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Green synthesis and characterization with *in vitro* antibacterial and antioxidant profiling of gold nano-conjugates using *Citrus limon* and *Citrus aurantifolia* leaf

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Abstract
Background: Nanotechnology, concerned with the nanoparticles having a size of 1-100 nm in one dimension is one of the ultimate expeditiously advanced areas of technology that has opened up many new edges of research for modern day society. These nano-conjugates can be synthesized via different methods. Although, the green approach is the most favoured one as it is way more natural, less hazardous, and eco-friendly in nature.
Method: Our present study gives an overview of two *Citrus* species plant (Family: Rutaceae) i.e., *Citrus limon* and *Citrus aurantifolia* leaf extract treated gold nano-conjugates synthesis. Subsequently after synthesis, gold nanoparticles were undergone through biophysical techniques; including Stability checking, UV-Vis Spectrophotometric analysis, DLS, and FT-IR followed by *in vitro* antibacterial and antioxidant profiling (HPLC-DAD screening of polyphenolics).
Results: According to the results via different characterization methods, it is revealed that each of the nano-conjugate displayed a single sharp peak within the range of 500-550 nm. As well as particle size ranges within 7 nm-20 nm. Whereas FT-IR data of both crude leaf extract and their respective synthesized gold-nanoparticles establish the amalgamation of various bioactive compounds of the extracts with the gold nanoparticles. Presence of sinapic acid as a potent phenolic acid emphasizes the antioxidative nature of the plant itself.
Conclusion: The *in vitro* antibacterial efficacy against two well-known bacteria and anti-oxidant activity using DPPH assay further enhances the stability of those green synthesized nano-conjugates.

Keywords: *Citrus Limon*, *Citrus aurantifolia*, green synthesis, gold nano-conjugates, HPLC-DAD profiling, anti-oxidant activity, antibacterial property

Introduction
Nanotechnology is one of the most widely used technologies in translational research where the size of particles should be within the range of 1 to 100 nm. [1, 2]. Synthesis of nanoconjugates having eco-friendly way has attracted a great attention [3, 4]. There are many more uses in the fields of food, drug delivery, environment and photo electrochemical applications [5-9]. It was found that to overcome the problem of nanoscale toxicity, researchers have incorporated the concept of "green chemistry" that involve bacteria, fungi, and plants [10, 11]. The use of plant extracts to synthesize large numbers of nanoparticles is the subject of this article as it is the most widely used method of environmentally friendly and environmentally friendly approaches in chemistry. Medicinal plants have long been treated as a source of remedies. Most of these plants contain certain phytochemicals referring to various compounds that occur naturally in plants which play an essential role in drug discovery. Although most people in developing countries rely on herbal medicines and their derived bioactive compounds to develop new antimicrobial compounds from alternative sources such as medicinal plants [12, 13]. Plants belonging to the family Rutaceae under the genus- Citrus are rich in these secondary bioactive metabolites [14]. Among many other citrus species plants, our study deals with two important species, *Citrus aurantifolia*, [Figure 1A] commonly known as lime, and the other *Citrus limon*, natively known as a lemon. *Citrus limon* (*C. limon*) [Figure 1B] is a spiny, evergreen shrub mostly native to tropical and subtropical Southeast Asia [15]. More precisely, lemons are known for their properties and their importance in relation to food and nutrition. But most importantly, it is of great medical and nutritional importance [16]. *Citrus* plants have been shown to have powerful analgesic, anti-inflammatory, antioxidant, anthelmintic, antibacterial, antifungal, and lipid-lowering properties.

They have been found to have significant amounts of anti-hyperglycaemic, anti-diabetic, and hypoglycaemic activity [17, 18]. *Citrus aurantifolia* (*C. aurantifolia*) is a small, dense, irregularly branched tree characterized by short, sharp thorns and alternating oval to oblong shaped leaves with serrated edges, native to hot subtropical or tropical regions India, Egypt and the West Indies [19]. The importance of *C. aurantifolia* in both domestic and ethnographic use cannot be exaggerated. It is valued for its multi health beneficiary properties including antivirals, antifungals, anthelmintics, astringents, diuretics, and treatment of stomach disorders, headaches, arthritis, colds, coughs, and sore throats. Traditionally used as a folk medicine for its role as appetite stimulant [20, 39].

Fig 1: Plant Body of *Citrus* Species [1A: *Citrus limon*; 1B: *Citrus aurantifolia*]

Here our current research investigation focuses on green synthesis and comparative biophysical characterization of gold nano conjugates as well as evaluation of *in vitro* antioxidant efficacy and antibacterial potential of *C. limon* and *C. aurantifolia* leaves aqueous extract and their synthesized gold nano conjugates. The overall work flow is represented through Figure 2.

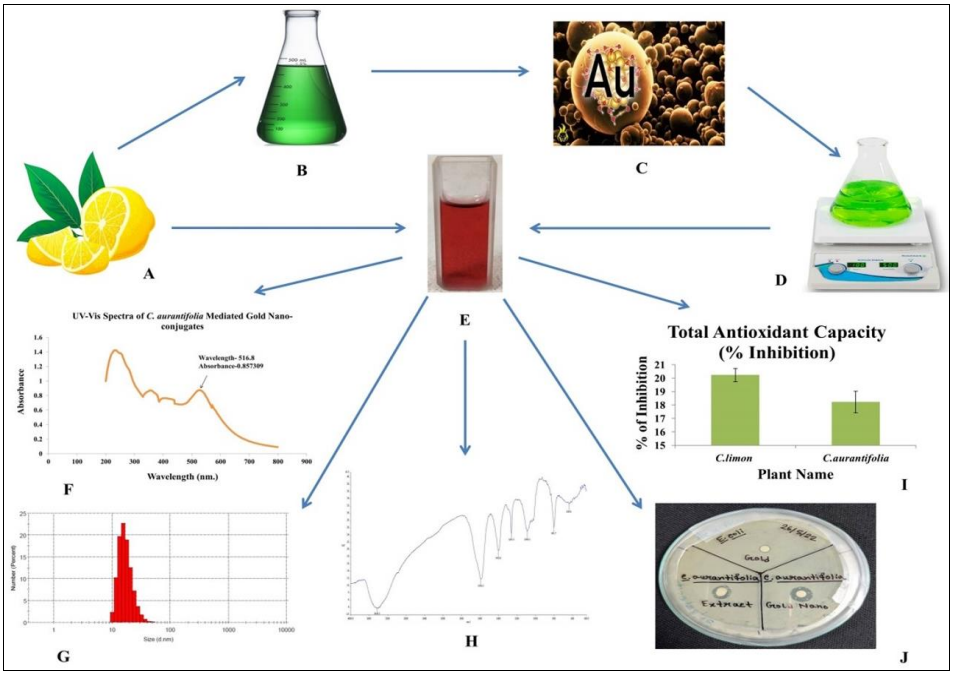


Fig 2: The Overall Workflow [A: Selection of Plant Species; B: Preparation of Leaf Extract; C: Application of Gold Solution; D: Initiation of Gold Nano-Conjugates Synthesis; E: Prepared Gold Nano-Conjugates; F-J: Biophysical Characterizations of Gold Nano-Conjugates Along With *In vitro* Evaluation of Anti-Oxidant and Antibacterial Efficacy]

Materials

Chemicals

All the analytical grade chemicals are used in the experiments. Ascorbic acid and tetrachloroauric acid trihydrate ($HAuCl_4$, $3H_2O$) were obtained from Merck Life Science, Mumbai. DPPH was bought from SRL Pvt. Ltd., Maharashtra. Trisodium citrate dihydrate from RFCL Ltd. Haryana. Besides these, both Luria Bertani agar and Luria broth were collected from Himedia Laboratories Pvt. Ltd., Mumbai.

Sample collection and preparation

The leaves of both plant species *Citrus limon* and *Citrus aurantifolia* [Figure 1A and 1B], were collected from N 24 PGS, West Bengal, in the month of March-2022. After collection, both the leaves were carefully washed and dried completely; next, each leaf sample was mechanically grounded to coarse powder.

Methods

Extraction technique

1gm of each leaf grounded powder was added to standard amount of solvent i.e., 20 ml. (double distilled water) in two conical flasks and kept at room temperature for overnight extraction. After overnight shaking extracts were filtered. The filtered solutions of both the extracts were stored at the refrigerator according to the need for the specific assay.

Extractive value

The percentages of extractive value were determined by the ideal method with slight modifications using distilled water as solvent [22].

HPLC-DAD Mediated Quantification

The HPLC-DAD based quantification of ten bioactive compounds was accomplished. The peak area was calculated by Open Lab CDS version 2.0 software [23, 24].

Preparation of 1mM Chloroauric acid Solution

Chloroauric acid aqueous solution was prepared by following the standard procedure with slight modifications. Accurately weighed tetrachloroauric acid trihydrate was diffused in HPLC graded water and stir for sometimes on magnetic stirrer for complete dissolution. After the solution was prepared, it was filtered using 0.2 µm. syringe filter.

Synthesis of Plant mediated Gold Nano-Conjugates

Initially, in a conical flask 20 ml. of 1mM gold solution was taken and kept on magnetic stirrer cum mantle heater for allowing the solution to boil up to at least 100˚. Meanwhile 1 ml. of plant extract (respectively *C. limon* and *C. auarntifolia* leaf aqueous extract) and 1 ml. of trisodium citrate dihydrate (1 gm. in 10 ml dH_2O) were merged in a beaker and from that mixture add 200 µl. to the boiling gold solution drop wise. As soon as the colour of the light yellow gold solution changes to red wine/ purple colour, stop adding the plant extract more and start the magnetic stirrer to assure mixing of nanoparticles throughout the solution. Next the prepared plant mediated green synthesized nano-conjugates were collected in a small glass bottles for further experiments.

Physical Characterization Techniques for Gold Nano conjugates

The biophysical characterization study of gold nano-conjugates comprises of 3 consecutive day-based stability checking, UV-Vis Spectrophotometric analysis, Fourier Transmission Infrared Spectroscopy and last but not the least Dynamic light Scattering. All of these techniques give the information about size, shape, quantity, stableness and presence of the functional groups of the nanoparticles.

Stability Checking

A three consecutive day's stability test was done employing UV-Vis spectrophotometric scanning analysis (200-800 nm.) and change in absorbance for every day that revealed the stability of the gold nano-conjugates.

UV-Vis Spectroscopy

The main objective of absorbance spectroscopy is to identify the optical properties of the solution. Lights of different wavelengths are passed through the sample and the amount of absorbed light is measured. This whole technique works on the rule of Beer-Lambert's Law for measuring the absorbance where the change in wavelengths usually arises because of the surface plasmon resonance of the particle. (UV-Vis: By SHIMAZDU MODEL: 2401PC) [25-27].

Dynamic Light Scattering (DLS)

Dynamic Light Scattering is one of the quantitative techniques that allows particle sizing down to 1 nm. In diameter. According to the procedure, a light from a laser source is sent through the solution and upon interacting with the moving particles of the solution, the light gets scattered in different direction which creates change in the frequency of light, directly related with the size of the particles. The smaller the size of the particle is, the greater the shift in the frequency to happen. (DLS: Zeta Size Nano-S. By MALVERN Instruments. MODEL: ZEN1600) [25-27].

Fourier Transmission Infrared Spectroscopy (FT-IR)

FT-IR is a chemical interpretive technique that determines infrared intensity against wavelength of the light by detecting the vibration characteristics of functional groups of light of the particles. (FT-IR: By PerkinElmer. MODEL: SPECTRUM 100) [25-27].

Determination of *In vitro* Antioxidant Efficacy of Gold Nano-Conjugates

DPPH radical scavenging assay

Estimation of *in vitro* antioxidant capacity via DPPH radical scavenging activity was checked following the typical procedure with minor alterations using ascorbic acid as standard [28] Absorbance was read at 517 nm. The % of inhibition was computed by the below formula:

% Inhibition of DPPH= OD of control-OD of sample/ OD of control*100

Determination of *In vitro* Antibacterial Potential of Gold Nano-Conjugates

Disk Diffusion method

Two bacterial strains used in the present study were obtained from the Department of Microbiology, Techno India University, West Bengal.

Gram-positive bacteria: *Staphylococcus aureus*
Gram-negative bacteria: *Escherichia coli*

The antibacterial susceptibility study was carried out via disc diffusion assay [29, 30]. The antibacterial affectivity was measured on the basis of zone of inhibition (mm.) surrounding the disc. All tests were carried out in triplicates.

Results and Discussion

Extractive Value

The current study has shown that the extractive value of *C. limon* aquoues extract was highest, i.e., 19.25±1.23%, whereas *C. aurantifolia* aquoues extract shows 9.63±0.51%, which is the lowest. Percentages of extractive value [Figure 3] are directly correlated with the number of bioactive constituents present in the extract using a specific solvent.

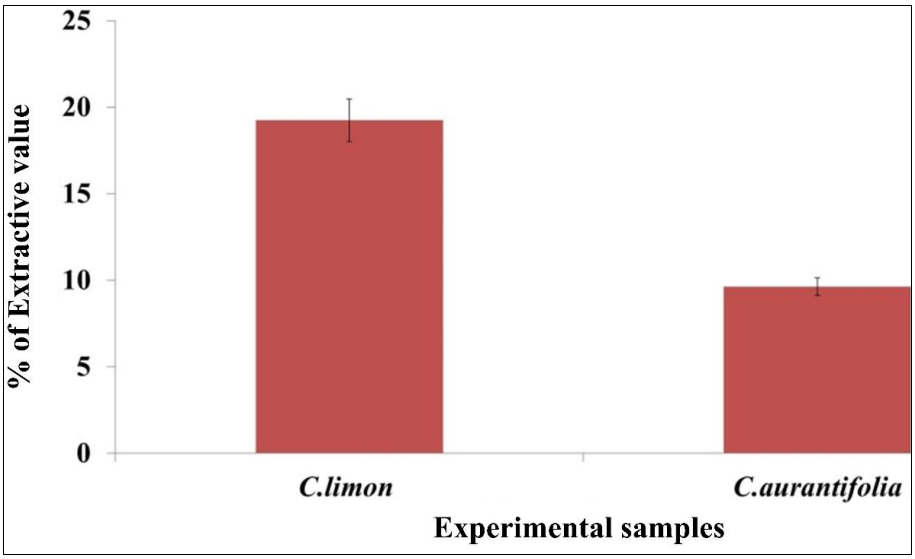


Fig 3: Graph Showing the Extractive Values in Percentages for *C. limon* and *C. aurantifolia* Aqueous Extract

HPLC-DAD Mediated Quantification

The HPLC-DAD based screening profile obtained from the *Citrus* species plant leaves aqueous extracts revealed ten bioactive polyphenolic compounds. Among them, sinapic acid was found to be present in the maximum amount in both leaf extracts; in case of *C. limon* it was 153.487 ng/µl. whereas in case of *C. aurantifolia*, it was 223.090 ng/µl. on the other hand quercetin was present in the lowest amount in both plant leaf extracts i.e., 1.531 ng/µl. in case of *C. limon* and 1.835 ng/µl. in case of *C. aurantifolia*. The overall HPLC-DAD quantification data is represented by both Figure 4 and Table 1. Sinapic acid which is signified as a potent antioxidant, has been investigated and reported to be effective in treating a number of clinical disorders, including infections [31], oxidative stress [32], inflammation [33, 34], cancer [35], diabetes [36], neurodegeneration [37], and anxiety [38].

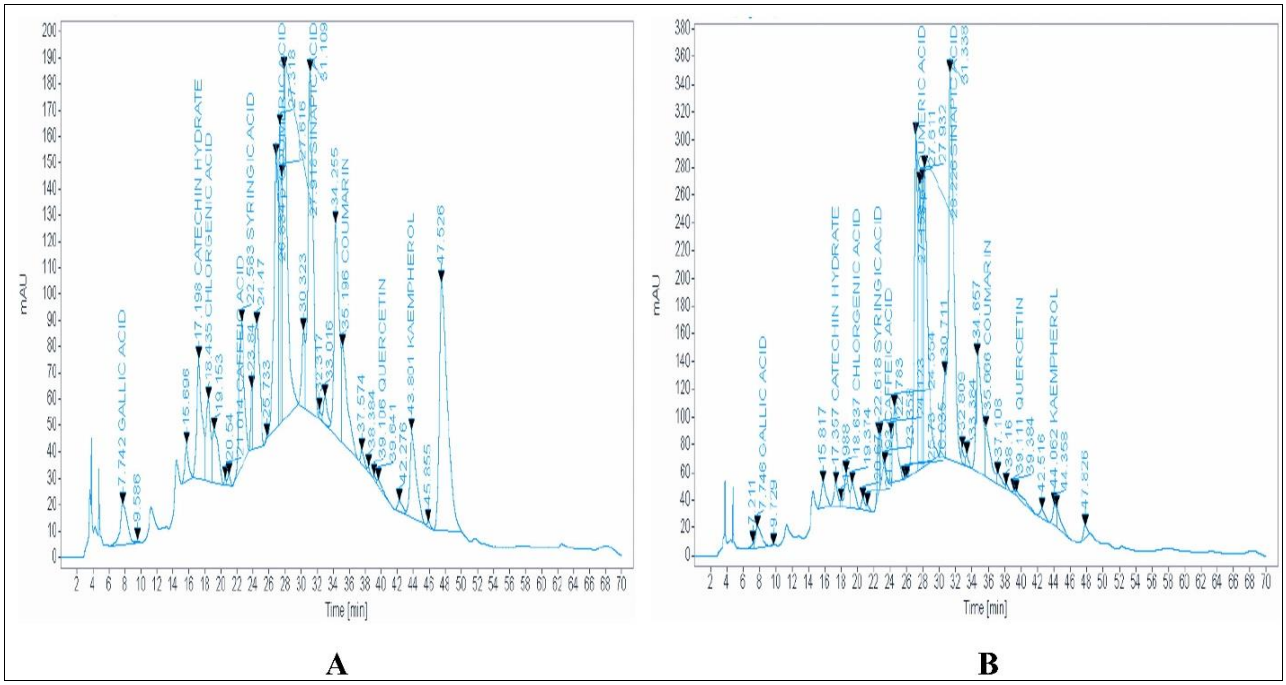


Fig 4: HPLC-DAD Mediated Quantification of the Bioactive Compounds Presents in the Aqueous Extracts of [A: *C. limon*; B: *C. aurantifolia*]

Table 1: HPLC-DAD Mediated Quantification of Bioactive Compounds Present in the Aqueous Extracts of *C. limon* and *C. aurantifolia*

Phenolic compounds detected	Amount to be present in *C. limon* Extract (ng/ µl.)	Amount to be present in *C. aurantifolia* Extract (ng/ µl.)
Gallic acid	17.307	14.457
Catechin hydrate	90.040	24.776
Chlorogenic acid	42.103	28.214
Caffeic acid	1.747	1.964
Syringic caid	28.497	8.324
p Coumeric acid	34.184	70.090
Sinapic acid	153.487	223.090
Coumarin	16.119	14.007
Quercetin	1.531	1.835
Kaempferol	52.076	11.615

Biophysical Characterization Results of the Gold Nano-Conjugates

Stability Checking

The results of three days-based stability analysis revealed that the prepared gold nano-conjugates of both *C. limon* and *C. aurantifolia* show their absorption peak more or less on similar wavelengths i.e., in case of *C. limon* it was around 531.2 to 540.8 nm. And in case of *C. aurantifolia* it was 516.8 to 526.4 nm. [Figure 5]

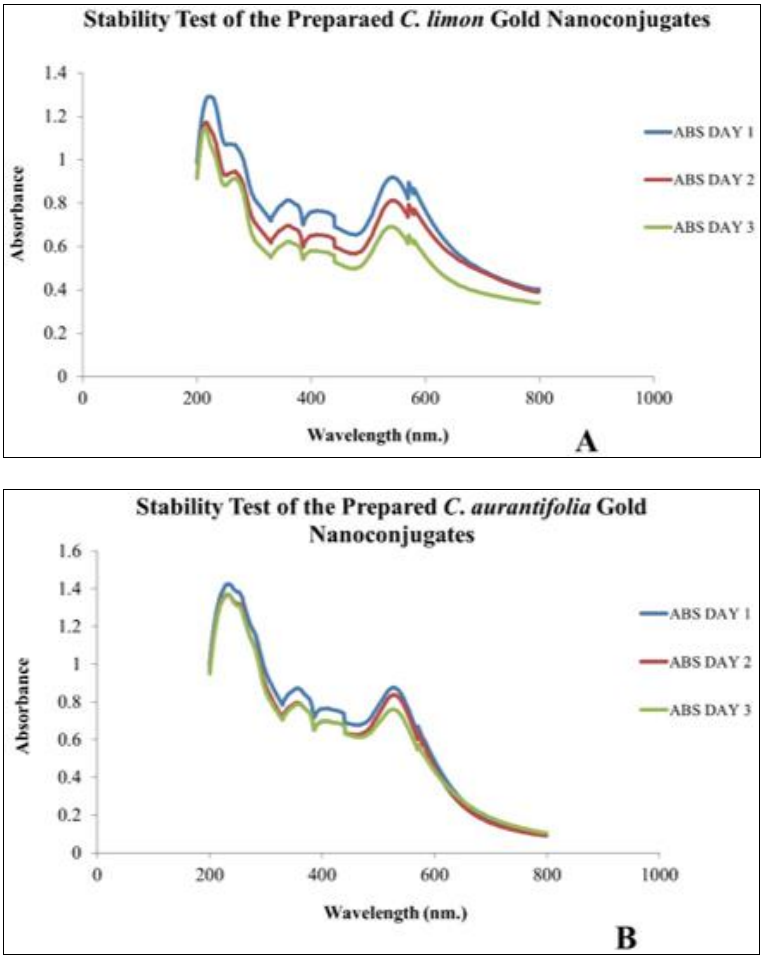


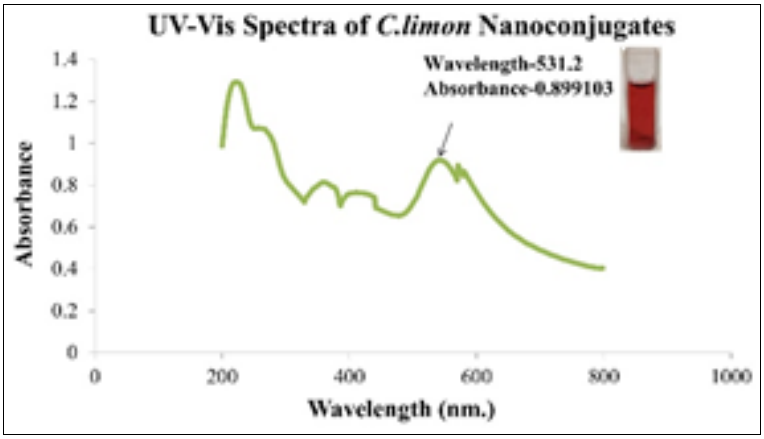
Fig 5: Stability Checking Spectral Data (3 Days) of Plant Mediated Gold Nano-Conjugates [A: *C. limon*; B: *C. aurantifolia*]

UV-Vis Spectroscopy

The UV-Vis absorbance spectrum of *C. limon* and *C. aurantifolia* leaf aqueous extract mediated gold nano-conjugates have shown single sharp peak at each of the respective wavelength of 531.2 nm. and 516.8 nm. The absorbance values of each of gold nano-conjugates are given in tabulated manner. [Table 2 and Figure 6] Giving absorption peak within the range of 500-600 nm. further confirms the formation of plant mediated green nano-conjugates.

Table 2: UV-Vis Absorption Spectral Data of *C. limon* and *C. aurantifolia* Leaf Extract Mediated Gold Nano-Conjugates

Plant Name	Absorption Peaks at	Absorbance Value
C. limon	531.2 nm.	0.899103
C. aurantifolia	516.8 nm.	0.857309



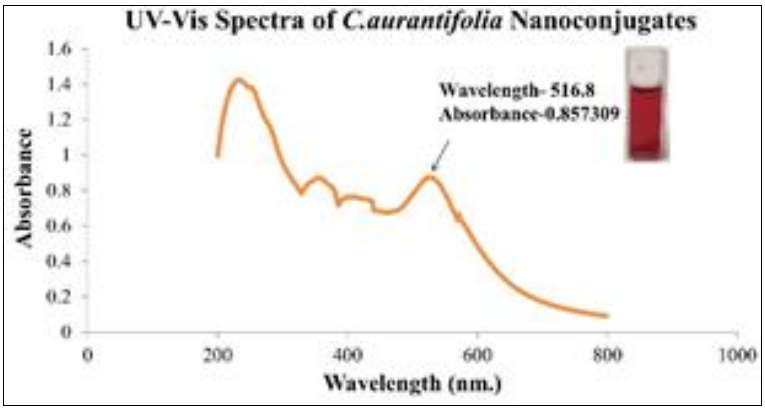
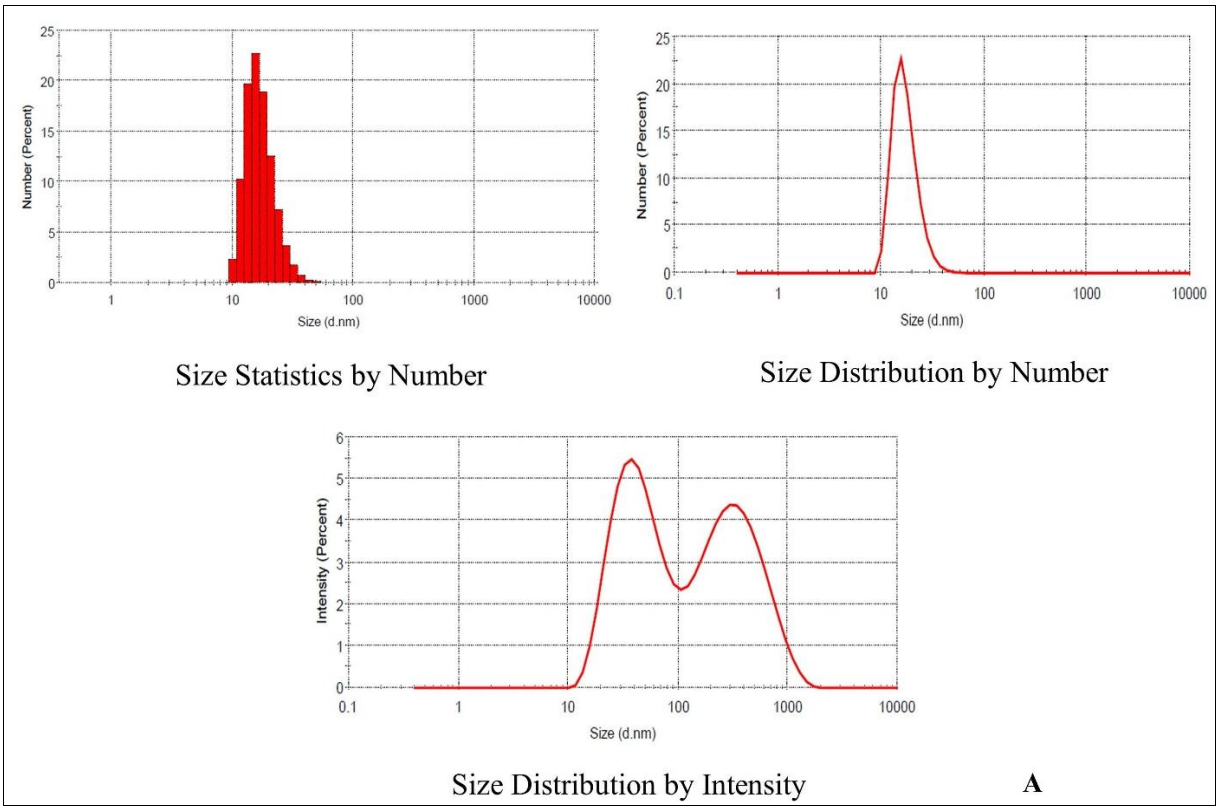


Fig 6: UV-Vis Spectra of Mediated Gold Nano-Conjugates [A: *C. limon*; B: *C. aurantifolia*]

Dynamic Light Scattering (DLS)

The histogram regarding the size distribution data of DLS indicates the size of *C. limon* and *C. aurantifolia* leaf aqueous extract mediated gold nano-conjugates varies from 7.123 nm to 17.54 nm. This particle size can be determined by aligning the irregular changes in the power of light dispersed from the suspension of nano-conjugates. The DLS result of the two gold nano-conjugates is represented through graphical manner. [Figure 7]



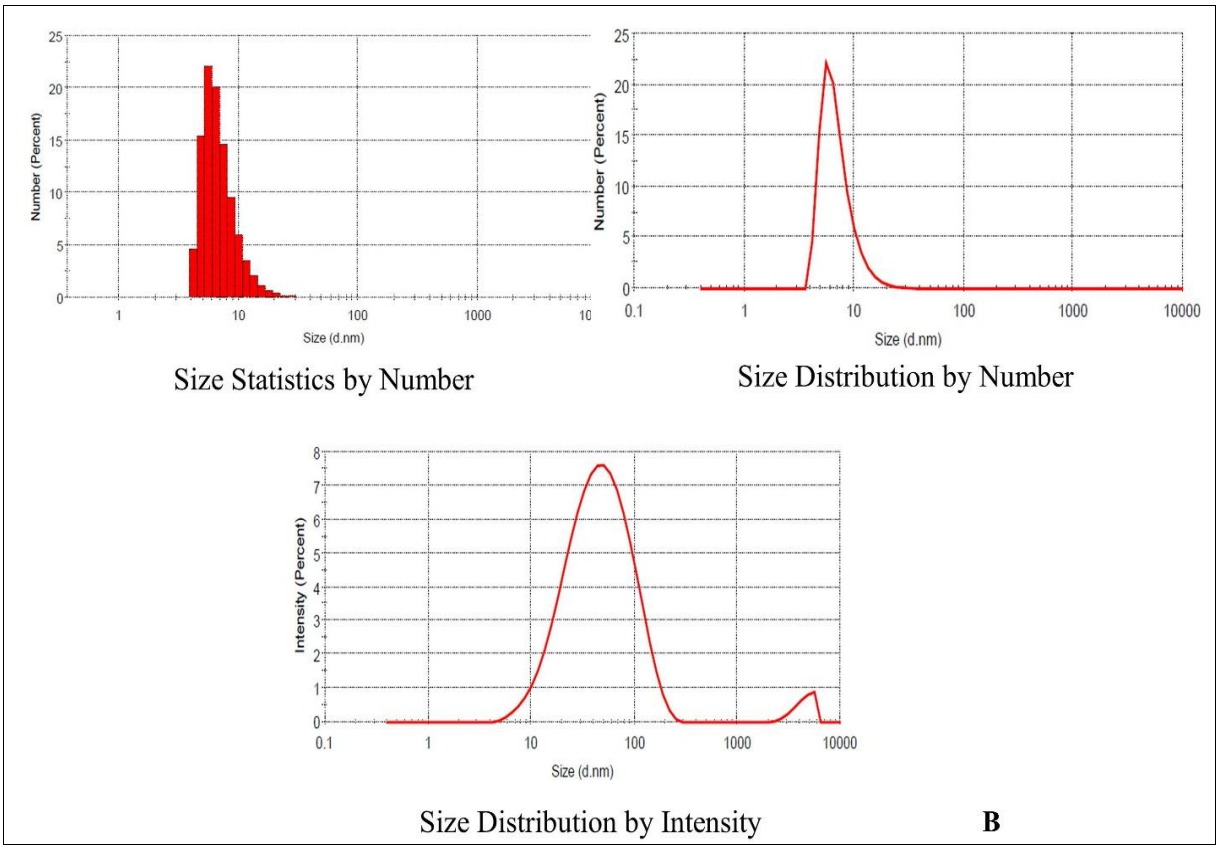


Fig 7: DLS Data of Mediated Gold Nano-Conjugates [A: *C. limon*; B: *C. aurantifolia*]

Fourier Transmission Infrared Spectroscopy (FT-IR)

FT-IR is most useful rapid, non-invasive and high resolution oriented analytical tool for determining organic or inorganic functional groups as well as identifying types of chemical bonds that is more like a molecular fingerprinting. FT-IR is an analytical procedure, and it does not interpret the particular amount of individual metabolites, but it gives the overviews of the metabolic composition of an extract residue at a particular time period. It has been documented that FT-IR spectrum study is proved a valuable and reliable procedure for detection, identification of metabolites of an unknown plant mixture. It has been used to know the structural composition, dynamics, conformational changes, structural stability, and aggregation of proteins. It has been used to identify functional groups such as polysaccharides and esters, as well as inter atom chemical bonds in several of the sample. The wavelength of absorbed light indicates the type of chemical bond that can be seen in the spectrum. The FT-IR data are represented through Figure 8A and 8B where, each of the peaks generated from the FT-IR suggests the presence of various well-known organic or inorganic functional groups that ensures the establishment of gold Nano-conjugates and enhances their bio utility.

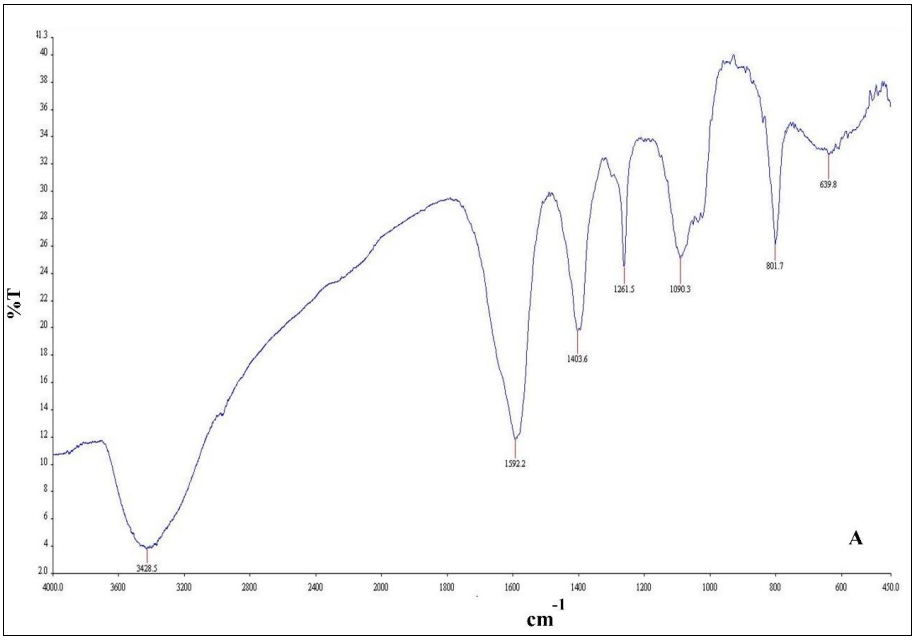


Fig 8A: FT-IR Data of *C. limon* Gold Nano-Conjugates

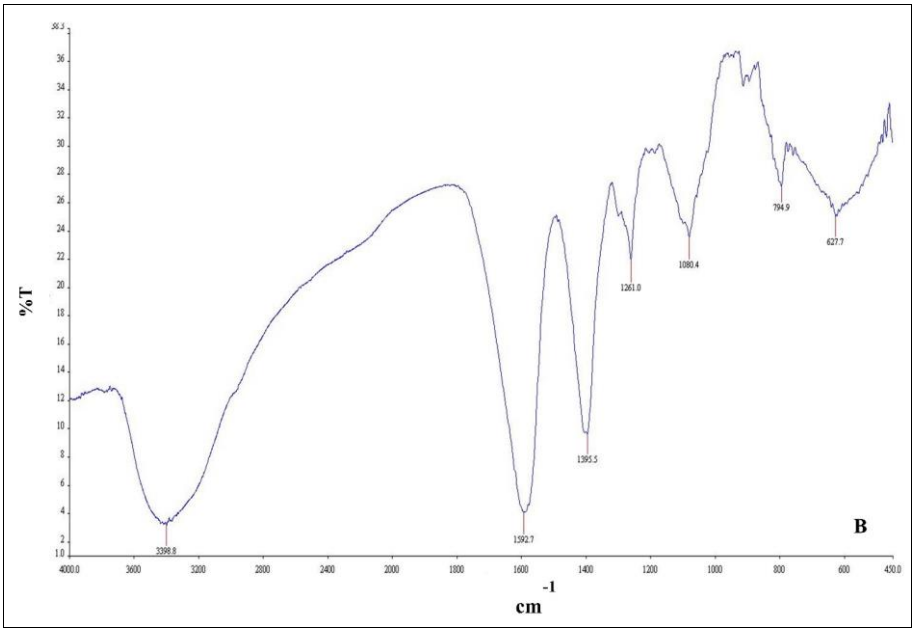


Fig 8B: FT-IR Data of *C. aurantifolia* Gold Nano-Conjugates

Determination of *In vitro* Antioxidant Efficacy via DPPH Free Radical Scavenging Assay

After performing biophysical characterization, we have done quantitative estimation of free radicals scavenging potential of *C. limon* and *C. aurantifolia* extract mediated gold nano-conjugates using the standard protocol of DPPH free radical assay with minimal alterations. The results obtained from the analysis have revealed that both the *Citrus* species extract mediated gold nano-conjugates show medium amount of free radical scavenging potential. In case of *C. limon* it is 20.23±0.48% whereas in case of *C. aurantifolia*, it is 18.22±0.79%. [Figure 9] This data may further enhance the procedure of green synthesis of nanoparticles and maintaining their stability.

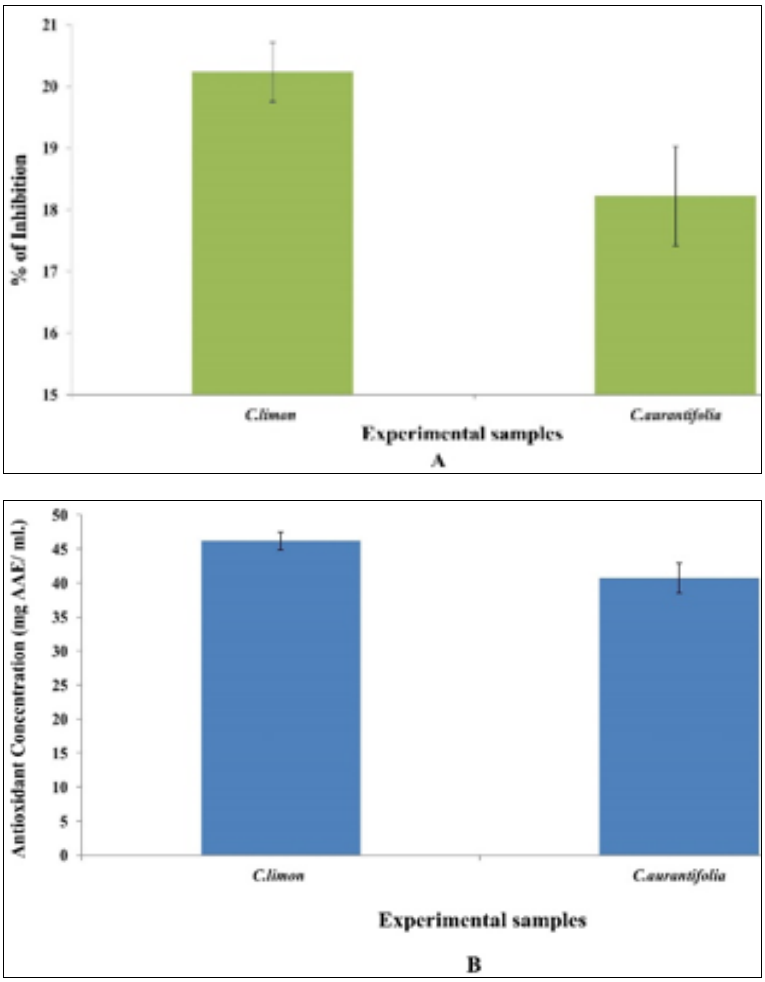


Fig 9: DPPH Free Radical Scavenging Data of *Citrus* species leaf Extract Mediated Gold Nano-Conjugates

Determination of *In vitro* Antibacterial Potential of Gold Nano-Conjugates

Antimicrobial activity of gold chloride solution, *C. limon* and *C. aurantifolia* leaf extract and green synthesized gold nano-conjugates of the two-plant leaf extract were tested against one Gram-Positive: *Staphylococcus aureus*; and one Gram-Negative: *Escherichia coli* pathogen using Kirby-Bauer disc diffusion assay. Results from the assay revealed that the pure gold chloride solution showed zero zone of inhibition. On the other hand, between *C. limon* and *C. aurantifolia* mediated gold nano-conjugates, the former gold nano-conjugates showed the highest inhibition than the latter one against Escherichia *coli* (14.67±0.57 mm.). Data of overall antibacterial potential is represented via both graphical and pictorial manner. [Figure 10]. From this antibiotic susceptibility study, we can assume that upon becoming a stable gold nano-conjugate, it will show or give further a better zone of inhibition comparative to the crude extract.

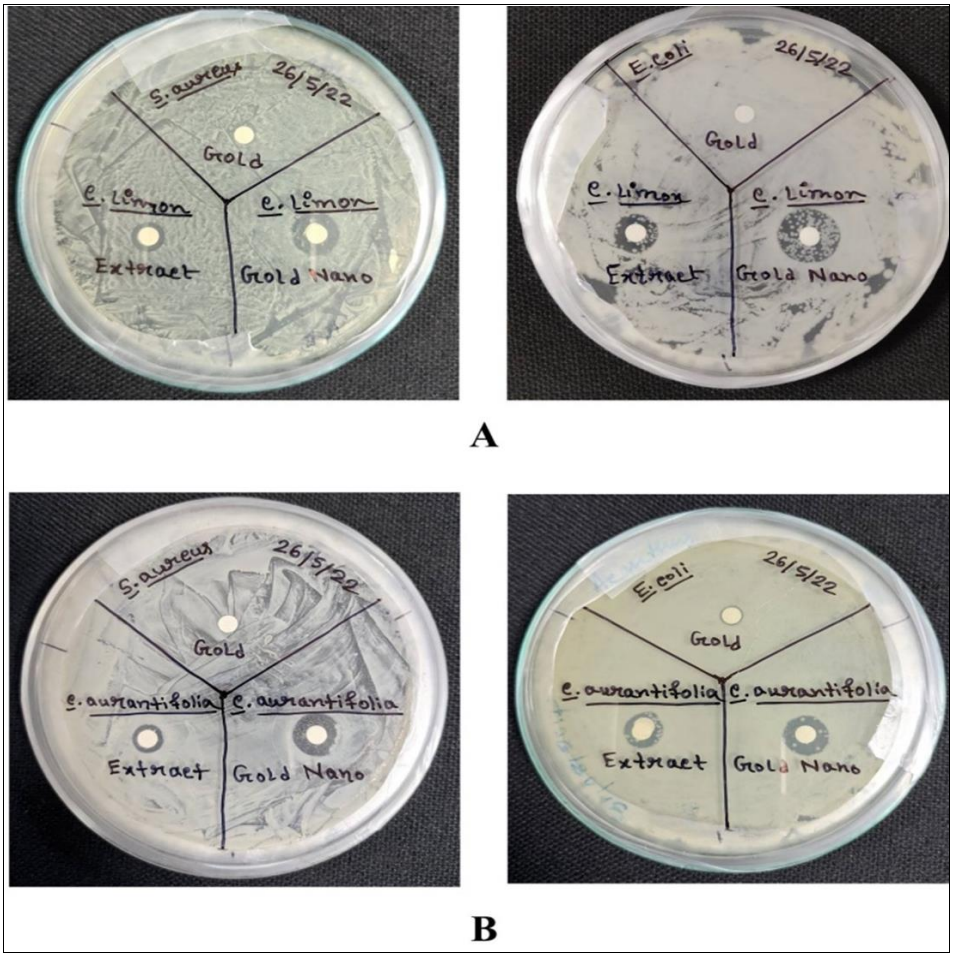


Fig 10: Agar plates showing zone of inhibition by Gold Nano-conjugates against different bacterial strains [A: *C. limon*; B: *C. aurantifolia*]

Conclusion

This study gives an environmental positive path for the synthesis of gold nano-conjugates employing two *Citrus* species i.e., *C. limon* and *C. aurantifolia* leaves aqueous extract. The extract indicates that the characteristics of both reducing and stabilizing agent in order to prepare stable nano-conjugates, owing to different compounds in the leaves of *C. limon* and *C. aurantifolia*. The utility based acceptance of the leaves from the plant takes full recognition of bioactive material which is equally eco-friendly, efficient, and safe. This easy and cost-effective procedure of generating the nano-conjugates are less toxic and thus nano-conjugates which are developed from the green origin are more steady, rational and therefore the rate of synthesis is also quicker than most of the other physical, chemical methods or sources. In recent years nano-biotechnology produced ethno-botanicals has received more attention to pharmaceutical, nutraceutical, and cosmetics industries as they are recommended for their competence and are believed to be secure for human consumption. From that context, it can conclude that *C. limon* and *C. aurantifolia* leaves extract mediated gold nano-conjugates have the probability to be utilized in different biomedical and other fields where non toxicity is crucial.

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