Anti-Inflammatory and Anti-Oxidant Activity of L. Angustifolia Mediated Carbon Nanoparticles Synthesized Using Microwave Heat Method

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This research aims to study the antioxidant, anti-inflammatory, antimicrobial, and cytotoxic activity of carbon nanoparticles biosynthesized using lavender extract. Green synthesis of CNPs using Lavandula angustifolia flower extract and is evaluated for antioxidant activity - DPPH, $\rm H_2O_2$, FRAP, ABTS assay, anti-inflammatory activity - bovine serum albumin, egg albumin denaturation, human RBC membrane stabilization assay, antimicrobial activity- agar well diffusion method and cytotoxic activity using brine shrimp lethality assay. Biosynthesized CNPs showed a maximum zone of inhibition of in the highest tested concentration of $100\mu \rm g/ml$. The results show that L. angustifolia-mediated CNPs are less cytotoxic and show excellent antioxidant, anti-inflammatory, and antimicrobial activity. Further research is needed on carbon nanoparticles to assess their biomedical activities to be used in future therapeutics.

Keywords: Anti-inflammatory, antioxidant, carbon nanoparticles, egg albumin, biomedical applications.

1. Introduction

The word "nano" originates from the Greek word - nanos meaning 'dwarf'. The main objective of the multidisciplinary field of nanotechnology is to govern matter at atomic and molecular scale. Nobel prize-winning physicist Richard P. Feynman introduced the initial idea of nanotechnological approaches in his lecture - "There's plenty of room at the bottom" in 1959. Professor Norio Taniguchi was the first to coin the term nanotechnology in 1974 (1).

Nanoparticles are the key component of nanotechnology. NPs have a size in the range of 1 to 100 nm. NPs have a high surface area to volume ratio, improved mechanical strength, and enhanced stability or reactivity in a chemical process, thus NPs in the nanoscale range exhibit distinct chemical, physical, and biological features than corresponding particles at a range exceeding nanoscale (2). Biomedical applications of NPs - bioimaging- surface-enhanced Raman spectroscopy (SERS), CT, fluorescence, MRI, photoacoustic, ultrasound imaging, positron emission tomography - PET, chemiluminescence imaging, therapy - magnetic hyperthermia, photodynamic and photothermal therapy, drug delivery, catalytic activity, and tumor tissue targeting (3), agriculture- fertilizer, sensors, pesticide, food packaging and preservation, medicine- antibacterial, antidiabetic, antifungal, anticancer, antioxidant, antiinflammatory and antiviral agents (4). Applications of nanotechnology are - in nanomedicine - targeted drug delivery, theranostics, imaging, photothermal therapy, cell repair, AIDS management vaccination, biosensors, tissue engineering, bone substitute material, dentistry, sensory aids, cosmetics (5), environmental remediation, wastewater treatment, solar cells, fuel cells, supercapacitors, rechargeable batteries, thermal insulation using aerogels, sensing - gas, heavy metal ion, electrochemical and optical sensor (6).

Nanoparticles are synthesized using two main approaches - top-down and bottom-up methods. In top down method, thin layered crystals are produced by breaking Vanderwaals force which holds bulk components together. The bottom-up method utilizes covalent or ionic bonding and involves the aggregation of atoms and molecules to produce NPs (7). Physical methods are classified under the top-down approach, and chemical and biological methods are classified under the bottom-up approach. Physical methods have drawbacks like cost of production is high, low yield, and high energy utilization. The chemical method is economical for largescale production and causes environmental damage by utilizing noxious chemicals and hazardous by-products for the synthesis of NPs. Traditional synthesis methods are timeconsuming and unsafe- carcinogenic, cytotoxic, genotoxic, and noxious chemicals are used, act as environmental pollutants, and biomedical applications are limited due to their toxic nature. Biosynthesized NPs are eco-friendly, feasible, rapid, economical, and non-toxic (8). Various organisms can be used for the biosynthesis of NPs like plants, bacteria, yeast, fungi, and actinomycetes. It has disadvantages like microbial culture needs to be preserved, and maintained to prevent contamination, and has advantages like - rapid reproduction, simpler culture methods, fast growth, and large-scale production of NPs mediated by plants without any contamination (9).

Carbon in a zero-valent state has the distinct ability to form C-C covalent bonds in varying hybridizations of sp, sp2, and sp3. This leads to various types of carbon allotropes such as graphite, diamond, carbon nanotubes, and graphene. Carbon allotropes can be classified based on the hybridization of carbon atoms, dimensions, and morphological features (10). Allotropes are elements which differ in physical properties but have identical chemical structure (11). Naturally occurring carbon allotropes are graphite - which has sp2 hybridization and diamond has sp3 hybridization. In 1985, Kroto et al. discovered the first synthetic allotrope - fullerene which is zero-dimensional. Then, one dimensional- carbon nanotube with sp hybridization is discovered by Iijima, and two dimensional- grapheme with sp2 hybridization is found by Novoselov (12). Carbon allotropes are classified based on morphology - with inner cavities-fullerene, CNT and without inner voids- carbon nano onions, graphene, carbon dots. Some

carbon allotropes - activated carbon, fullerene, graphene oxide, CNTs, nanohorns, mesoporous CNPs (MCNs) nanodiamonds, and carbon and graphene quantum dots (13). Applications of carbon-based nanomaterials are in automobile, aerospace, household applications, electrochemical energy storage- fuel cell, electrocatalyst, and supercapacitors. Biomedical applications- delivery of anticancer drugs, biomolecules detection, biosensors, immunosensors (11), antimicrobial activity, bioimaging, tumor targeting, tissue engineering, drug and gene delivery (12).

Lavender belongs to the family Lamiaceae, its generic name originates from the Latin verb 'layare' meaning to 'wash'. L. angustifolia has narrow leaves and is known as garden layender. Lavender oil contains more than 100 components. Lavender oil is used to treat skin inflammation, and psoriasis, used in an ointment to treat burns, has antimicrobial activity and thus fastens the rate of healing, in aromatherapy it is used as a sedative and has a moodenhancing effect (14). The Lamiaceae family has more than 200 genera which contains nearly 6000 species. Essential oil of Lamiaceae species has pharmacological properties like antiinflammatory, antidepressant, anticancer, antiseptic, sedative, antifungal, antimicrobial, antiviral, antibacterial, anxiolytic, and antioxidant activity (14,15). The main constituents found in most lavender essential oils are linalool, 1,8-cineole, camphor, α-pinene, borneol, βpinene, thymol, and ρ-cymene (15). Genus Lavandula is native to regions near the Mediterranean Sea. Lavandula angustifolia is commonly known as English lavender, it is frostresistant, has a beautiful color, and is a main species of lavender. Lavender is used in perfumes, food, aromatherapy, and cosmetics. The main components of lavender are volatile oils, limonene, linalyl acetate, coumarin, terpene, perillyl alcohol, tannin, cis-smine, and caffeic acid. Used in the treatment of depression, sleep disorders, dysmenorrhea, reduces labor pain, blood sugar levels, and episiotomy healing (16). Lavandula angustifolia flower extractmediated CNPs are assessed for antioxidant and anti-inflammatory activity using hydrogen peroxide, DPPH, FRAP, egg-albumin, and bovine serum albumin denaturation assays.

2. Materials and Methods:

Synthesis of carbon nanoparticles:

5g of dried Lavandula angustifolia flower is weighed and taken in a conical flask. 100 ml of distilled water is added to it and it is kept for boiling in a heating mantle. The extract is filtered using muslin cloth and the filtered extract is again kept for boiling to reduce it to 2ml. Then it is poured onto petri plates and burnt using a microwave oven. The burnt powder is scraped and stored for further use.

Antioxidant activity:

Hydrogen peroxide assay:

1 ml of hydrogen peroxide was added to 5 test tubes. L.angustifolia extract-mediated CNPs and standard ascorbic acid are taken in concentrations ranging between 10 to 50 μ g/ml. Test tubes are kept for incubation in a dark place for 10 minutes. Absorbance values are measured using a spectrophotometer at 530 nm wavelength. The percentage of inhibition is calculated using the formula (17):

Percentage of inhibition = [(absorbance of control - absorbance of sample) / absorbance of control] \times 100

DPPH assay:

In 10 ml of ethanol, 0.03g of DPPH powder is added and dissolved to prepare the stock solution. 0.05 ml of this prepared solution is mixed with 49.5 ml of methanol to form the working standard solution. 1 ml of working standard solution is taken in each test tube. L. angustifolia extract-mediated CNPs and standard ascorbic acid are taken in concentrations of 10, 20, 30, 40, and 50 μ g/ml. It is kept in dark incubation for 10 minutes. Changes in the color of the solution are observed and absorbance readings are taken at 517 nm wavelength. The percentage of radical scavenging activity is calculated using the formula (18):

% of radical scavenged = [(absorbance of control - absorbance of sample) / absorbance of control)]×100

FRAP assay:

In 5 centrifuge tubes, 0.5 ml of ferric chloride is taken. CNPs are added in concentrations ranging between 10 to 50 μ g/ml, 2.5 ml of phosphate buffer and potassium ferricyanide is added to all tubes. Kept for incubation at room temperature for 30 minutes. Add 2.5 of TCA and incubate at room temperature for 10 minutes. Then, samples are kept in a water bath for 10 minutes at 30°C and it is centrifuged for 10 minutes at 1200 rpm. 2.5 of distilled water and supernatant of the sample are taken in a test tube and incubated for 10 minutes. UV spectroscopy readings are taken at a wavelength of 700 nm. Antioxidant activity is calculated using :

% of radical scavenging of FRAP = [1 - (absorbance of sample/absorbance of control)] x 100 ABTS assay:

ABTS•+ (ABTS radical cation) reagent is produced by a reaction of 7.0 mM ABTS in 50% ethanol with 2.45 mM potassium persulfate (in distilled water). The prepared reagent is stored in the refrigerator for at least 24 hours. Before using, the reagent is diluted with 50% of ethanol until its absorbance reaches 1.0 (\pm 0.02) at a wavelength of 734 nm. Different concentrations of biosynthesized CNPs - 10, 20, 30, 40, and 50 $\mu g/ml$ are added to 250 μL of ABTS reagent. 200 μL of each concentration of NPs and reagent solution is added in a 96-well microplate. 200 μL of ethanol is used as a blank. After 10 minutes of dark incubation, its absorbance readings are measured at a wavelength of 734 nm using a microplate reader. The radical scavenging activity of CNPs is calculated using the formula :

% of radical scavenged = [(Absorbance of blank - Absorbance of sample in different concentrations) / Absorbance of blank] x 100

Nitric oxide assay:

Griess Ilosvay reaction is used to estimate radical inhibition of nitric oxide. Instead of 1-naphthylamine (5%), naphthyl ethylenediamine dihydrochloride (0.1% w/v) is used to modify the Griess Ilosvay reagent. 3 mL of the reaction mixture is made using 2 mL of sodium nitroprusside (10mm), 0.5 mL of phosphate buffer saline, and 10 to 50 μ g/ml of CNPs or standard rutin (0.5 mL). The reaction mixture is incubated for 150 minutes at 25°C. After

incubation, 1mL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) is mixed with 0.5mL of the reaction mixture and kept still for 5 minutes to complete diazotization. 1 mL of naphthyl ethylenediamine dihydrochloride is added, mixed, and kept still for 30 minutes at 25°C. In diffused light, a pink-coloured chromophore is formed. At 540 nm, the absorbance of the samples is measured against blank solutions. The percentage of inhibition is calculated using:

% of inhibition = [(absorbance of control - absorbance of sample) / absorbance of control] x 100

Anti-inflammatory:

Egg-albumin denaturation assay:

2.8 ml of phosphate buffer and 0.2 ml of egg white are added to test tubes. CNPs are added in concentrations ranging between 10 to 50 μ g/ml and incubated at room temperature for 10 minutes. After that, samples are kept in a water bath at 50°C. Changes in color in the sample are observed and UV spectroscopy readings are taken at 660 nm. The percentage of inhibition is calculated using the formula (19):

% of inhibition = (Absorbance of sample/ Absorbance of control) -1×100

Bovine serum albumin denaturation assay:

In 50 ml of distilled water, 0.05g of commercial bovine serum albumin is added and dissolved. 3 ml of this solution is added to test tubes and CNPs are added in concentrations ranging between 10 - 50 $\mu g/ml$. Incubated for 10 minutes at room temperature and kept in a water bath. UV spectroscopy readings are taken at 660 nm. Its percentage of inhibition is calculator using .

% of inhibition = [Absorbance of control - (Absorbance of sample / Absorbance of control)] $\times 100$

Membrane stabilization assay:

In a sterile test tube with anticoagulant, fresh human blood is collected and centrifuged at 8000 rpm for 10 minutes at room temperature to separate RBCs from other cellular components. After the supernatant is discarded, RBCs are washed with phosphate buffer for 3 times. Resuspending RBCs make 10%(v/v) of RBC suspension in 50 mM Tris-HCl buffer (p.H 7.4). In each centrifuge tube, 1 ml of RBC suspension is pipetted and different concentrations of CNPs ranging from 10 to 50 μ g/ml are added. It is mixed gently and incubated at 37°C for 30 minutes. To pellet RBCs, it is centrifuged at 8000 rpm for 10 minutes at room temperature. Using a UV-vis spectrophotometer, readings of supernatant are measured at a wavelength of 540 nm. The percentage of inhibition is calculated using the formula :

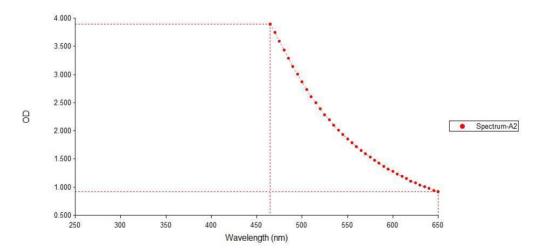
% of inhibition = [(absorbance of control - absorbance of sample) / absorbance of control] x 100

3. Results:

Figure 1: Lavender extract is prepared using dry lavender flower and distilled water



Figure 2: UV spectra analysis of carbon nanoparticles synthesized using lavender flower



The optical characteristics of the nanoparticle formed from carbon were investigated by measuring their UV-Vis absorption spectra throughout a wavelength range of 250 to 650 nm. The presence of molecules bonded to the surface or surface functional groups may have an impact on the electrical structure of the nanodots, as evidenced by the maximum absorption peak seen at 465 nm as shown in Figure 2.

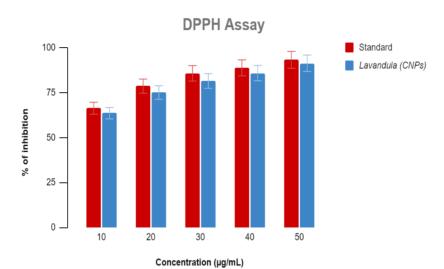
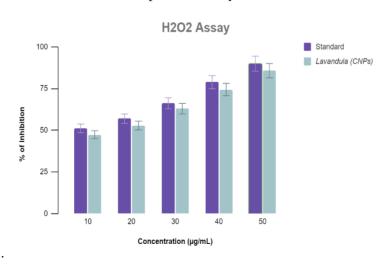


Figure 3: Antioxidant activity of green synthesized CNPs evaluated using DPPH assay

In Figure 3, Lavandula angustifolia flower extract-mediated CNPs are assessed for antioxidant activity using DPPH assay. CNPs showed antioxidant activity of 63.51% at 10 μ g/ml concentration, the values for concentrations of 20, 30, 40, and 50 μ g/ml are 74.96%, 81.39%, 85.76%, and 91.24%. Standard showed antioxidant activity of 66.25, 78.52, 85.63, 88.68, and 93.15% for the tested concentrations respectively.

Figure 4: Free radical scavenging activity of biosynthesized CNPs assessed using hydrogen peroxide assay



CNPs biosynthesized using L.angustifolia flower extract exhibited radical scavenging activity of 47.3, 52.8, 62.9, 74.4, and 85.7% in the tested concentrations of 10, 20, 30, 40 and 50 μ g/ml. Standard shows values of 51.1%, 56.9%, 66.1%, 78.8% and 89.9% respectively for the above mentioned concentrations as shown in Figure 4.

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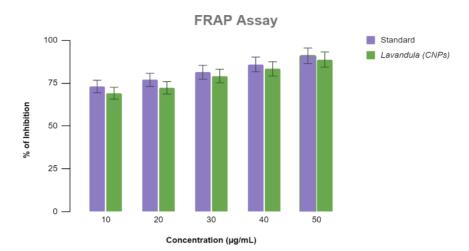
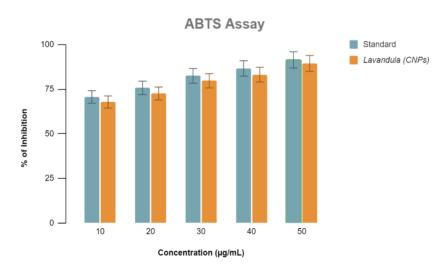


Figure 5: Antioxidant activity of green synthesized CNPs assessed using FRAP assay

L. angustifolia flower extract mediated CNPs showed maximum radical scavenging activity of 88.67% at the highest tested concentration of $50~\mu g/ml$. The values for concentrations of 10, 20, 30 and $40~\mu g/ml$ are 69.07, 72.18, 79.08 and 83.24%. Standard exhibited antioxidant activity of 90.89% at the highest tested concentration. The values for remaining concentrations are 72.98, 76.84, 81.31 and 85.84% represented graphically in Figure 5.

Figure 6: Antioxidant activity of L.angustifolia mediated CNPs assessed using ABTS assay.



In Figure 6, green synthesized CNPs showed antioxidant activity of 67.77, 72.53, 79.64, 83.11 and 89.38% in tested concentrations ranging from 10 to 50 μ g/ml. Standard showed values of 70.56, 75.68, 82.43, 86.57, and 91.39% in tested concentrations respectively.

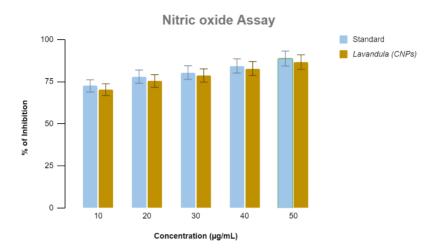
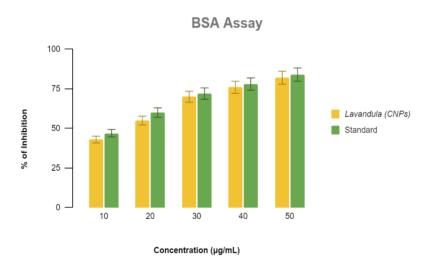


Figure 7: The radical scavenging activity of CNPs evaluated using nitric oxide assay.

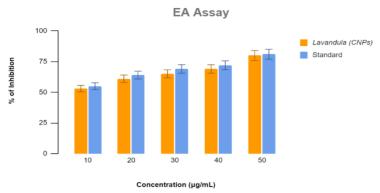
Biosynthesized CNPs exhibited radical scavenging activity of 70.14, 75.36, 78.62, 82.76 and 86.59% for the tested concentrations of 10, 20, 30, 40 and 50 μ g/ml in nitric oxide assay. Standard exhibited values of 72.43, 77.94, 80.37, 84.28, and 88.67% respectively as shown in Figure 7.

Figure 8: Anti-inflammatory activity of biosynthesized CNPs assessed using BSA assay.



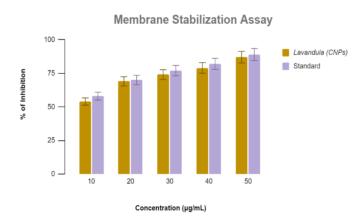
Green synthesized CNPs are taken in concentrations of 10, 20, 30, 40, and 50 μ g/ml and showed protein denaturation inhibition activity of 43, 55, 70, 76 and 82%. Standard exhibited values of 47, 60, 72, 78, and 84% as shown in Figure 8.

Figure 9: Anti-inflammatory activity of biosynthesized CNPs evaluated using egg albumin denaturation assay.



Biosynthesized CNPs are taken in concentrations of 10, 20, 30, 40 and 50 μ g/ml and it shows percentage of inhibition of 53, 61, 65, 69 and 80% for these tested concentrations in egg albumin denaturation assay. Standard taken in tested concentration showed values of 55, 64, 69, 72, and 81% respectively for anti-inflammatory activity as shown in Figure 9.

Figure 10: Anti-inflammatory activity of CNPs assessed using membrane stabilization assay.



L. angustifolia flower extract-mediated CNPs show membrane stabilizing activity of 54%, 69%, 74%, 79%, and 87% in tested concentrations of 10, 20, 30, 40 and 50 μ g/ml in human RBC membrane stabilization assay as shown in Figure 10. The values exhibited by the standard are 58%, 70%, 77%, 82%, and 89% in tested concentrations respectively.

4. Discussion:

In the DPPH assay, L.angustifolia-mediated CNPs exhibited anti-oxidant activity for the tested concentrations ranging from 10 to 50 μ g/ml in the range of 63.51 to 91.24% and standard exhibited values in the range of 66.25 to 93.15% respectively. Previously done studies show

microwave-synthesized carbon dots (U-dots) tested in concentrations ranging between 0.02 to 0.09 mg/mL exhibited free radical scavenging activity ranging from 23.30 ± 0.08 to $87.26 \pm 0.02\%$ (20). Nanocomposite containing multiwalled carbon nanotubes (MWCNTs) and hydroxyapatite NPs are biosynthesized using Beta vulgaris leaf extract. Synthesized nanocomposite, B.vulgaris extract, and standard butylated hydroxyl toluene (BHT) exhibited antioxidant activity of 30%, 70%, and 90% respectively (21). Carbon dots (C-dots) and polyethyleneimine surface passivated C-dots (CDP) are synthesized using mint leaves. For the tested concentrations ranging from 5 to 60 μ g mL⁻¹, CD exhibited antioxidant activity in the range of 4.7 to 21.95% and CDP showed values ranging from 12.0 to 64.94% respectively (22).

At the lowest tested concentration of 10 μ g/ml, L.angustifolia flower extract mediated CNPs and standard exhibited radical scavenging activity of 47.3 and 51.1%. At the highest tested concentration of 50 μ g/ml, their corresponding values are 85.7 and 89.9% respectively in hydrogen peroxide assay. Some previously done studies showed similar activity. The nanocomposite of vanadium oxide and graphene oxide (V₂O₅/GO) is synthesized using coprecipitation. At the concentration of 350 μ g/mL, V₂O₅/GO1:3, V₂O₅/GO1:4, and ascorbic acid exhibited antioxidant activity of 60, 53, and 75% respectively (20). GO with ratios of 1:2, 1:3, and 1:4 of graphite: potassium permanganate mass ratios are synthesized and decorated with magnesium oxide NPs. At a tested concentration of 400 μ g/mL, GO1:4, MgO-rGO1:4, and ascorbic acid exhibited radical scavenging activity of 58.36±5.29, 44.97±1.30 and 83.61±0.25% respectively (23).

CNPs biosynthesized using L.angustifolia flower extract exhibited antioxidant activity in the range of 69.07 to 88.67% for the tested concentration ranging from 10 to 50 μg/ml and standard exhibited values ranging from 72.98 to 90.89% in FRAP assay. A previously done study showed similar results. Aerial parts - leaf, stem, and flower of the Acetosa sagittata plant are used to biosynthesize carbon dots. For tested concentrations ranging from 50 to 200 μg mL⁻¹, the antioxidant activity of leaf-mediated CDs ranged from 80.26 to 92.62%, stem-mediated CDs ranged from 86.82 to 93.62% and flower-mediated CDs ranged from 83.07 to 90.37% respectively (24). At the highest concentration of 50 μg/ml, green synthesized CNPs and standard showed values of 89.38 and 91.39% respectively for antioxidant activity in the ABTS assay. In a similarly done research, Crocus sativus petal extract-mediated ZnO-NPs are decorated by graphene (G-ZnO). For tested concentrations ranging from 31.2 to 500 μg.ml⁻¹, G-ZnO nanocomposite showed values ranging from 55.76 to 95.1% for antioxidant activity (25). GO is incorporated with Psidium guajava leaf extract mediated selenium microparticles (SeNPs) forming (Se/GO) and it exhibited antioxidant activity of 90.9% (26).

In nitric oxide assay, L. angustifolia flower extract mediated CNPs and standard exhibited percentage of inhibition of 86.59 and 88.67% for antioxidant activity at highest tested concentration of 50 μ g/ml. In the bovine serum albumin denaturation assay, CNPs biosynthesized using L. angustifolia flower extract exhibited anti-inflammatory activity ranging from 43 to 82% and the values for standard ranged from 47 to 84% for the tested concentration ranging from 10 to 50 μ g/ml. Research conducted using carbon allotropes showed the following results. For the tested concentrations ranging from 50 to 250 μ g mL⁻¹, fluorescent carbon dots biosynthesized using leaf extract of Centella asiatica (Ca-CDs) exhibited anti-inflammatory activity ranging from 32.96 \pm 0.4411 to 85.84 \pm 0.7321% and

values for standard aspirin ranged from 38.03 ± 0.5023 to $91.04 \pm 1.9049\%$ (27). At a concentration of 50 µg mL⁻¹, Hibiscus rosa-sinensis leaf extract-mediated carbon quantum dots (CQDs), plant extract, and standard aspirin inhibited protein denaturation by 23.78 ± 2.2001 , 18.28 ± 2.9017 , and $35.47 \pm 2.1572\%$. At the highest tested concentration of 250 µg mL⁻¹, their corresponding values are 89.42 ± 3.7487 , 73.33 ± 2.3999 , and $94.03 \pm 2.908\%$ respectively (28). Carbon nanodots produced from biomass waste exhibited anti-inflammatory activity of 62% (29).

At the highest tested concentration of 50 μ g/ml, standard and biosynthesized CNPs exhibited values of 81 and 80% for anti-inflammatory activity in egg albumin denaturation assay. CNPs biosynthesized using L. angustifolia flower extract and standard exhibited membrane stabilization activity of 87 and 89% in human RBC membrane stabilization assay. Research conducted using carbon allotrope showed the following result. Young leaf (YC) of Coffea arabica extract-mediated rGO is synthesized. At the highest tested concentration of 1200 μ g/ml, YC/rGO, GO and aspirin exhibited membrane stabilizing activity of 75.15 \pm 0.14, 34.00 \pm 0.18, and 60.24 \pm 2.11% respectively (30,31).

5. Conclusion:

Green-synthesized nanoparticles are cost-effective, eco-friendly, non-toxic as NPs are not synthesized using harmful reducing agents, and bioactive compounds of plant extract elevate the biomedical activity of nanoparticles. In this research, carbon nanoparticles are biosynthesized using Lavandula angustifolia flower extract and the NPs exhibited efficient antioxidant and anti-inflammatory activity. Further research must be conducted on carbon nanoparticles to study their biomedical applications and whether they can be utilized in therapeutic applications to cure diseases than conventional treatment.

Funding Details:

There is no funding acquired for carrying out this research.

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