid are added in the range of 10, 20, 30, 40 and 50µg/ml. Samples are kept for incubation in a dark place for 10 minutes. UV visible spectroscopy readings are taken at a wavelength of 530 nm (13).

DPPH assay:

To make a stock solution, DPPH powder is weighed to the nearest 0.03g and dissolved in 10 ml of ethanol. 49.5 ml of methanol and 0.05 ml of the produced solution are combined to create the working standard solution. Each test tube receives one millilitre of the working standard solution before CNPs at concentrations of 10, 20, 30, 40, and 50 µg/ml are added. Let it sit in

the dark for ten minutes. UV spectroscopy is used to measure absorbance at 517 nm and record any colour changes in the material (14).

FRAP assay:

Ferric chloride (about 0.5 ml) and CNPs (10–50 µg/ml) at varying quantities are added to each centrifuge tube. Potassium ferricyanide and 2.5 ml of phosphate buffer are then added, and the mixture is allowed to sit at room temperature for 30 minutes. Re-incubate for 10 minutes after adding 2.5 ml of trichloroacetic acid. Following ten minutes at 30°C in a water bath, the samples are centrifuged for ten minutes at 1200 rpm. Test tubes are filled with 2.5 ml of distilled water and 2.5 ml of centrifuged sample supernatant. They are then incubated for 10 minutes at room temperature. UV spectroscopy is used to measure absorbance at 700 nm (15).

ABTS assay:

A reaction between 7.0 mM of ABTS in 50% ethanol and 2.45 mM of potassium persulfate (in distilled water) produces the ABTS•+ radical cation. The prepared reagent is kept for at least a day in the refrigerator. Before using, the reagent is diluted with 50% ethanol until the absorbance at a wavelength of 734 nm is 1.0 (±0.02). Various quantities of CNPs (10, 20, 30, 40, and 50µg/ml) are added to 250µL of ABTS. 200 µL of a reagent combination and NPs at various concentrations are applied to 96-well microplates, whereas 200 µL of ethanol is used as a blank. Using a microplate reader, absorbance measurements are made at a wavelength of 734 nm following ten minutes of dark incubation.

Nitric oxide assay:

An assessment of nitric oxide radical inhibition can be made with the Griess-Ilosvay reaction. Instead of utilising 5 percent 1-naphthylamine, 0.1% w/v of naphthyl ethylenediamine dihydrochloride is used in this work to modify the GriessIlosay reagent. To make 3 mL of reaction mixture, add 2 mL of sodium nitroprusside (10 mm), 0.5 mL of phosphate buffer saline, and different doses of CNPs (10 to 50 µg/ml) or 0.5 mL of standard rutin. Incubate for 150 minutes at 25 °C. Following incubation, 0.5 mL of reaction mixture is combined with 1 mL of sulfanilic acid reagent (0.33% in 20% of glacial acetic acid). This combination is then allowed to stand still for 5 minutes to finish the diazotisation process. Afterward, 1 mL of naphthyl ethylenediamine dihydrochloride is added and the mixture is allowed to stand still for 30 minutes at 25°C. In scattered light, a pink coloured chromophore is formed. Absorbance of the samples are measured at 540 nm.

Anti-inflammatory assays:

Egg albumin denaturation assay:

Add 2.8 ml of phosphate buffer and 0.2 ml of egg white to each test tube. CNPs are added in the range of 10 - 50µg/ml and incubated at room temperature for 10 minutes. Samples are kept in water bath at 50°C. Any changes in color of the sample is observed and absorbance readings in UV spectroscopy for samples are taken at 660 nm(16).

Bovine serum albumin denaturation assay:

Add 0.05g of commercial bovine serum albumin to 50 ml of distilled water. Add 3 ml of this solution to test tubes and varying concentrations of CNPs ranging between 10 to 50µg/ml are

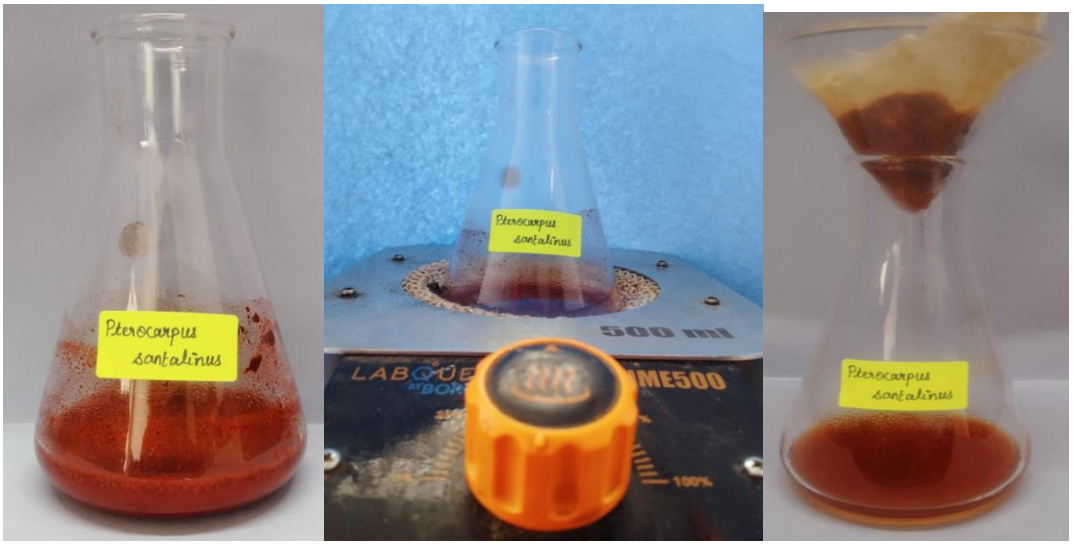
added. Incubate for 10 minutes at room temperature and the samples are kept in a water bath. After that, absorbance readings are taken using UV visible spectroscopy at 660 nm.

Membrane stabilization assay:

In a sterile tube with anticoagulant, human blood is freshly collected and centrifuged for 10 minutes at 1000 rpm to separate RBCs from other components. Supernatant is discarded and RBCs are washed with PBS for three times. Resuspending RBCs in Tris-HCl buffer make 10% (v/v) RBC suspension. 1 ml of prepared suspension is added to centrifuge tubes and varying concentrations of CNPs ranging from 10 to 50µg/ml are added to each tube. Gently mix the contents and incubate for 30 minutes at 37°C. Centrifuge it at room temperature for 10 minutes at 1000 rpm to pellet the RBCs. Absorbance of supernatant is measured at 540 nm using a UV-vis spectrophotometer.

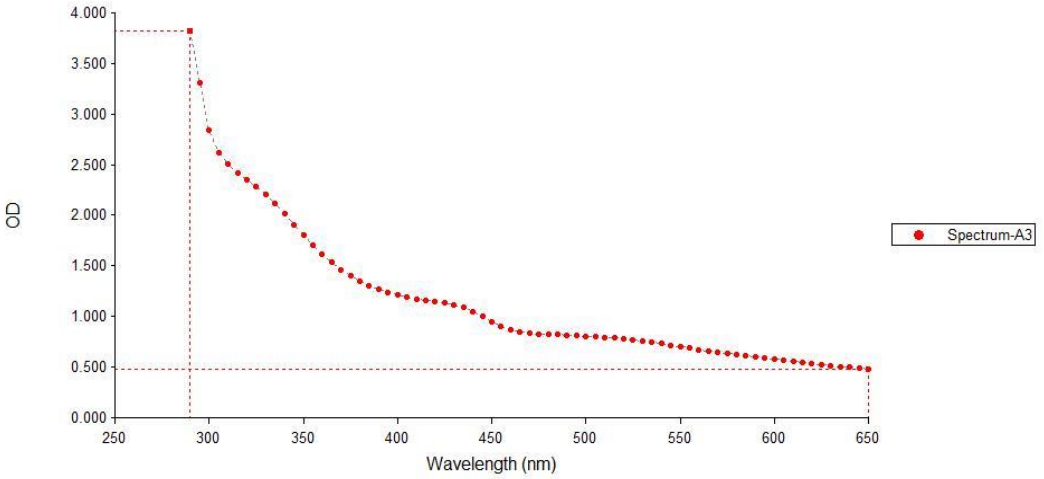
3. Results:

Figure 1: a) Red Sandalwood powder mixed with distilled water, b) the mixture is placed for boiling in the heating mantle, and c) Theresultant extract is filtered.



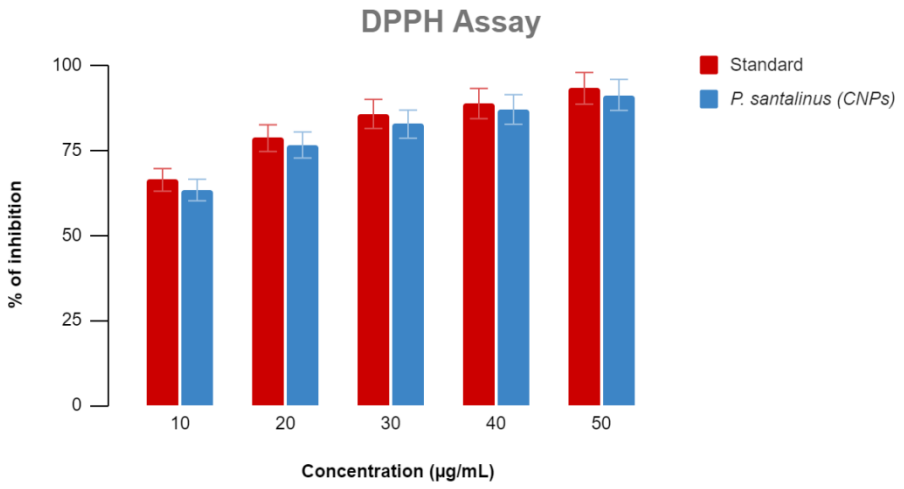
(a) (b) (c)

Figure 2: UV spectra analysis of CNPs synthesized using red sandalwood



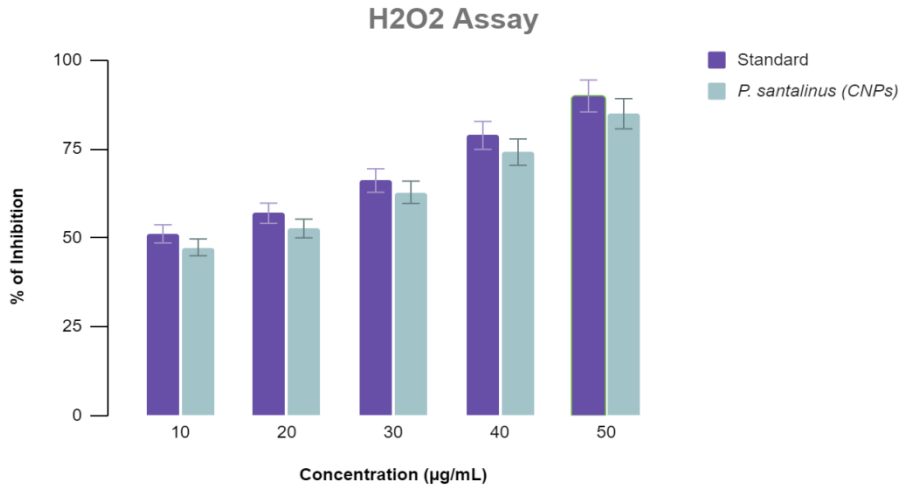
The overall synthesis of CNPs is represented in Figure 1. The UV-Vis absorption spectra of the synthesized carbon nanoparticles were recorded over the wavelength range of 250 to 650 nm. The analysis revealed several notable features. A prominent absorption peak was observed around 290 nm, which is attributed to the π-π* transitions within the carbon nanodots' conjugated structures where the maximum absorption peak was shown in Figure 2.

Figure 3: Radical scavenging activity of P.santalinus mediated CNPs assessed using DPPH assay.



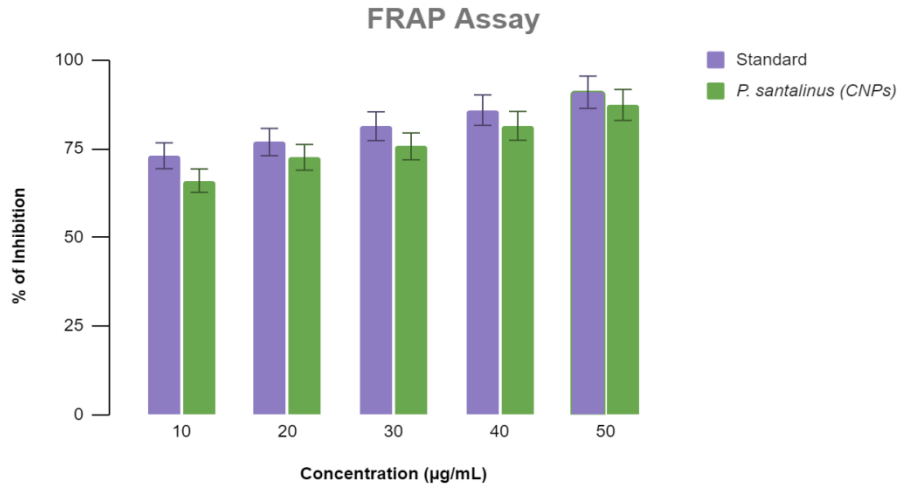
In DPPH assay, carbon nanoparticles biosynthesized using P.santalinusbark extract are taken in concentrations ranging from 10 to 50 µg/ml and the radical scavenging activity exhibited for the tested concentrations are 63.28, 76.51, 82.63, 86.94 and 91.22%. The corresponding values for standard are 66.25, 78.52, 85.63, 88.68 and 93.15% represented in Figure 3.

Figure 4: Antioxidant activity of biosynthesized CNPs evaluated using hydrogen peroxide assay



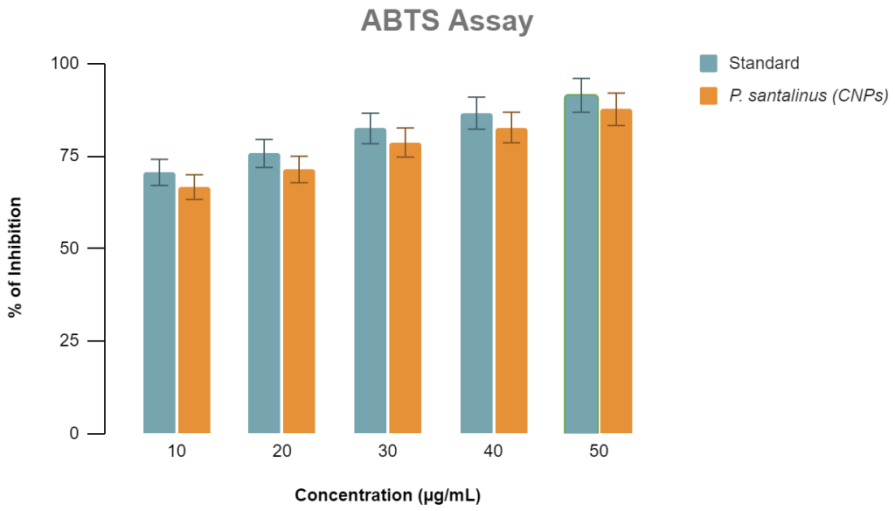
Green synthesized CNPs are assessed using hydrogen peroxide assay. For the concentration of 10µg/ml, CNPs showed an antioxidant value of 47.3% and its activity increased to 52.6% and 62.8% at the concentration of 20 and 30 µg/ml. At concentrations of 40 and 50µg/ml, CNPs showed antioxidant activity of 74.1 and 84.9%. For tested concentrations, the standard showed values of 51.1, 56.9, 66.1, 78.8, and 89.9% as shown in Figure 4..

Figure 5: Radical scavenging ability of CNPs assessed using FRAP assay



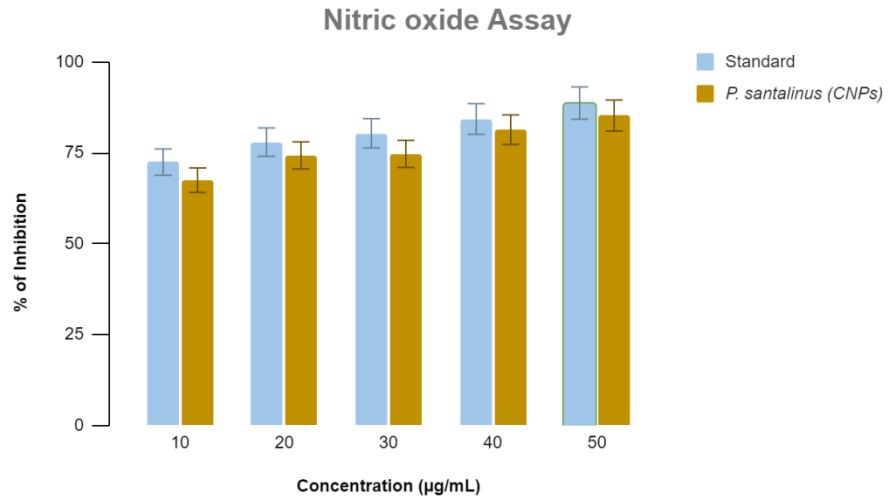
CNPs synthesized using P.santalinus extract exhibited radical scavenging activity of 65.96% at 10µg/ml, 72.54% at 20µg/ml, 75.63% at 30µg/ml, 81.41% at 40µg/ml and 87.31% at 50µg/ml. The corresponding values for standard are 72.98, 76.84, 81.31, 85.84 and 90.89% respectively as shown in Figure 5.

Figure 6: Antioxidant activity of CNPs evaluated using ABTS assay



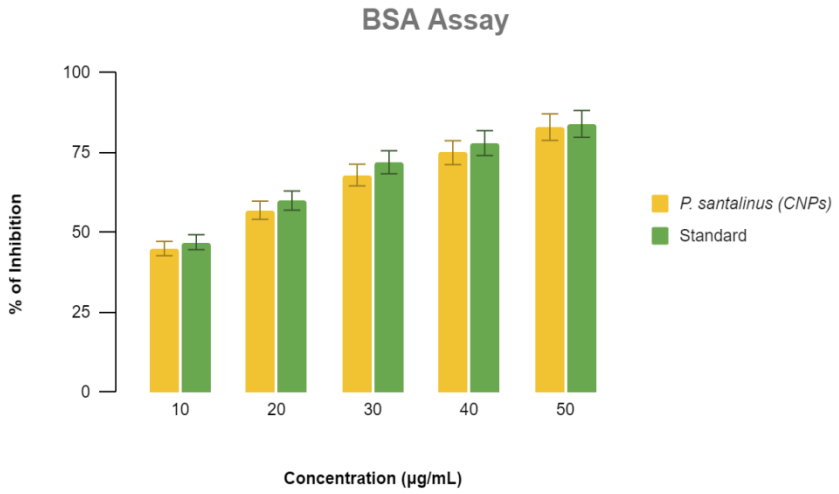
In Figure 6, biosynthesized CNPs exhibited a percentage of inhibition of 66.59%, 71.34%, 78.63%, 82.69%, and 87.61% at the concentrations of 10, 20, 30, 40 and 50µg/ml respectively. Standard shows values of 70.56, 75.68, 82.43, 86.57, and 91.39% respectively.

Figure 7: Radical scavenging activity of biosynthesized CNPs using nitric oxide assay



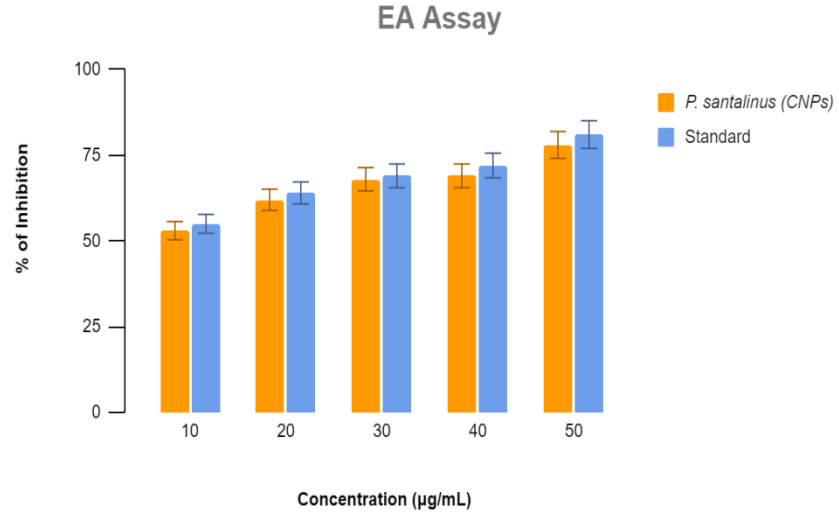
P.santalinus mediated CNPs showed nitric oxide scavenging activity of 67.45, 74.28, 74.69, 81.36 and 85.24% for the tested concentrations of 10, 20, 30, 40 and 50 µg/ml. Standard showed antioxidant activity of 72.43, 77.94, 80.37, 84.28 and 88.67% respectively for the tested concentrations as shown in Figure 7.

Figure 8: Anti-inflammatory activity of CNPs evaluated using bovine serum albumin denaturation assay



In Figure 8, green synthesized CNPs showed protein denaturation inhibition activity of 45% at 10µg/ml, 57% at 20µg/ml, 68% at 30µg/ml, 75% at 40µg/ml and 83% at 50µg/ml. Standard exhibited anti-inflammatory activity of 47, 60, 72, 78 and 84% respectively in tested concentrations.

Figure 9: Protein denaturation inhibition activity of CNPs assessed using egg albumin denaturation assay



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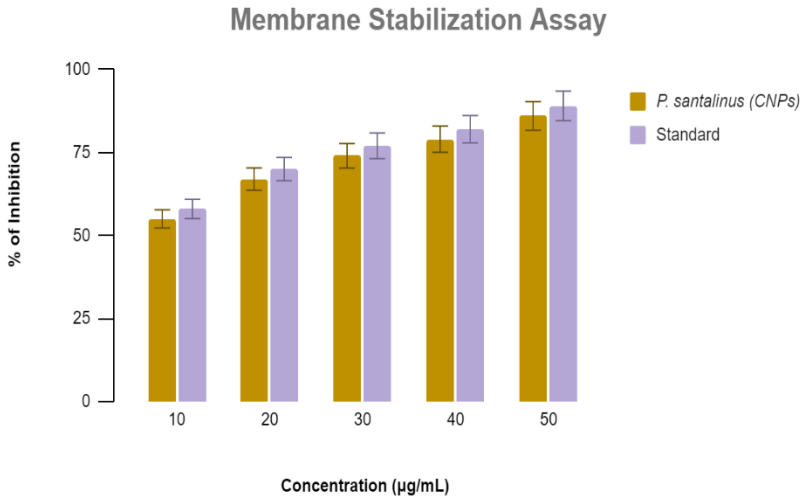
P.santalinus extract mediated CNPs showed anti-inflammatory activity of 53, 62, 68, 69 and 78% in the tested concentrations of 10, 20, 30, 40, and 50µg/ml. In the tested concentrations,

standard exhibited values of 55, 64, 69, 72, and 81% as represented in Figure 9.

Figure 10: Membrane stabilizing activity of biosynthesized CNPs assessed using human RBC membrane stabilization assay.



CNPs synthesized using P.santalinus extract showed membrane stabilizing activity of 55, 67, 74, 79, and 86% in the tested concentrations ranging from 10 to 50µg/mL. For the tested concentrations, the standard showed values of 58, 70, 77, 82, and 89%, which is graphically presented in Figure 10.

4. Discussion:

At the highest tested concentration of 50 µg/ml, biosynthesized CNPs exhibited antioxidant activity of 91.22% and standard exhibited a value of 93.15%. At the lowest tested concentration of 10µg/ml, CNPs, and standard exhibited values of 63.28 and 66.25% respectively in DPPH assay. Similar values are seen in previously done research, carbon dots (CDs) are synthesized using Azadirachtaindica (neem) leaf and it exhibits maximum antioxidant activity of 77% at the highest tested concentration of 50 µg/mL and minimum activity of 16% at the lowest tested concentration of 5µg/mL (17).Carbon Quantum Dots (CQDs) are synthesized using Ananas comosus (pineapple) peel and are taken in concentrations of 1 to 5 mg/ml. CQDs and standard ascorbic acid exhibited maximum antioxidant activity of 23.3 and 33.9% respectively at the highest tested concentration of 5 mg/ml (18). The leaf of Catharanthus roseus is used to synthesize CQD and is taken in concentrations ranging from 50 to 300 µg/mL. At a concentration of 200 µg/mL, CQD exhibited radical scavenging activity of 67.3% and after this concentration, it reached the saturation limit (19).

Antioxidant activity exhibited by synthesized CNPs ranges from 47.3% to 84.9% and values for a standard range from 51.1% to 89.9% for the tested concentration of 10 to 50µg/ml in hydrogen peroxide assay. Results from previously done studies show that at the concentration

of 1000 µg/mL, RGO-ZnO nanocomposite exhibited antioxidant activity of 62.1% (20). Graphene quantum dots magnesium hydroxide nanocomposites (GQDs/$Mg(OH)_2$) exhibited antioxidant activity of 62.18% at the concentration of 100 µg/mL (21). GQDs/ZnO nanocomposite is synthesized using Sarcopoteriumspinosum. At the concentration of 100 µg/mL, GQDs/ZnO and standard ascorbic acid exhibited antioxidant activity of 62.58 and 68.20% respectively (22).

Green synthesized CNPs and standard exhibited maximum radical scavenging activity of 87.31% and 90.89% at the highest concentration of 50µg/ml. At the lowest tested concentration of 10 µg/ml, CNPs exhibited values of 65.96% and 72.98% respectively in the FRAP assay.

Biosynthesized CNPs are assessed using ABTS assay, it exhibited maximum radical scavenging activity of 87.61% at highest tested concentration of 50µg/ml and showed a value of 66.59% at lowest tested concentration of 10µg/ml. The corresponding values exhibited by the standard are 91.39% and 70.56% respectively. Previously done studies show that RGO synthesized using green tea leaves (Camellia sinensis), GO and standard Trolox exhibited antioxidant activity of 88.87 ± 1.74, $4.38 \pm 2.53\%$, and $94.96 \pm 1.87\%$ respectively at the highest tested concentration of 600 µg/ml (23). GO-SNC is formed by fabricating rGO with tri-metallic nanocomposite - SnO_2-NiO-CuO (SNC). At the highest tested concentration of 0.020 mg/ml, GO, SNC, GO-SNC, and standard ascorbic acid exhibited radical scavenging activity of 98.25%, 96.78%, 93.86%, and 44.15% respectively (24).

P. santalinus-mediated CNPs are taken in concentrations ranging between 10 to 50µg/ml and are assessed using nitric oxide assay. In the tested concentrations, the radical scavenging activity of CNPs ranges from 67.45 to 85.24%. The values exhibited by the standard ranges from 72.43 to 88.67% respectively. Previously done research on GO shows that RGO-ZnO nanocomposite exhibited radical scavenging activity of 42.7% at the concentration of 1000 µg/mL (25).

Synthesized CNPs exhibited minimum protein denaturation activity of 45%at 10µg/ml and maximum activity of 83% at 50µg/ml concentration. For the same concentrations, values of the standard are 47% and 84% respectively in the bovine serum albumin assay. Previously done research shows that titanium-doped graphene oxide NPs (Ti/GO-NPs) are taken in concentrations ranging between 20 to 100 µg/mL. At the concentration of 80 µg/mL, Ti/GO-NPs and standard diclofenac sodium exhibited anti-inflammatory activity of 80 and 70%. At the concentration of 20µg/mL, their corresponding values are 19 and 38% respectively (26).

Green synthesized CNPs exhibited anti-inflammatory activity ranging from 53 to 78% for tested concentrations of 10 to 50µg/ml. The corresponding values of standard are 55 to 81% respectively in egg albumin denaturation assay. Research done on carbon allotrope shows that CQDs are synthesized using Z.officinaleand are used to prepare gold (GCQD-Au) nanocomposite. Anti-inflammatory activity exhibited by synthesized gold nanocomposite is 84.44% in the highest tested concentration (27). In the tested concentration of 50 µg/mL, CdO/GO and Ag_2O/GO nanocomposites exhibited protein denaturation inhibition activity with values of 30.6 and 30.5% respectively (28). In tested concentration of 250µg/mL, GO, $MG_{0.0}$ (0% of GO), $MG_{0.4}$ (4.0% of GO) and $MG_{0.5}$ (5.0% of GO) nanocomposites exhibited anti-inflammatory activity of 11.42%, 35.68%, 44.9%, and 12.2% respectively (29).

CNPs synthesized using P.santalinus bark extract exhibited membrane stabilizing activity of 86% at the highest tested concentration of 50µg/mL. For the same concentration, the standard exhibited a value of 89% in the human RBC membrane stabilization assay. Similarly done research on carbon allotropes shows that N-doped carbon nanodots (CNDs) are synthesized using citric acid, which exhibited membrane stabilizing activity of 40% at the concentration of 25 µg mL^{-1} and 82% at highest tested concentration of 200 µg mL^{-1} (25). Carica Papaya leaf is used to synthesize fluorescent carbon dots (CDs). For the tested concentrations of 0 to 45 µg/µL, CDs exhibited membrane stabilizing activity ranging from 58% to 97% and standard diclofenac exhibited values of 91% to 98% respectively (30). Carissa carandas is used to synthesize carbon dots and is doped with nitrogen (N-CDs) which are taken in concentrations of 20 to 100 µg mL^{-1}. The anti-inflammatory activity of N-CDs ranges from 86.13 to 97% and standard diclofenac exhibited values ranging from 78.67 to 96.71% for the tested concentrations (31).

5. Conclusion:

Carbon nanoparticles biosynthesized using Pterocarpus santalinus bark extract showed efficient antimicrobial, antioxidant, anti-inflammatory activity and non-toxic. 100% live nauplii are seen after 2 days of incubation with highest tested concentration - 80 µg/mL of CNPs. Green synthesis of nanoparticles makes nanoparticles free from toxic chemicals and improves its biomedical activity. There is only limited research available on carbon nanoparticles, thus it requires further study to assess its potential biomedical applications.

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