Research Article

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CHARACTERIZATION AND COMPARATIVE EVALUATION OF ANTIBACTERIAL AND CYTOTOXIC EFFICACY BETWEEN TWO QUERCETIN-AU-NANOCONJUGATES SYNTHESIZED USING PURE TRI-SODIUM CITRATE AND ITS NATURAL ALTERNATIVE - LEMON EXTRACT

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ABSTRACT

Green synthesis of AuNPs that have potential anticancer properties is relatively simple, cheap and eco-friendly compared to the conventional chemical/physical approaches. Quercetin is known for its antioxidant and anticancer properties, i.e., induction of apoptosis, tumour suppression, etc. This study aims to characterize and compare between two differentially synthesized Quercetin-Au-Nanoconjugates, Q-Au-NC_{TSC} and Q-AU-NC_{LE} using a pure biochemical reductant, trisodium citrate and its natural alternative, citrus lemon extract respectively. Antibacterial and anticancer effects of both the nanoconjugates would also be checked and compared to analyze whether the use of a lemon extract has any impact on its structure and functional properties. A series of physicochemical characterizations viz. UV-Vis spectrophotometry, DLS, Zeta Potential, FT-IR, and SEM of the nanoconjugates were done. Further, evaluation of *in vitro* antibacterial activity was done against two Gram-positive bacteria: *Staphylococcus aureus*; *Bacillus Subtilis*; and two Gram-negative bacteria: *Pseudomonas aeruginosa*; *Klebsiella pneumonia* and cytotoxicity efficacy were checked on breast cancer (MCF7) cell line. Effective reduction of Au^{+3} to Au^{0} with quantum confinement in nano-regime was confirmed by a change of bulk colour of the $HAu^{+3}Cl_4$ solution, whereas conjugation of Quercetin to AuNPs was confirmed by FTIR. DLS showed the average size of the Q-Au-NC_{TSC} and Q-Au-NC_{LE} are 30 nm and 35.6 nm, respectively. The Q-Au-NC_{LE} has shown comparatively better stability and antibacterial activity. In the case of cytotoxicity study on MCF7 cell line, the Q-Au-NC_{LE} showed better efficacy (cell death ~ 75%) with respect to Q-Au-NC_{TSC} (cell death ~66%). Natural sources rich in citric acid would serve as the best alternative to tri-sodium citrate in the synthesis of Au-NPs and different nanoconjugates for biomedical applications.

Keywords: Quercetin-Au-Nanocojugates, Antibacterial, Anticancer, Sodium citrate, Lemon pulp, MCF7 Cell line.

INTRODUCTION

In modern medicine, nanoconjugates have emerged as essential players in the recent era. Metal-nanoconjugates can be made by conjugating metal nanoparticles with drugs or any therapeutically essential molecules. Among the several metallic nanoparticles, gold nanoparticles find its applications against several diseases including cancer due to its nontoxic nature and laser sensitiveness. Gold nanoparticles are commonly synthesized by reducing tetrachloroauric acid with tri-sodium citrate, a method pioneered by Turkevich *et al*. (1951). Recently green synthesis of nanoparticles which is an effective alternative to chemical and physical methods is known to be an eco-friendly way of manufacturing nanoparticles. Besides, this process does not require expensive, harmful and toxic chemicals.[1] Gold nanoparticles (AuNPs) are significantly used in various fields, including medical, food, healthcare, consumer and industrial purposes, due to their exceptional physicochemical properties.

Quercetin (3,3',4',5,7-pentahydroxyflavone) (Figure 1) is a yellow, crystalline stable structure with astringent properties and is insoluble in water. However, it is soluble in glacial acetic acid and aqueous alkaline solutions and sparingly soluble in alcohol.[2,3]

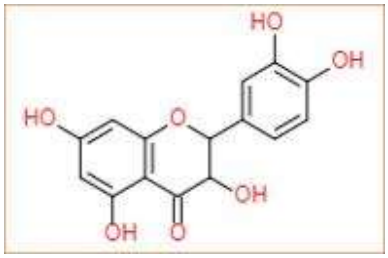


Figure 1: Chemical Structure of Quercetin

Quercetin and likewise 2,000 other flavonoids exist as condensation products of p-glycoside. It is one of the most extensively used bioflavonoids found to be present in more than twenty plant materials including fruits (mainly citrus), green leafy vegetables as well as many seeds, barks, broccoli, olive oil, apples, onions, green tea, red grapes, red wine, dark cherries and berries such as blueberries and cranberries.[4,5] Being a plant-derived aglycone form of flavonoid glycosides, it has many nutritional benefits against a variety of disorders, including cardiovascular protection, has anticancer, antitumor, anti-ulcer, anti-allergy, anti-viral, anti-inflammatory activity, anti-diabetic, gastro protective effects, it is also antihypertensive, immunomodulatory and anti-infective.[6] Plant extracts/ compounds are considered ideal sources of bioactive molecules due to their ample availability and wide array of reducing

metabolites. Plant secondary metabolites such as polyphenols provide rich resources as potential drugs, nutraceuticals and food additives. Nanoparticles because of their tunable size, shape and morphology can have immense diagnostic and therapeutic prospects.[7] However controlling the size/shape and attaining the monodispersity are some of the significant challenges which are often encountered by the researchers in the preparation of nanoparticles besides the toxicity problems associated with it. To address all these issues, plant extracts/ compounds have been evolved as one of the most promising options in the synthesis of nanoparticles. The plant-derived compounds have both protective and reducing properties, which are essential for the reduction of metal ions to their corresponding nanoparticles. The key player[8] Quercetin has potential antioxidant properties due to its capacity to scavenge free radicals and bind transition metal ions. These characteristics of Quercetin concede it to inhibit lipid peroxidation,[9] a process of free radicals' generation from unsaturated fatty acids via the abstraction of hydrogen.[10] Free radicals have deleterious effects throughout the body, and it is responsible for several diseases such as cancer, cardiovascular and neurodegenerative diseases etc. However, antioxidants can deactivate free radicals by reacting with it.[11] Free radicals trigger the secretion of the pro-inflammatory cytokine that are responsible for chronic inflammatory diseases.[12,13] Quercetin, can also reduce inflammation and anti-oxidative stress by scavenging free radicals.

Quercetin is known to exhibit antibacterial effects against almost all strains of bacteria, significantly affecting the gastrointestinal, respiratory, urinary and dermal system. Oxidative DNA damage is an established liability factor of cancer. Antioxidants, such as Quercetin, are thought to play an essential role in protecting cells from oxidative stress induced by reactive species[14], e.g., reactive oxygen species (ROS) and reactive nitrogen species (RNS) that play a crucial role in human cancer development, significantly since, antioxidants delay the onset of some types of cancer. ROS is a collective term often used by biologists to include oxygen radicals, superoxide, hydroxyl, peroxyl and alkoxyl and certain non-radicals that are oxidizing agents.[15] Comprehensive experimental studies have demonstrated that it can control allergic symptoms, improve blood flow and increase glucose levels and can also reduce the absorption of fat.[16]

The present course of study aims at the comparative evaluation of Quercetin-Au-Nanoconjugates, synthesized using trisodium citrate as a synthetic reducing agent and lemon extract, as an alternative natural origin of Citric acid. In addition to it, the antimicrobial and anticancer activities of both the gold-nanoconjugates, will be checked and compared to understand their therapeutic efficacy.

MATERIAL AND METHODS

Chemical and instruments

The components used for the synthesis of Gold Nanoparticles include Auric-chloride ($HAuCl_4$) (from SRL Pvt. Ltd.), Tri-sodium citrate from (RFCL Ltd.), Quercetin (MP Biomedicals India Pvt Ltd.), lemon extract, deionized water, and methanol as solvents. The bacterial strains *Staphylococcus aureus, Bacillus Subtilis, Pseudomonas aeruginosa, Klebsiella pneumoniae* were obtained from Department of Microbiology, Calcutta University, West Bengal, India. The MCF7 cell line is obtained from NCCS, Pune, India and maintained in Dulbecco's Modified Eagle Medium with 10% Fetal Bovine Serum (Gibco, Waltham, MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Waltham, MA, USA). The types of equipment used during the synthesis purpose include a heating mantle, magnetic stirrer, magnetic bead and different glass-wares.

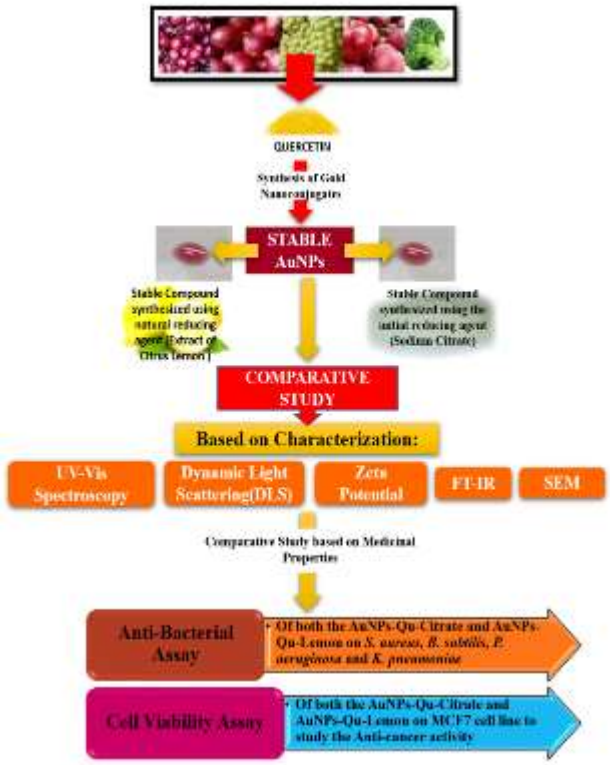


Figure 2: Schematic Diagram of The Work-Flow Followed for The Study

Synthesis and Characterization of AuNPs

Initially, 20 mg of 1 mM $HAuCl_4$ was dissolved in 50 ml of distilled water to prepare the stock solution and from that 10 ml was taken in a conical flask and placed on a heating mantle to allow the solution to boil. Meanwhile, 200 µl of Quercetin dissolved in methanol (1 mg/1 ml concentration), and 200 µl of Sodium Citrate (1 g in 10 ml dH20) were mixed in a beaker and 200 µl of the mixture was added to the boiling Gold solution dropwise. As an alternative to this chemical method, 40 µl of the lemon extract was mixed with 200 µl of Quercetin solution and 200 µl of this mixture was added to the boiling gold solution dropwise.

Development of gold nanoparticles was confirmed by the change in colour of the solution from light yellow to red wine/purple colour. The flask was then placed on a magnetic stirrer to ensure proper mixing and dispersion of nanoparticles.

The solution containing gold nanoparticles was stored in small glass bottles to carry out their stability test, characterization and further *in vitro* studies as well.
The comparative evaluation between the Q-Au-NC_{TSC} and Q-Au-NC_{LE} was done based on the UV-VIS Spectroscopy,[17] Dynamic Light Scattering Particle Size Analyzer,[18] Stability Test, Zeta Potential,[19] Fourier-transform infrared spectroscopy (FTIR),[20] Scanning Electron Microscopy (SEM)[21] results and the medicinal properties, that were deciphered by antimicrobial assay on Gram (+) and Gram (-) strains and cytotoxicity assay on breast cancer (MCF7) cell line. The workflow that is being followed in the present course of study is given in Figure 2.

Assessment of *in vitro* antibacterial and anticancer activity of Q-Au-NCs

Antimicrobial activity study was carried out by using the Kirby-Bauer disc diffusion assay. The pure compounds and the corresponding synthesized Q-Au-NCs have tested for their antibacterial activities against Gram-Positive bacterial strains: *Staphylococcus aureus*; *Bacillus Subtilis*; and Gram-Negative bacterial strains: *Pseudomonas aeruginosa*; *Klebsiella pneumonia*. In the agar plates, sterile paper discs of diameter 5 mm were placed, and gold solution as control along with Quercetin, sodium citrate or lemon extract and the respective gold nanoconjugates were added. The plates were kept for 16-18 h of incubation at 37°C, and the antimicrobial activity of pure extracts and individual gold nanoparticle were analyzed by Kirby-Bauer's disk-diffusion assay method. [22]

Cell viability was assayed using a modified colourimetric technique that is based on the ability of live cells to cleave a tetrazolium salt, WST by mitochondrial dehydrogenases to form formazan in viable cells.

MCF-7 was cultured in DMEM with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mmol/L in a humidified atmosphere (5% CO2, 37°C). Cells (1×10^5) were seeded in a 96-well plate for Control and five different experimental groups with cell density 2×10^4. After 24 h incubation, MCF7 cells were treated with different concentration of nanoconjugates respectively and again incubated for 24 h respectively. After the incubation period was over, the cell viability was determined by the WST-1 reagent. Whole experiments were performed in triplicate. Cellular morphology of all the groups was investigated by an inverted phase-contrast microscope.[23]

RESULT AND DISCUSSION

Phytochemical Screening and Determination of Free radical scavenging activity of Citrus Lemon extract

The total phenolics and flavonoids were assessed using standard Folin-Ciocalteu and Aluminium chloride methods with some modifications, respectively. Antioxidant activity was estimated by 2,2-diphenyl-1-picrylhydrazyl (DPPH), i.e., radical scavenging activity assay with little modifications. The total phenolic and flavonoid content in the lemon extract is presented in Table 1 and the antioxidant concentration along with %DPPH scavenging activity is given in Table 2.[24-26]

Table 1: Total Phenolic Content and Total Flavonoids Content

TPC (mg GAE/ml)	TFC (mg QE/ml)
0.699 ± 0.005	0.463 ± 0.049

Table 2: Free Radicals Scavenging

Antioxidant Concentration (mg AEE/ ml)	% DPPH Scavenging Activity
0.645 ± 0.022365226	41.26 ± 1.213960461

Green synthesis and characterization of AuNPs

The formation of the nanoparticles was confirmed by the wine-red colour. Relationship between particle size and the colour was revealed by transparency and turbidity. Turbid solutions indicate the aggregate formation of different sizes while clear solutions indicate the formation of nanoparticles of uniform size. A wine-red colour was observed in the case of both the samples Q-Au-NC$_{TSC}$ (Figure 3a) and Q-Au-NC$_{LE}$ (Figure 3b).

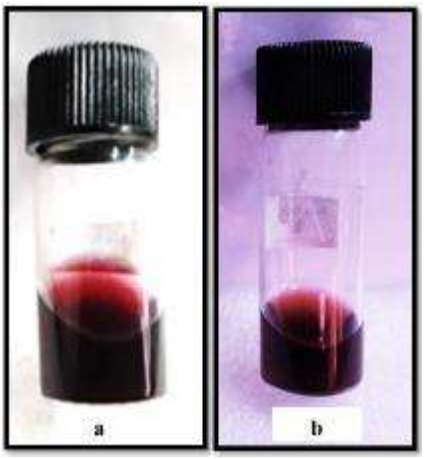


Figure 3: Quercetin Conjugated AuNPs; (a) Synthesized using Sodium Citrate; (b) Synthesized using Lemon Extract

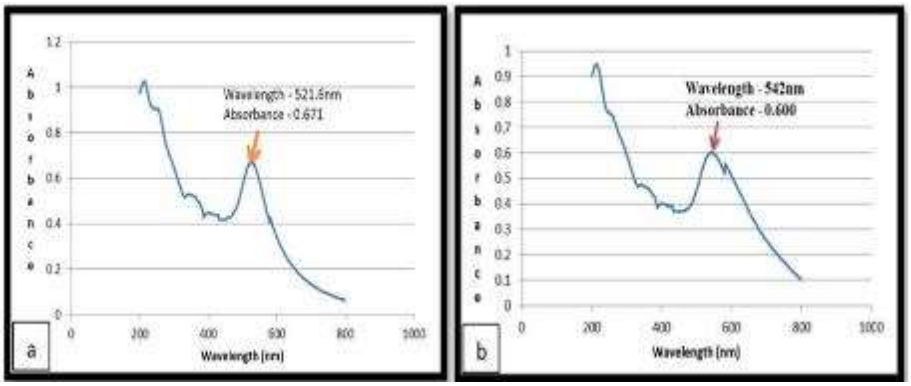


Figure 4: UV-Vis Spectra of Quercetin Conjugated AuNPs (a) Synthesized using Sodium Citrate; (b) Synthesized using Lemon Extract

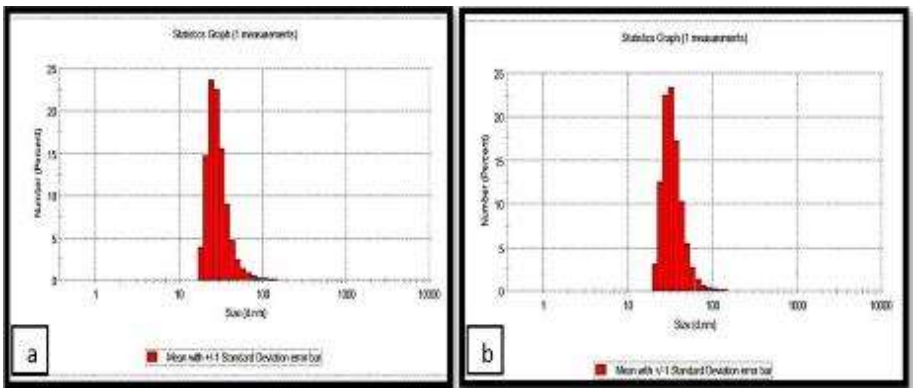


Figure 5: DLS Analysis of Quercetin Conjugated AuNPs (a) Synthesized using Sodium Citrate; (b) Synthesized using Lemon Extract

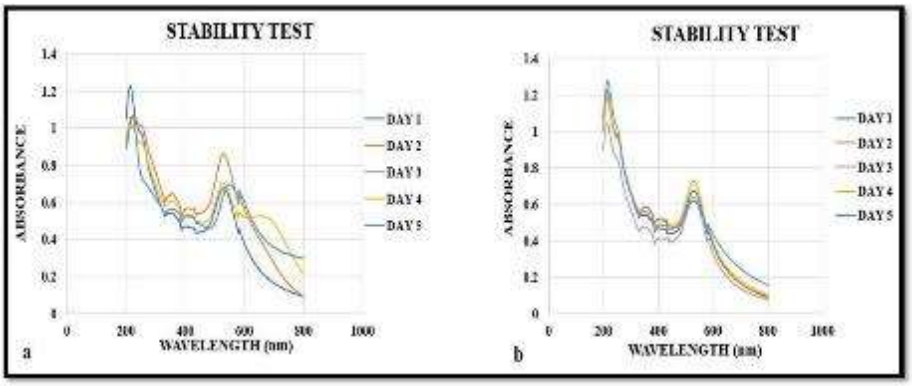


Figure 6: 5 Days Stability Test of The Quercetin Conjugated AuNPs (a) Synthesized using Sodium Citrate; (b) Synthesized using Lemon Extract

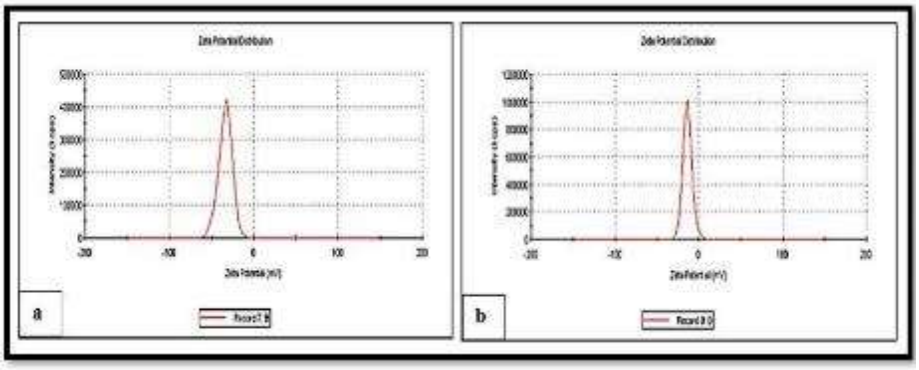


Figure 7: Zeta Potential Measurement of Quercetin Conjugated AuNPs (a) Synthesized using Sodium Citrate; (b) Synthesized using Lemon Extract

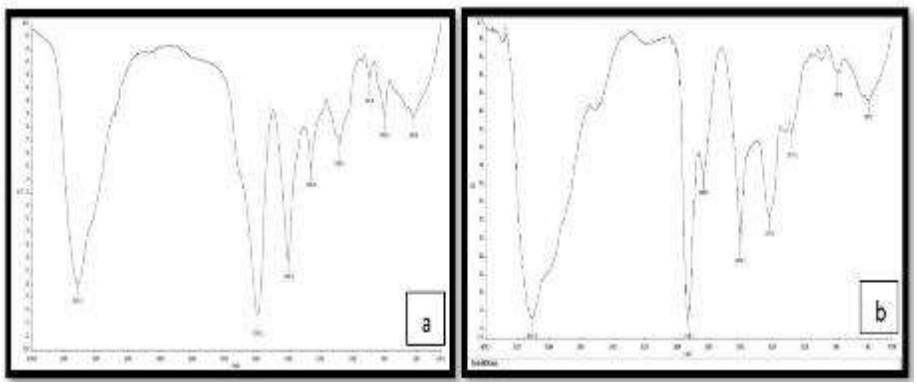


Figure 8: FT-IR Analysis of Quercetin Conjugated AuNPs (a) Synthesized using Sodium Citrate; (b) Synthesized using Lemon Extract

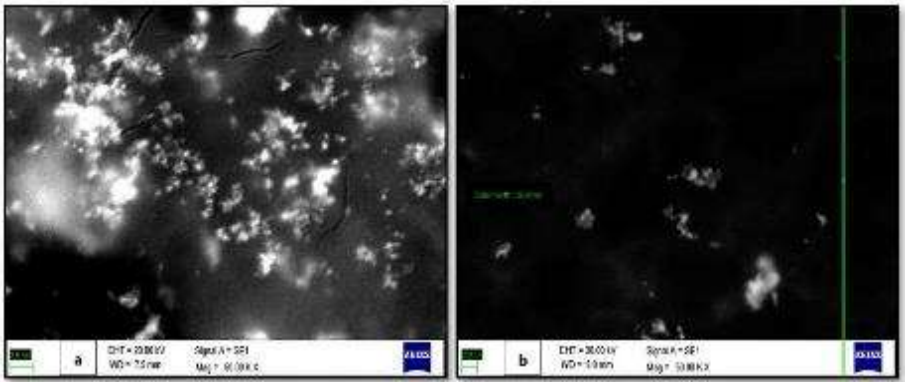


Figure 9: SEM Analysis of Quercetin Conjugated AuNPs (a) Synthesized using Sodium Citrate; (b) Synthesized using Lemon Extract

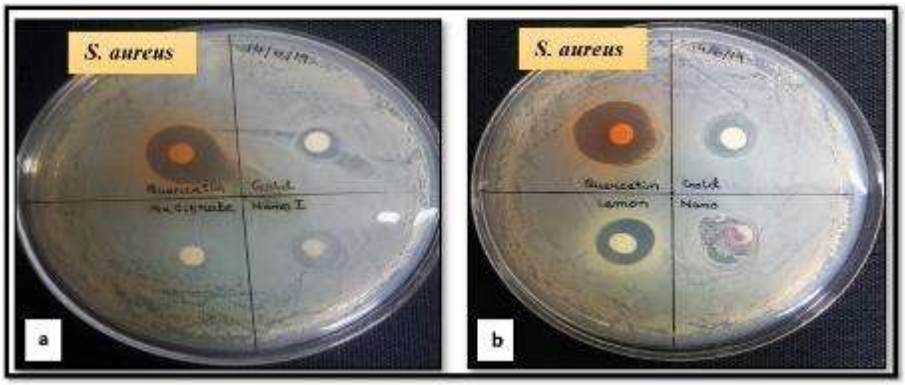


Figure 10: Antibacterial efficacy of Quercetin Conjugated AuNPs (a) Synthesized using Sodium Citrate; (b) Synthesized using Lemon Extract on *S. aureus*

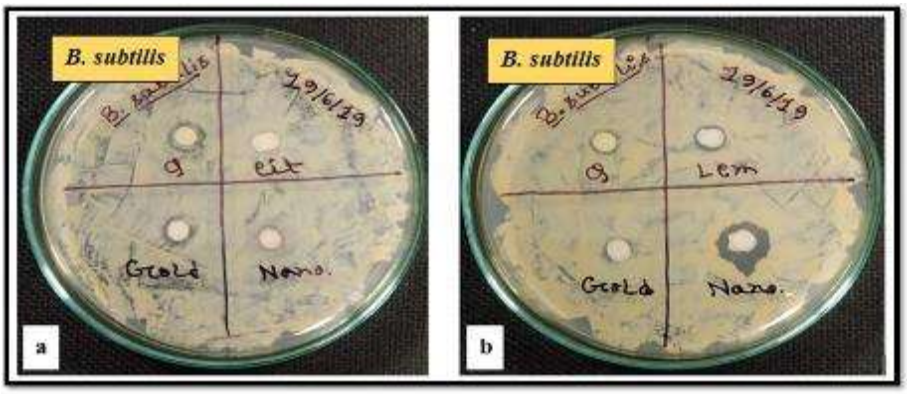


Figure 11: Antibacterial efficacy of Quercetin Conjugated AuNPs (a) Synthesized using Sodium Citrate; (b) Synthesized using Lemon Extract on *B. subtilis*

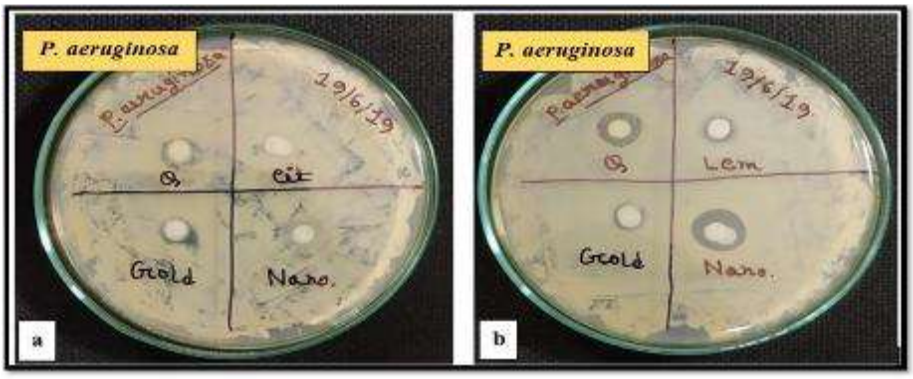


Figure 12: Antibacterial efficacy of Quercetin Conjugated AuNPs (a) Synthesized using Sodium Citrate; (b) Synthesized using Lemon Extract on *P. aeruginosa*

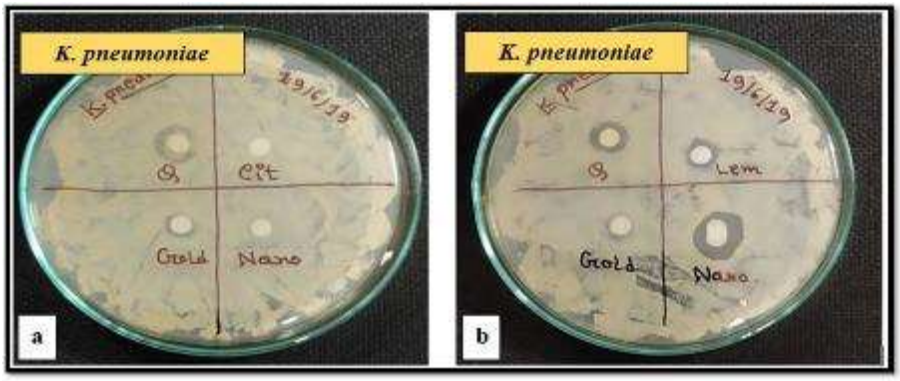


Figure 13: Antibacterial efficacy of Quercetin Conjugated AuNPs (a) Synthesized using Sodium Citrate; (b) Synthesized using Lemon Extract on *K. pneumoniae*

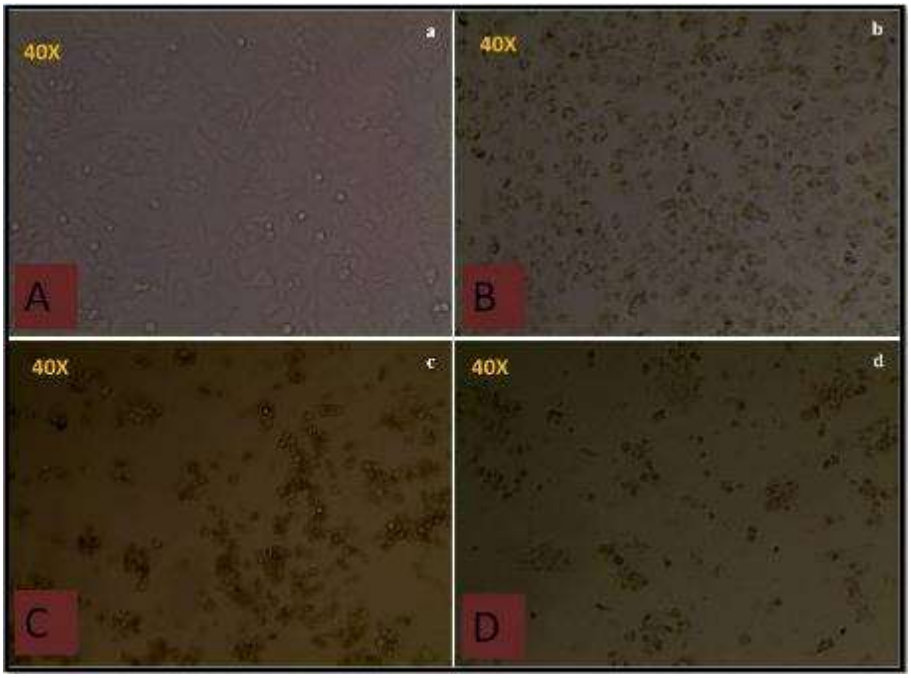


Figure 14: Cytotoxic efficacy of Quercetin Conjugated AuNPs (a) Synthesized using Sodium Citrate; (b) Synthesized using Lemon Extract on MCF7 cell line

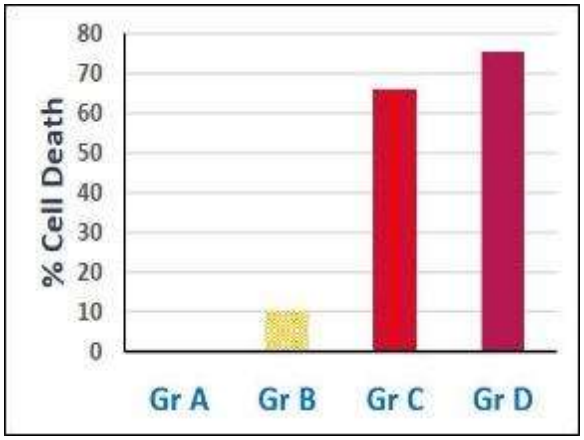


Figure 15: Cell Viability assay of Quercetin Conjugated AuNPs (a) Synthesized using Sodium Citrate; (b) Synthesized using Lemon Extract on MCF7 cell line

Physico-chemical characterization and comparative study of AuNPs with Quercetin synthesized using Sodium Citrate and Lemon Extract

UV-VIS Spectroscopy

The reduction of pure Au^{3+} ions to Au^0 was monitored by measuring the UV-Vis spectrum using UV-Vis Spectrophotometer (SHIMADZU Model: 2401PC). The absorption peak was observed at 521.6 nm (while using Sodium Citrate) (Figure 4a) and 542 nm (using lemon extract) (Figure 4b).[17]

Dynamic Light Scattering Particle Size Analyzer

The AuNPs synthesized with Quercetin using sodium citrate and lemon extract have a particle size of 30 nm **(**Figure 5a) and 35.68 nm (Figure 5b) respectively, analyzed using the DLS instrument (Zetasizer Nano-S. MALVERN Instruments, Model: ZEN1600).[18]

Stability Test

Again, a five days stability test was done using spectrophotometric scanning analysis to compare the shift in λ_{max} and change in OD values, that revealed the stability of the two samples of AuNCs, i.e., Q-Au-NC_{TSC}, and Q-Au-NC_{LE} t (Figure 6a and 6b).

Zeta Potential

As reported by Michael R *et al*. (2010), zeta potential measurements reveal the stability of nanoparticles. Data reveals the nanoconjugates are highly stable and have surface charge of (-) 33.5 mV for Q-Au-NC_{TSC} and (-) 13.5 mV for Q-Au-NC_{LE} (Figure 7a and 7b). It must be noted that as the zeta potential values approach zero, stability decreases.[19]

Fourier-transform infrared spectroscopy (FTIR)

FTIR spectral analysis was done for the pure extract, gold solution, as well as Quercetin, conjugated AuNPs. The main objective was to identify absorption peaks that exhibited notable shifts/ present in the two media. The high similarity between extract and Quercetin conjugated AuNPs indicated that the same compounds existed in both media. FTIR spectrum of the Q-Au-NC_{TSC} shows eight principal absorptions at 3433.1, 1591.3, 1400.4, 1260.9, 1081.4, 895.2, 802.0 and 620.4 cm^{-1} (Figure 8a) and Q-Au-NC_{LE} show eight principal absorptions at 3414.9, 1729.1, 1630.7, 1402.3, 1222.0, 1077.2, 789.1, 597.0 cm^{-1} (Figure 8b). The analysis was done using FT-IR RX1-Perkin Elmer (PerkinElmer Model: Spectrum 100) with a transmittance in the range of 4000 to 450 cm^{-1} at a resolution of 4 cm^{-1}.[20]

Scanning Electron Microscopy (SEM)

SEM image shows various characters like morphological character, size and surface of the AuNCs. SEM microscopy revealed that the AuNCs synthesized, in both the cases are in the range of 30-45 nm in size (Figure 9a and 9b) having the mixture of many shapes, i.e., triangle, rhombus and spherical are observed. However, spherical shaped AuNCs are predominant. SEM analysis showed the formation of AuNCs, which were well dispersed, and the aggregation of the particles could be seen. SEM measurements were performed on EVO 18 Carl Zeiss – United Kingdom.[21]

Evaluation of antibacterial activities of AuNPs

Antimicrobial activity of Quercetin solution, gold chloride solution and two Au-NCs were tested against Gram-Positive: *Staphylococcus aureus; Bacillus Subtilis*; and Gram-Negative: *Pseudomonas aeruginosa; Klebsiella pneumonia* bacteria using Kirby-Bauer disc diffusion assay. The Quercetin and gold chloride (on increasing concentration) solutions had given small zones of inhibition when tested for their antibacterial activities towards both the gram-positive and gram-negative bacterial strains. On the basis of the zone of inhibition obtained, between Q-Au-NC_{TCS} and Q-Au-NC_{LE}, the latter showed better inhibition (Figure 10a, 10b, 11a, 11b, 12a, 12b, 13a and 13b). We suspect that on increasing the concentration of the nanoparticles, it will show or give a further better zone of inhibition.

Table 3: Observations for the Antibacterial Activity for Gold-Quercetin-Sodium Citrate Nano-conjugates

***S. aureus* (Gram +ve)**	
Sample	Zone of inhibition (mm.)
Gold	9.0
Quercetin	16.3
Gold-Quercetin-Citrate Nano	7.3
Sodium citrate	No zone
***B. subtilis* (Gram +ve)**	
Gold	6.3
Quercetin	7.6
Gold-Quercetin-Citrate Nano	8.3
Sodium citrate	4
***P. aeruginosa* (Gram -ve)**	
Gold	6.8
Quercetin	7.6
Gold-Quercetin-Citrate Nano	7
Sodium citrate	4.7
***K. pneumonia* (Gram -ve)**	
Gold	5.3
Quercetin	8.3
Gold-Quercetin-Citrate Nano	7.6
Sodium citrate	5

Table 4: Observations for the Antibacterial Activity for Gold-Quercetin-Lemon extract Nano-conjugates

***S. aureus* (Gram +ve)**	
Sample	Zone of inhibition (mm.)
Gold	11.6
Quercetin	19.3
Gold-Quercetin-Lemon extract Nano	11.2
Lemon extract	11.4
***B. subtilis* (Gram +ve)**	
Gold	5.7
Quercetin	6
Gold-Quercetin-Lemon extract Nano	11.6
Lemon extract	6.3
***P. aeruginosa* (Gram -ve)**	
Gold	6.7
Quercetin	9
Gold-Quercetin-Lemon extract Nano	11.3
Lemon extract	6.9
***K. pneumonia* (Gram -ve)**	
Gold	6
Quercetin	8
Gold-Quercetin-Lemon extract Nano	12
Lemon extract	7

The zone of inhibitions obtained for AuNCs synthesized using Quercetin and Sodium Citrate are given in Table 3.

The zone of inhibitions obtained for AuNCs synthesized using Quercetin, and Lemon Extracts are given in Table 4.

Cell Viability Assay

Untreated MCF7 cell is shown in Figure 14A, and the gold chloride solution treated cell is shown in Figure 14B. Cell viability against the equal amount (1.75 mg) of Q-Au-NC_{TCS} and Q-Au-NC_{LE} were studied on MCF7 cell line; shown in Figure 14C and 14D respectively.

The bar graph (Figure 15) represents per cent cell death observed in cytotoxicity assay on breast cancer cell line (MCF7) for all the four experimental groups viz. Untreated cell (Group A), Gold Chloride solution treated (Group B), Q-Au-NC_{TCS} treated (Group C), and Q-Au-NC_{LE} treated (Group D).

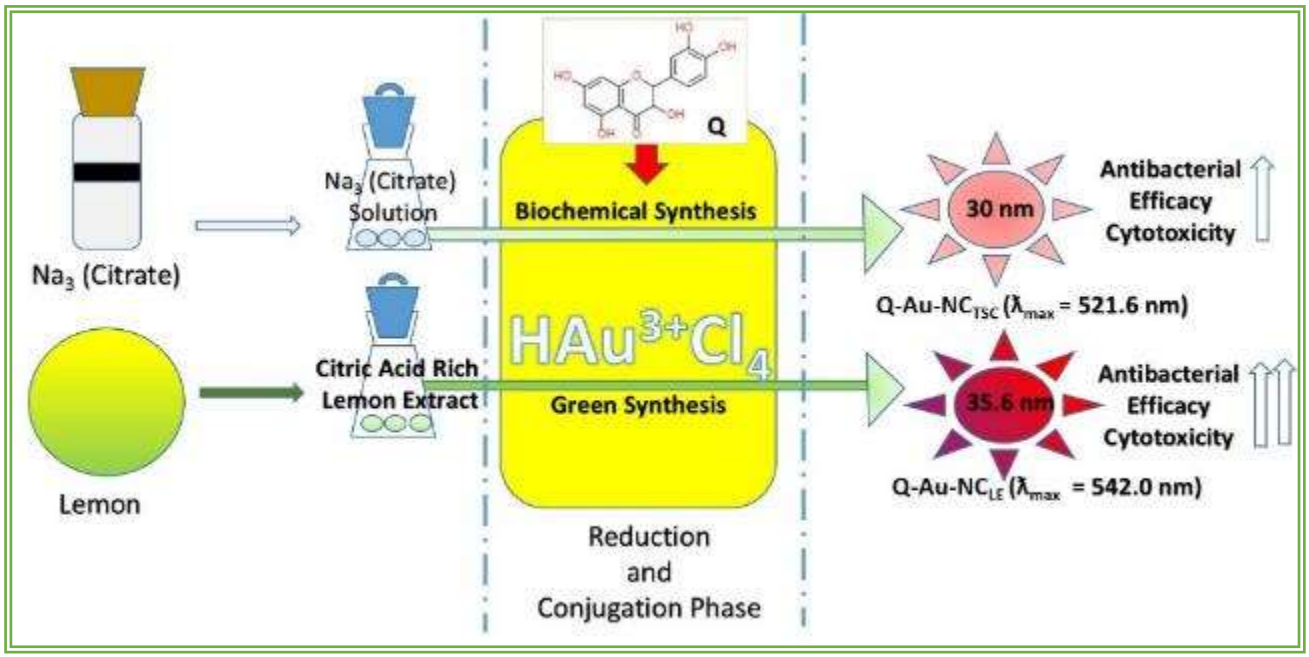
The Figure 15 showed that among the AuNCs, Q-Au-NC_{LE} showed the highest percentage of cell death (~ 75.37%) whereas Q-Au-NC_{TCS} and gold chloride solution showed the 66.081%, and 10.31% of cell death respectively with respect to the untreated cell, observed after 24 hours of incubation.

In a nutshell, the Quercetin conjugated AuNPs were synthesized using Citrus Lemon Extract as reducing agent in place of conventional biochemical, tri-sodium citrate. Physico-chemical characterization showed their almost comparable efficacy in the synthesis of gold nanoparticles. Five days stability test of the AuNCs showed that both of them are reasonably stable as evident from spectral data. However, Zeta potential data indicates slight less stability of the Q-Au-NC_{LE}. However, the Q-Au-NC_{LE} give better results for antibacterial activity. Zhang Y *et al.* (2015) reported that gold nanoparticles have conflicting antibacterial activity.[27] In this study, the antibacterial activity of the gold nanoconjugates may be attributed to conjugated Quercetin molecules. As reported by Rattanata *et al.* (2015), gold nanoparticles enhance the anticancer activity of the bioactive compound, Gallic Acid. Hence, Quercetin was added to the reaction mixture during the reduction phase for better conjugation with gold nanoparticles. It was observed that Q-Au-NC_{LE} showed better anticancer activity against MCF7 cell line with respect to chemically/ biochemically produced Quercetin-Au-Nanoconjugates, Q-Au-NC_{TSC}. This is maybe due to better reducing and capping effect of the natural lemon extract.[28]

CONCLUSION

Since Lemon extract contains a fair amount of natural citric acid with other polyphenol and flavonoids, it possesses high radical scavenging activity. Being a food material, it is nontoxic to a normal cell. Hence it can be used as a better alternative to tri-sodium citrate and may find extensive use in gold nanoparticles and gold nanoconjugates synthesis for biomedical applications.

In the present research, two differentially synthesized Quercetin-Au-Nanoconjugates, Q-Au-NC_{TSC} and Q-AU-NC_{LE} were synthesized using tri-sodium citrate and lemon extract (its natural alternative), characterized and compared.



The Q-Au-NC_{LE} has shown better stability and antibacterial activity compared to Q-Au-NC_{TSC}. On the MCF7 cell line, the Q-Au-NC_{LE} showed better cytotoxicity (cell death ~ 75%) with respect to Q-Au-NC_{TSC} (cell death ~66%). Natural sources with a high content of citric acid can serve as the best alternative to tri-sodium citrate in synthesizing Au-NPs and different nanoconjugates for biomedical applications. In addition, Q-Au-NC_{LE} can be considered a potential candidate for drug discovery.

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ABBREVIATIONS

AuNPs: Gold Nanoparticles
AuNCs: Gold Nanoconjugates
Q-Au-NCTSC: Quercetin nanoconjugates prepared using tri-sodium citrate
Q-Au-NCLE: Quercetin nanoconjugates prepared using Lemon extract
DLS: Dynamic Light Scattering
FTIR: Fourier-transform infrared Spectroscopy
SEM: Scanning Electron Microscopy

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