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Green synthesis of gold nano-conjugates using commonly used citrus species and evaluation of its *In-vitro* antibacterial efficacy against *Staphylococcus aureus*: A comparative study

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Abstract
Nanotechnology belongs to one of the areas of modern science, demonstrated numerous positive developments in the realm of medical research, basically deals with the particle size ranging between 1-100 nm. Out of so many methods for developing the metal nanoparticles, the green approach for metal nanoparticles preparation is less hazardous, eco-friendly and cost effective. According to various literature studies, plants belonging to *Citrus* species have enormous potential as anticancer and antioxidant rich food items. Therefore our present research work aims at the *Citrus* species (lemon, pummelo and the aroma king lemon) mediated Metal nano-particle synthesis followed by their characterization and determining its antibacterial property of against *Staphylococcus aureus* bacterium. According to the results of physical characterizations, all the three *Citrus* mediated nanoparticles show UV-Vis spectra within 520-530 nm. And have the particle size generated within 1-10 nm. Several functional groups are present in the produced nanoparticles, according to an FTIR analysis. The *in-vitro* antioxidant efficacy was maximum shown by the aroma king lemon. This overall results supports the innate antioxidant properties of *Citrus* species plants that may further supports its reducing property for nanoparticle synthesis.

Keywords: *Citrus* fruits, green approach, gold nano-conjugates, antibacterial, antioxidant

1. Introduction
Modern drug development markets are growing their efficacy in utilizing medicinal plants and natural products that have maximum affordability and acceptability as bio active constituents for the treatment of various diseases. Nowadays the green synthesis approach of nanotechnology has brought a revolution in the field of drug delivery system. Due to its less expenditure, high output, low toxicity and recyclable nature it has become a modern-day practice in laboratories for the synthesis of nano sized particles. Methods of green synthesis of nanoparticles deal with the preparation of nanoparticles from plants, microorganisms or any other green origin organisms [1-3]. Medicinal plants are a vital source of varieties of potent and powerful drugs and which is used worldwide for their multi-beneficiary roles against varieties of illness [21]. Moreover, the depiction of plants' ability to heal diseases and their significance in religious texts has strongly encouraged people to utilize natural remedies and scientists to go through researches its pharmacological potential [5].
Some epidemiologic studies also suggests that *Citrus* fruits contains significant amount of biologically active compounds and shows nutritional and health benefits, plays crucial antagonist role in tumor development and progression and the consumption of less-fat diets, high amount of fibers, that we can easily get from fruits like lemon, pummelo, Gandhoraj help us to improve our immunity and to fight against different chronic diseases [6-8]. Fruit antioxidant activity is thought to be especially important in combating a variety of degenerative diseases. Fruit pericarp contains many phenolic compounds, such as catechin, epicatechin, p-coumaric acid, caffeic acid, ferulic acid, and their esters, throughout addition to sugars, phenolic compounds, essential minerals, and vitamins A, B, and C [9]. Vitamins are organic substances vital for body function and indispensable to our life. Among the 13 vitamins reported in literature, six of them are found in Citrus fruits, including vitamin A, vitamin B1, vitamin B2, vitamin C, vitamin E and vitamin B3 [6]. Of these vitamins, vitamin A, vitamin C and vitamin E were evaluated for their antioxidant activities [10].

Vitamin C, their antioxidant activities [10]. Vitamin C, chemically known as L-ascorbic acid or simply ascorbate, is a water soluble substance and is a major vitamin found in *Citrus* and rich in the flesh and peel of fruits [11]. Vitamin C is a natural free radical scavenger, which can effectively scavenge a variety species of reactive oxygen species (ROS) and give off semi dehydro-ascorbic acid, and reducing sulfur radicals [10]. In the last couple of decades, it is evident that there has been a continuous growth in research and promotion of plants-based drugs. Thus the interest of people has inclined towards herbal medicines. This is the primary reason behind our selection of plant sample i.e. different *Citrus* species (lemon, pummel and Gandhoraj), which are easily available in the state of West Bengal. Therefore, our course of study is designed on the basis of these previously mentioned *Citrus* species juice mediated gold nanoparticle synthesis, followed by their major physical characterizations techniques including UV-Vis spectrophotometric analysis for peak identification, DLS study for size determination and FTIR study for identification of various functional groups in the synthesized nanoparticles. The synthesized nanoparticles were then undergone for their *in-vitro* evaluation of antibacterial efficacy against gram positive bacterium *S. aureus*. The workflow is represented in a schematic manner. [Figure 1]

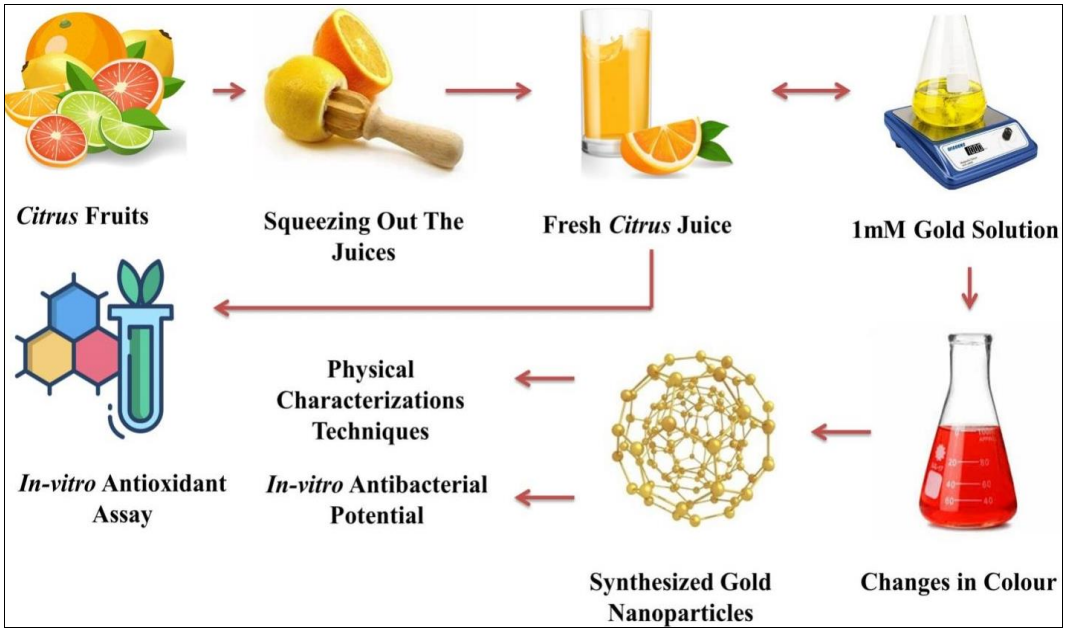

Fig 1: The Schematic Workflow

2. Materials and Methods

2.1 Collection and Preparation of *Citrus* Fruits

All the three *Citrus* species i.e. common lemon (*Citrus limon*), pummelo (*Citrus maxima*) and the lemon king or Gandhoraj [Figure 2] were collected from the local market of South Kolkata. After buying, they were cleaned properly with running water so that no dust is left. Next after cleaning, the outer layers of the fruits were peeled off and they were cut into pieces. Simultaneously, their juices were collected by squeezing out. The juices were stored at cool temperature for further use.

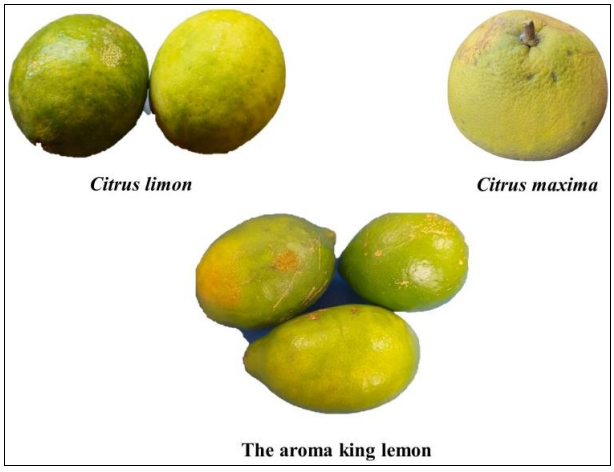

Fig 2: The Selected *Citrus* Fruits

2.2 Collection of Chemicals

Gold (III) Chloride trihydrate ($HAuCl_4$, $3H_2O$), Luria bertani agar and Luria broth were bought from HIMEDIA, DPPH, Ethanol and Sodium citrate were obtained from SRL chemicals private limited. All are analytical grade.

2.3 Collection of Microorganism

The gram positive bacterial strain *Staphylococcus aureus* was obtained from Department of Biotechnology, Techno India University, West Bengal.

2.4 Free Radical Scavenging Assay by DPPH Method

All the fresh juice samples were prepared for determining their free radical scavenging activities, which is measured by DPPH (α, α-diphenyl-β-picryl hydroxyl) method. Ascorbic acid was used as standard. The absorbance was recorded after 30 minutes incubation at 517 nm. The results were represented through percentage of inhibition [12].

Percentage of Inhibition = [(A0 – A1)/A0] ×100

[A0 and A1 represent the absorbance of the control and of sample]

2.5 Synthesis of Gold nano-conjugates

At first 1mM. Gold solution was prepared in deionized HPLC graded water in a large beaker and passed through 37 µm syringe filter (Serum Acrodisc). Next the solutions are taken in a conical flask and keep it on a heating mantle, so that the

solution starts to boil.
Meanwhile, freshly squeezed lemon, pummelo and gondhoraj juices are mixed with sodium citrate solution in an equal volume within a small beaker. In meantime when the gold solution starts boiling, the mixtures of respective lemon juices along with sodium citrate were added to it in drop wise.
The initial color of gold solution was light yellow but after adding respective lime juices and sodium citrate mixture change in the color is noticed which tends to red wine purple coloration. All the three conical flasks are then kept in magnetic stirrer for 15 mins. at highest rpm to ensure proper mixing and dispersion of nanoparticles [13].

2.6 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.

2.6.1 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) [14-16].

2.6.2 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) [14-16].

2.6.3 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) [14-16].

2.6.4 Determination of *In-vitro* Antibacterial Potential of Gold Nano-Conjugates
2.6.4.1 Disk Diffusion method
Two bacterial strain used in the present study were obtained from the Department of Biotechnology, Techno India University, West Bengal.

2.6.4.2 Gram-positive bacteria: *Staphylococcus aureus*
The antibacterial susceptibility study was carried out via disc diffusion assay [17-18].
The antibacterial affectivity was measured on the basis of zone of inhibition (mm.) surrounding the disc. All tests were carried out in triplicates.

3. Results and Discussions
3.1 Free Radical Scavenging Assay by DPPH Method
DPPH radical scavenging capacity (%) was used to calculate antioxidants' ability to capture reactive oxygen species; among these lemons the aroma king lemon (Gandhoraj) has shown the maximum inhibition percentage i.e. 48.64±0.97%, followed by pummelo (47.92±1.18%) and lemon shows the lowest percentage (25.39±0.48%). [Figure 3]

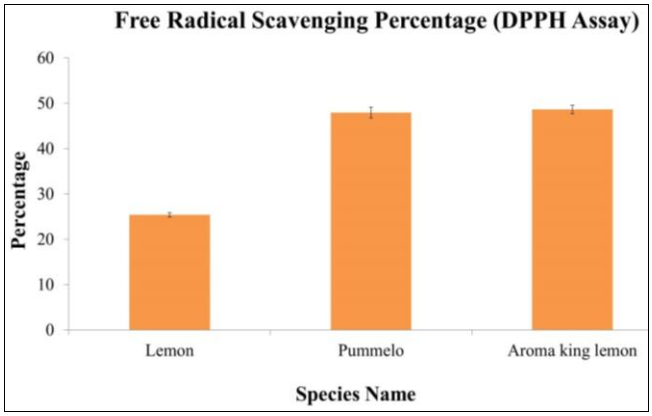

Fig 3: Results of Inhibition Percentage Assay [DPPH Method]

3.2 Synthesis of Gold nano-conjugates
The distinct wine red color [Figure 4A, 4B, and 4C] gave as confirmation that nanoparticle formation had actually occurred. Transparency and turbidity showed a correlation among particle size and color. Whereas clear solutions show the creation of nanomaterials of uniform size, turbid solutions show the aggregate development of varied sizes.

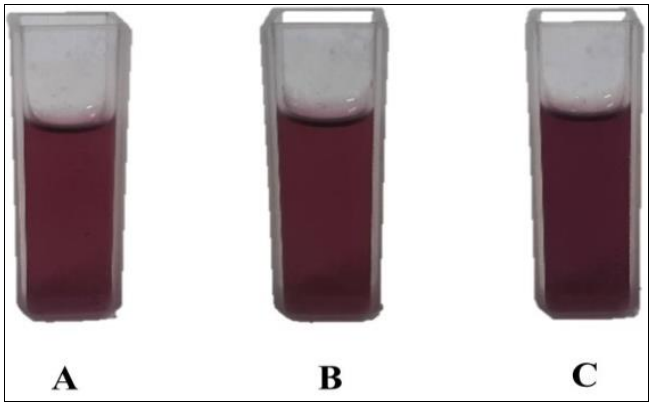

Fig 4: The Synthesized Gold Nanoconjugates [A: Lemon mediated, B: Pummelo mediated, C: Aromatic king lemon mediated]

3.3 Results of Physico-Chemical Characterization
3.3.1 UV-VIS Spectroscopy
Using a UV-Vis Spectrophotometer to measure the UV-Vis spectrum, the conversion of pure Au3+ ions to Au0 was seen (SHIMADZU Model: 2401PC). The absorption peaks were observed at 528 nm (Pummelo) 530 nm (Lemon and Aroma king lemon) [Figure 5A, 5B and 5C] [19].

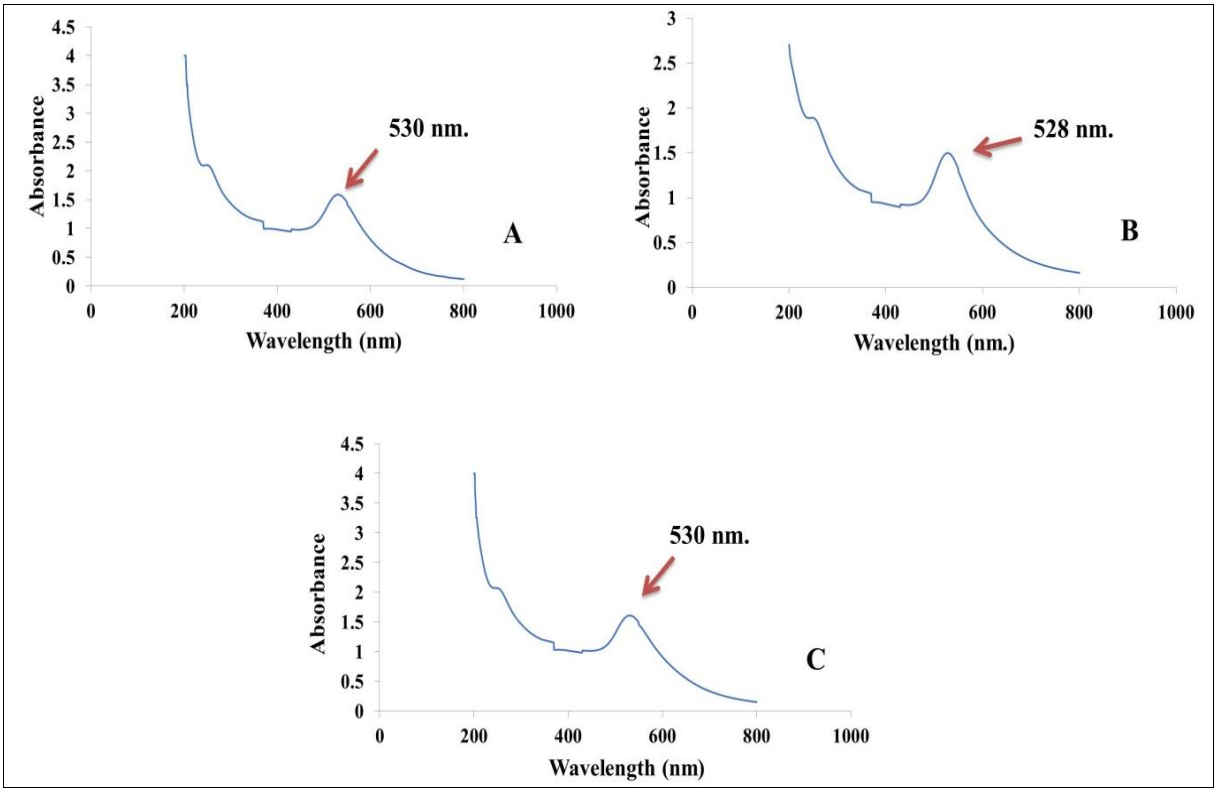

Fig 5: Results of UV-Vis Spectra Analysis of Gold nanoconjugates [A: Lemon mediated, B: Pummelo mediated, C: Aromatic king lemon mediated]

3.3.2 Dynamic Light Scattering Particle Size Analyzer

The AuNPs synthesized with three different Citrus fruits juice using sodium citrate and have a particle size ranges within 1 to 10 nm. **[**Figure 6A, 6B and 6C] respectively, analyzed using the DLS instrument (Zetasizer Nano-S. MALVERN Instruments, Model: ZEN1600) [14].

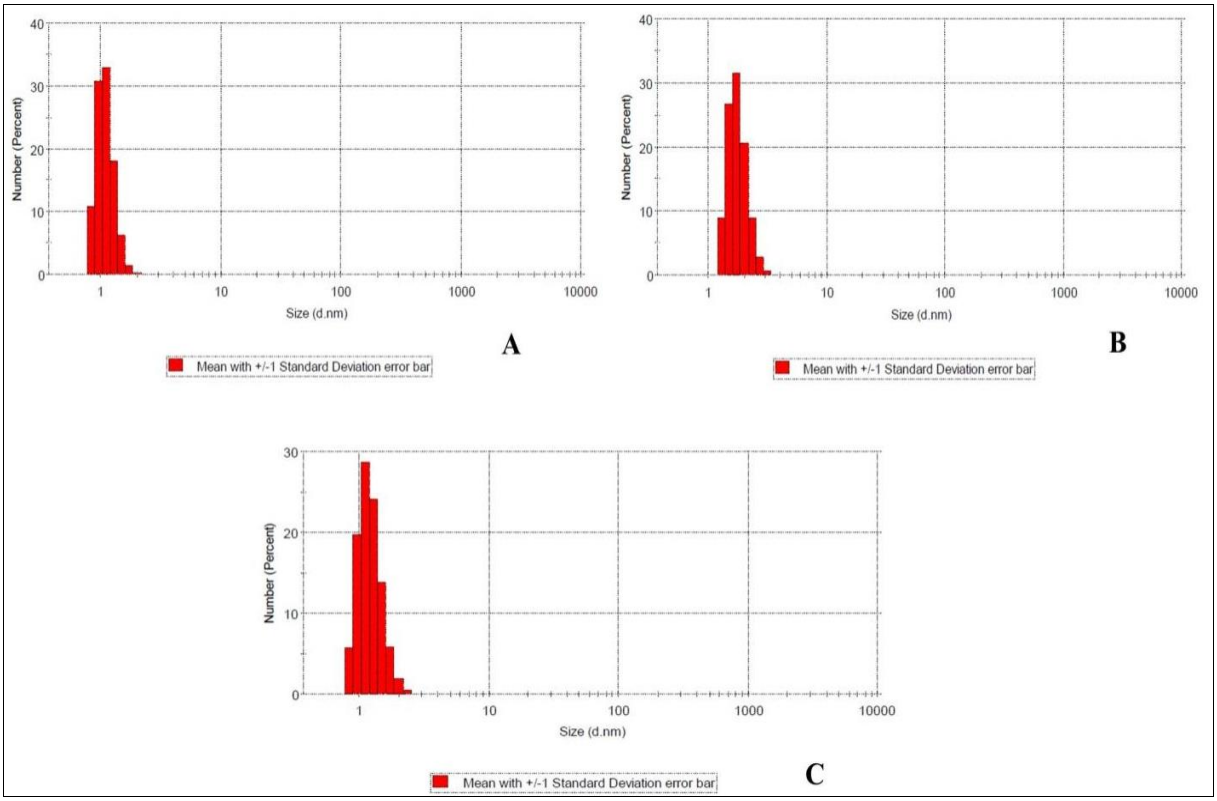

Fig 6: Results of DLS Analysis of Gold nanoconjugates [A: Lemon mediated, B: Pummelo mediated, C: Aromatic king lemon mediated]

3.3.3 Fourier-transform infrared spectroscopy (FTIR)

FTIR spectral analysis was done for the synthesized gold nano-conjugates exhibited notable shifts in absorption spectra [Figure 7A, 7B and 7C]. Moreover, there is a similarity in between the peaks generated in case of different nano-conjugates. Each peaks of absorption indicate the presence of specific functional groups, for eg. Amines, aldehydes, alkyls, amides, carboxyl groups and so on [20].

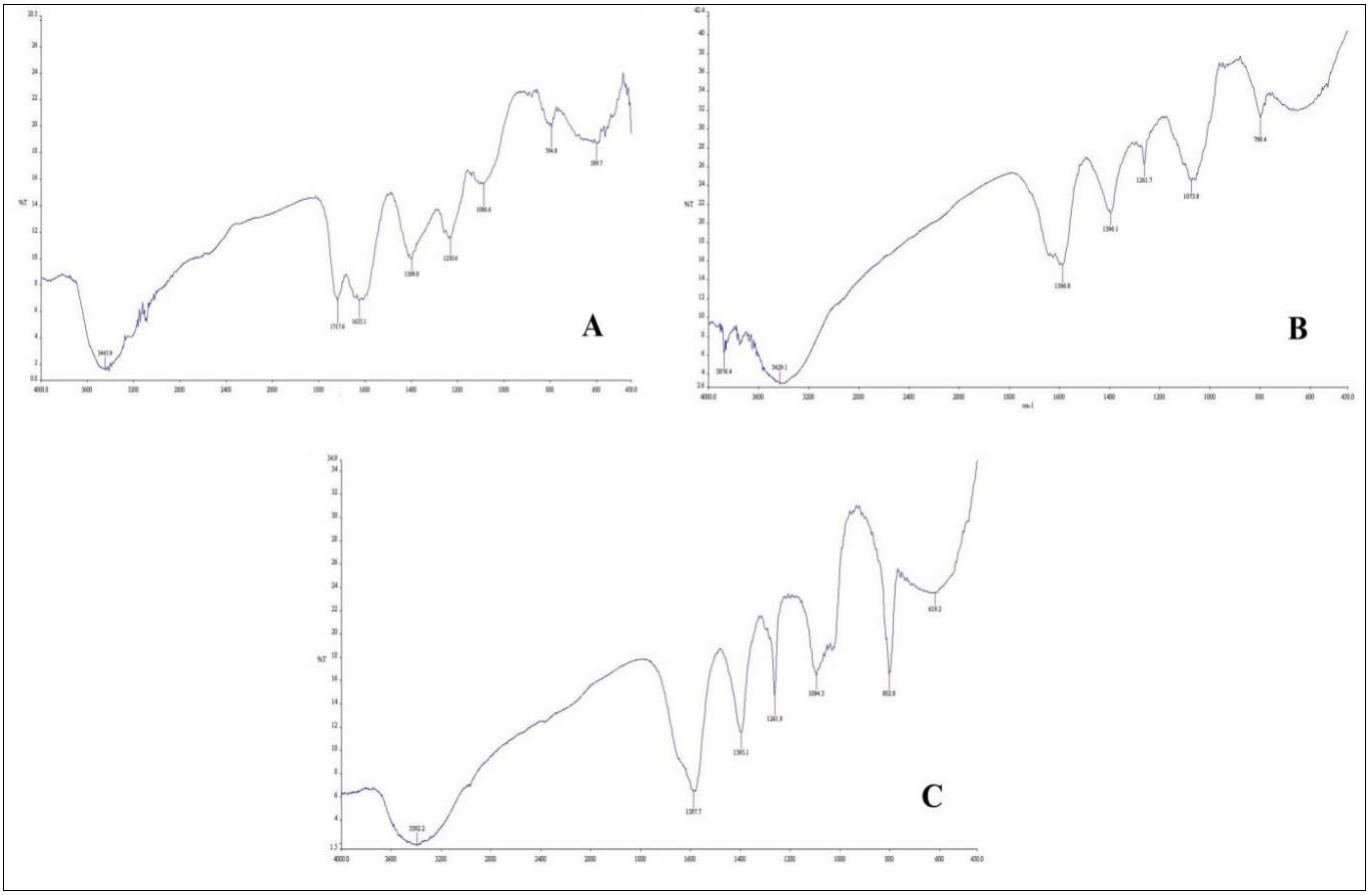

Fig 7: Results of FTIR Spectral Analysis of Gold nanoconjugates [A: Lemon mediated, B: Pummelo mediated, C: Aromatic king lemon mediated]

3.3.4 Results of *In-vitro* Antibacterial Potential of Gold Nano-Conjugates

Using the Kirby-Bauer disc diffusion assay, the *in-vitro* antimicrobial property of pure lemon juices, gold chloride solution, and gold nano-conjugates were evaluated against the Gram-Positive *Staphylococcus aureus* bacterium. When examined for their antibacterial activity toward the gram-positive bacterial strain, each crude lemon juice, manufactured gold nano-conjugates, and gold chloride (on increasing concentration) treatments had given tiny zones of inhibition [Figure 8A, 8B, and 8C]. We believe that when the nanoparticle concentrations increase, a better zone of inhibition will be visible or provided.

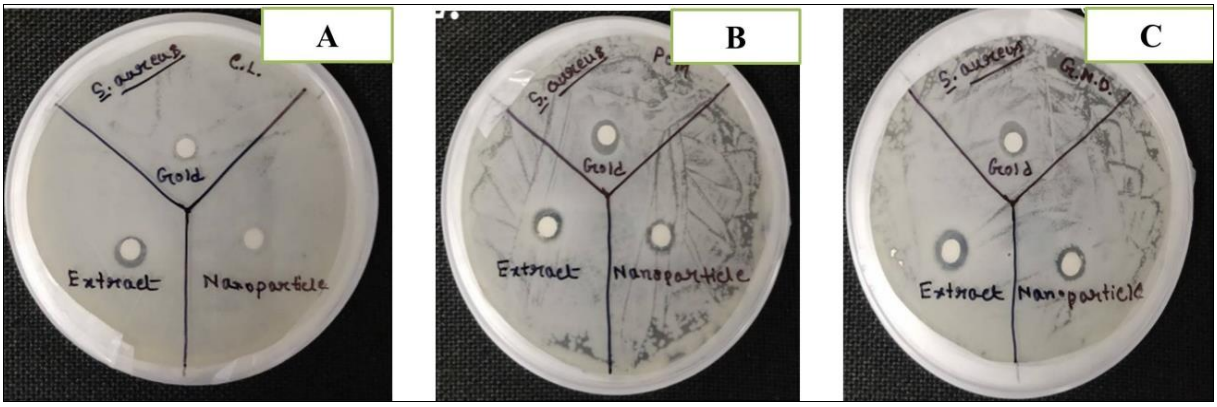

Fig 8: Results of *In-vitro* antibacterial activity of Gold nanoconjugates [A: Lemon mediated, B: Pummelo mediated, C: Aromatic king lemon mediated]

4. Conclusion

Lemon extract has significant radical scavenging action because it contains a fair amount of naturally occurring citric acid together with other bioactive phytomolecules. As a result, it can be utilized as a superior substitute for the synthetic chemical sodium citrate ($Na_3C_6H_5O_7$) and may be widely employed in the production of gold nanoconjugates for use in biomedicine. So even though the use of nano-models will help delivery systems in food and pharmaceutical sciences has many benefits, they may also cause toxic effects problems due to their shape, size, tissue accumulation, immunological reactions, and degradation substances. The structure of nano-carriers is crucial. Nano-carriers characterization is required to comprehend the capability of using these systems in food. Like this present work is showing three differentially synthesized AuNPs from the

juices of *Citrus limon* (lemon), *Citrus maxima* (pummelo) and the aroma king lemon or Gandhoraj significantly depicts the *in-vitro* antioxidant and antibacterial activity of the aroma king lemon on gram-positive *S. aureus* bacterium in comparison to the Gold Chloride solution as well as crude *Citrus* juices. In order to summarize the entire work, it can be concluded that nano-phyto molecules have enough benefits to create a decent candidate for industrial production (at the pharmaceutical as well as industrials) for trying to cover therapeutic purposes such as treating cancer with working to develop meditative foods.

5. Acknowledgement
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6. Conflict of Interest
The authors declare no conflict of interest.

7. Author's Contribution
SS and MS conceptualized the study plan, assisted and supervised the laboratory work. SS, MS and NRG conducted data analysis and interpretation, wrote the manuscript. MB and SC edited the manuscript.

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