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Research Article

Bio-inspired Synthesis of Ag, Au, and Ag-Au Bimetallic Nanoparticles using *Capsicum annuum* Aqueous Leaf Extract and Assess their *In-vitro* Antibacterial and Anticancer Potential

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ABSTRACT

Noble bimetallic nanoparticles (BNPs) exhibit strong anticancer and antibacterial activities. They are known to be stable, less toxic, environmentally friendly and biocompatible in nature. Due to their enhanced biological properties, they are well-suited for biomedical applications such as cancer therapy, gene therapy and drug delivery. In the current study, we examine the anticancer and antibacterial potential of bio-mediated mono and bimetallic nanoparticles. Here, aqueous *Capsicum annuum* leaf extract was employed as a good reducing and capping agent for producing Ag, Au monometallic, and Ag-Au bimetallic nanoparticles. The formation of C.A- Ag, Au MPNs and Ag-Au BNPs was initially confirmed by a visible color change of the reaction mixture and UV-visible spectra show the surface plasmon resonance (SPR) band observed at 543 nm. Furthermore, phytofabrication, crystallinities, structural alignments, particle size and elemental composition were studied by following standard physicochemical techniques such as fourier transform infrared (FTIR), X-ray diffraction (XRD), high resolution-transmission electron microscopy (HR-TEM), scanning electron microscopy and energy- dispersive X-ray spectroscopy (SEM-EDX), respectively. The results obtained from various characterization techniques confirmed that the C.A. mediated Ag, Au MNPs and Ag-Au BNPs were spherical in shape and face-centered cubic (FCC) structure with a nanoscale range (10–25 nm). The BNPs exhibit strong efficacy against bacterial strains. These nanoparticles were subjected to investigate the anticancer activity against human lung cancer cells (A549 cell line) through MTT assay. The cell viability was determined by this assay. The occurrence of cell apoptosis and necrotic were quantified by using dual fluorescent staining (AO/EB) and flow cytometry analysis. However, Ag-Au bimetallic nanoparticles showed the highest cytotoxic potential with low IC_{50} - 57.35 ± 0.05 µM values. This IC_{50} value is comparatively lower than, Ag, Au MNPs and C.A. aqueous leaf extract. IC_{50} values of C.A- Ag-Au BNPs predominantly induced cell apoptosis, necrotic and the death of A549 cells suggested the anticancer potential of C.A. mediated Ag-Au BNPs to treat the lung cancer cells.

Introduction

Cancer is one of the most severe categories of chronic diseases due to the uncontrolled cell growth that raises the mortality rate globally. For the treatment of cancer, there is no satisfactory medication or drug.[1] Noble metal nanoparticles have gained massive attention in the field of biomedicine, pharmacology and catalysis due to their distinctive properties such as large fraction of surface atoms, a high surface-to-volume ratio, physico-chemical properties, easy synthesis, chemical stability, and electrical conductivity. Their selective drug delivery, less toxicity and biocompatible nature, serve noble metal nanoparticles as an excellent anticancer and antimicrobial agent. They were intensively used to generate a more potent and effective antineoplastic drug for the treatment of cancer. The combination of both metals such as Ag-Au noble bimetal nanoparticles had significant characteristics so that they were employed in cancer therapy, drug delivery,

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gene therapy, biosensing and catalysis.[2-4] The production of noble metal nanoparticles can be accomplished by using a variety of chemical and physical methods. However, these methods are expensive and uneconomical and they require complicated procedures, and toxic chemicals.[5] Due to these disadvantages, we consider the biological methods for the synthesis of metal nanoparticles. This process generates nontoxic, biocompatible nanoparticles and is economical and environmentally beneficial. Plants and microbes are used as a bio-reductant for generate the bio-mediated nanoparticles.[6] Due to the accessibility, environmental friendliness, and safety for living organisms, phytochemical-mediated synthesis is particularly more advantageous than microorganism-mediated synthesis.[7] As a bio-reductant for the synthesis of noble metal nanoparticles, we here select the *Capsicum annuum* aqueous leaf extract. After the plant was used to cultivate chilies, it was thought of as agricultural waste, yet it is a good source of phytochemicals, including alkaloids, flavonoids, phenols, polysaccharides, terpenoids, and tannins.[8] The stability and surface fabrication of nanoparticles are accomplished by these organic moieties.[9] According to the literature, there hasn't yet been a report on the green synthesis of Ag-Au BNPs utilizing aqueous *C. annuum* leaf extract. In this study, we mainly attempt to demonstrate how *C. annuum* aqueous leaf extract is used to generate monometallic Ag, Au and bimetallic Ag-Au nanoparticles. Here, the aqueous leaf extract serves as a reducing, stabilizing, and capping agent. Through the utilization of UV-visible spectroscopy, fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD), scanning electron microscopy (SEM), and energy dispersive X-ray spectroscopy analysis (EDX), the optical, structural, and morphological characteristics of synthesized Ag, Au MNPs, and Ag-Au BNPs were investigated. Additionally, we use the well diffusion method to report their antibacterial activity against human pathogenic bacteria such *Escherichia coli* (ATCC10536) and *Staphylococcus aureus* (ATCC33591) and the MTT assay to evaluate their cytotoxicity against A549 human lung cancer cell lines. Apoptosis and necrotic of cancer cells were quantified through the flow cytometry analysis and (AO/EB) fluorescence labeling method.

Materials and Methods

Materials

The required chemicals were purchased form the following sources: Silver nitrate ($AgNO_3$), tetrachloroauric acid ($HAuCl_4$) from Sigma Aldrich. All the chemicals were used without further purification as obtained.

Preparation of *C. annuum* Leaf Extract

Fresh leaves of *C. annuum* free from disease were collected from the surrounding area of Madurai, washed 2 to 3 times with tap water, followed by DI water to remove unwanted dust particles, and dried at room temperature. Approximately 20 grams of leaves were finely chopped and transferred to a 400 mL beaker with 100 mL of DI water and stirred at 60°C for 1-hour. The mixture was cooled to room temperature, followed by filtering through Whatman filter paper no. 1. The collected filtrate was stored at 4°C for the synthesis of Ag, Au, and Ag-Au bimetal nanoparticles.[10]

Phytochemical Screening

Healthy and fresh leaves were selected for phytochemical analysis. The phytochemical screening of *C. annuum* leaf extract was carried out by the standard method that was previously reported.[11,8]

Biosynthesis of Ag, Au, and Ag-Au Bimetallic Nanoparticles

The Ag, Au, and Ag-Au BNP nanoparticles were synthesized by using the aqueous extract of *C. annuum* leaves. For the synthesis of Ag nanoparticles, about 10 mL of extract was added to the 40 ml of 1-mm $AgNO_3$ solution at room temperature. A similar procedure was followed for the synthesis of Au nanoparticles using a 1-mm $HAuCl_4$ precursor. But in the case of Ag-Au BNPs, 10 mL of extract was added to the 40 mL mixture of (1 mm) $AgNO_3$ and (1 mm) $HAuCl_4$ in a 1:1 ratio at room temperature. Here, the change in color of the reaction mixture indicates the reduction of metal salts to nanoparticles. These synthesized nanoparticles were centrifuged at 10,000 rpm for 20 minutes. The process of centrifugation and pellet redispersion is repeated three times and followed by oven drying at 60°C. Then the dried samples were used for further study.[3,12,13]

In-vitro Biological Activities

Anti-bacterial activity

Antibacterial activity of C.A. aqueous leaf extract-mediated Ag, Au MNPs, and Ag-Au BNPs was studied with bacterial strains such as *Escherichia coli* (ATCC10536) gram-ve and *Staphylococcus aureus* (ATCC33591) gram-ve by using the standard well diffusion method. The test solutions were dissolved in distilled water. The nutrient broth (NB) media and agar were autoclaved. The sterile media was used to culture the bacterial cells overnight at 37°C. The autoclaved NB agar was plated on a standard petridish. Once the agar was solidified, both bacterial strains were spread and plated using an L-rod. A sterile well cutter was used to punch holes in the agar plate. The test solutions of samples at different concentrations were spread onto the punched-in wells, and chloramphenicol was used as a control. These bacterial strains were used to evaluate the bactericidal effect of C.A. extract, Ag, Au MNPs, and Ag-Au BNPs, as well as the zone of inhibition. The zone of inhibition was directly proportional to the bactericidal effect of biomediated MNPs and BNPs.[14]

Anti-cancer MTT assay

The anticancer potential of phytofabricated MNPs and BNPs inhibiting the growth of A549 lung cancer cells was evaluated by a standard MTT assay. In this assay, the water-soluble yellow MTT tetrazolium salt is reduced to insoluble purple formazan crystals by the cleavage of its tetrazolium ring by the mitochondrial dehydrogenase enzymes of viable cells. The water in soluble formazan was solubilized by DMSO. The resulting purple solution is spectrophotometrically measured. For this assay, 100 µl of culture media containing 5000 cells were seeded in a 96-well plate and incubated for 12 hours at 37°C in a 5% CO_2 incubator. After incubation, 100 µl of C.A. extract, MNPs, and BNPs test solutions (10–100 g/mL) were added into wells accordingly, followed by incubation at the same conditions for 24 h. 20 µL of MTT solution (5 mg/mL stock solution prepared in phosphate buffered saline) was added and kept for 4 hours at the same conditions for incubation. Then the cell contents were discarded without disturbing the formazan crystals formed, followed by adding 200 µL of DMSO. The color intensity is inversely proportional to the dead cells. The absorbance was measured at 570 nm, and the percentage (%) of dead cells was quantified using eq. (1).[15,16]

$$(\%)\text{Inhibition} = 100 - \frac{\text{OD of Sample}}{\text{OD of Control}} \text{ X } 100 \qquad (1)$$

Assessment of Cell Apoptosis

Fluorescent staining method

The apoptosis of cells was evaluated by the dual AO-EB (Acridine orange-ethidium bromide) staining method. Here, cells were seeded in 24-well plates at a density of 4000 cells per well and incubated at 37°C for 24 hours. Synthesized MNPs and BNPs were incubated with cells, and after the incubation period, both stainings were added to each well. Immediately, cells were visualized using a fluorescence microscope, and the percentage (%) of cells was quantified.[17]

Table 1: Phytochemical analysis of *C. annuum* leaf extract

Phytochemicals	*Aqueous C.A leaf extract*
Alkaloids	+
Carbohydrates	+
Terpenoids	+
Cardiac glycosides	-
Phlobatannins	-
Tannins	+
Glycoside	-
Flavonoids	+
Saponins	-
Phenols	+

+ = Presence and - = Absence

Flow cytometry analysis

Flow cytometry is a versatile technique that is used to study cell apoptosis. It offers the ability to analyze large numbers of cells. Moreover, cell apoptosis is not a static process. It is specific and time-specific. The cells were seeded in a 6-well plate (10^5 cells per well) and cultured at 37°C for 24 hours. After that, the cells were incubated with the concentrations of the synthesized MNPs and BNPs for 24 hours. Cells were trypsinized and washed with PBS. The cells without any treatment were used as blank controls. Next, the cells were stained with annexin V-FITC/PI according to the annexin V-FITC apoptosis detection kit. Finally, apoptosis was evaluated by a flow cytometer (SYSMEX, Japan), and the data were analyzed by Flow Jo software.[18]

Characterization methods

The synthesized mono and bimetallic nanoparticles were characterized using UV-vis, FTIR, XRD, TEM and SEM-EDX. The formations of nanoparticles were monitored using the UV-vis spectroscopy technique (Beckman-Model No.DU-50, Fullerton). The spectra were recorded in the range between 200 and 900 nm. The phytochemical fabrications of synthesized nanoparticles were investigated by the FTIR spectrometer (Perkin Elmer model 10,300). The scanning range was 400–400 cm^{-1}. The crystalline purity, phase, and size of synthesized nanoparticles were examined by the XRD machine (panalytical, Philips PW 1830) using CuKα radiation in the 2θ ranges from 10–80°. The morphology and particles size of the synthesized nanoparticles were analyzed through the HR-TEM analysis (HRTEM-JEOL-3010). The surface morphology and the elemental composition were analyzed using the SEM-EDX technique (Quanta FEG 250).

Results and Discussion

Phytochemical Screening of *C. annuum* Leaf Extract

The C.A. leaf extract was analyzed by phytochemical screening for the evaluation of bio active compounds. Ten phytochemical tests were tested, out of which six were conformed in the Table 1. The positive sign indicates the presence of phytochemicals and the negative sign indicates the absence of phytochemicals. The identified bioactive compounds were flavonoids, phenols, alkaloids, carbohydrates and tannins, respectively.[19,20] These phytochemical constituents of the C.A. leaf extract were responsible for the process of bio-reduction as well as they are act as good reducing, capping and stabilizing agent for the synthesis of MNPs and BNPs.

Biosynthesis of Ag, Au and Ag-Au Bimetallic Nanoparticles

The reduction of metal salts by phytochemical constituents of C.A. leaf extract was monitored by the color change of solution from initial reaction mixture to final color change

which indicates the formation of metal nanoparticles. This color changes was observed for Ag nanoparticles from colorless solution to brown and for Au nanoparticles from pale yellow to violet color and for Ag-Au bimetallic nanoparticles were from pale yellow to reddish brown color. These observed color changes were indicates the process of bio reduction. The formation of nanoparticles was initially confirmed by the visual color change of the solution and it was further confirmed by the UV-vis spectroscopy technique.[12]

Physico-chemical Characterization of Ag, Au MNPs and Ag-Au BNPs

UV-vis spectroscopy analysis

The UV-Visible spectroscopy is a preliminary characterization tool for confirmed the formation of MNPs and BNPs. Fig. 1 shows the UV-vis spectra of C.A. leaf extract, Ag, Au MNPs and Ag-Au BNPs. The UV-vis spectra of color changed solution gives surface plasmon resonance (SPR) absorption band. The absorption band was observed at 260 nm for aqueous extract of C.A leaves. The surface plasmon bands observed at 433, 560 and 543 nm indicates the formation of Ag, Au MNPs and Ag-Au BNPs, respectively[12] which proves the bio reduction of metal solution into nanoparticles as well as the interaction of bio molecules with nanoparticles. The intensity of red shift peaks were indicates the reduction of $Au^{3+} \rightarrow Au^{0}$ and $Ag^{1+} \rightarrow Ag^{0}$. Thus proves the plant sources can be used as a natural reducing agent. The peak at 543 nm indicates the formation of homogeneous Ag-Au bimetallic nanoparticles. [21]

FTIR spectroscopy analysis

The presence of chemical composition and the functional groups of C.A. leaf extract and the synthesized MNPs and BNPs were analyzed by the technique FTIR. The intensity peaks were observed at 3445, 2930, 1637 and 1054 cm^{-1} indicates the presence of functional groups in plant extract (Fig. 2). These functional groups were accountable for the process of bio reduction also these peaks were indicating

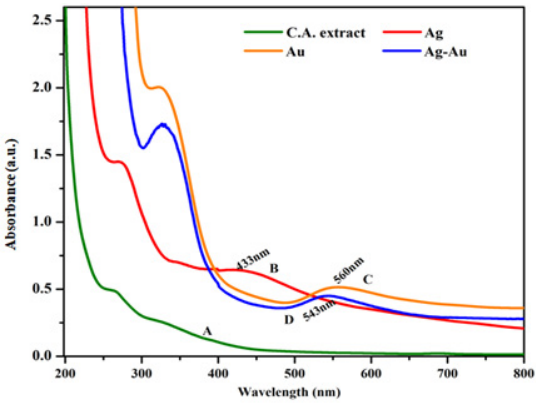

Fig. 1: UV-vis Absorption spectra of A- C.A leaf extract B- Ag MNPs, C- Au MNPs and D-Ag-Au BNPs

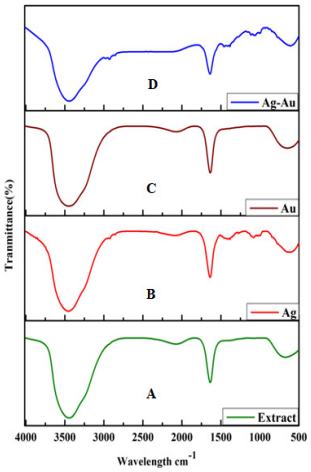

Fig. 2: FTIR spectra of A- C.A. leaf extract B-Ag MNPs, C- Au MNPs and D-Ag-Au BNPs

them as a reducing and capping agent. These characteristic peaks were shifted in the synthesized MNPS and BNPs. The intensive peaks at 3600 to 3400 cm^{-1} were represents the stretching frequency of O-H and N-H groups. The peaks were observed at 2800 to 2900 cm^{-1} indicates the presence of symmetric and asymmetric stretches of H-C-H. The peaks observed at 1650 to 1600 cm^{-1} characterize the anti-symmetric stretching of (COO^{-}) carboxylate groups. The stretching of (C-OH) was observed at 1020 to 1040 cm^{-1}.[8,11]

XRD analysis

The crystalline nature, phase purity and the size of C.A mediated MNPs and BNPs were investigated by X-Ray Diffraction analysis. Fig. 3. demonstrated the XRD spectra of Ag, Au MNPs and Ag-Au BNPs. The average crystallite size was determined by using Debye–Scherrer equation.

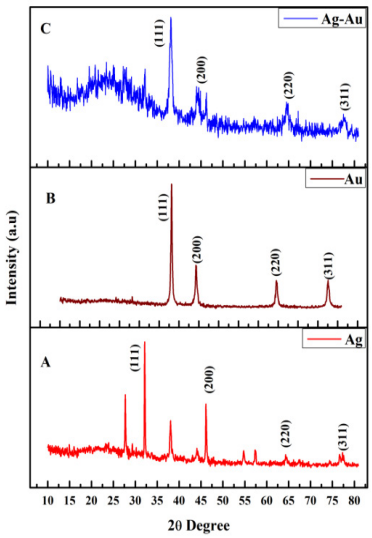

Fig. 3: XRD of A-Ag MNPs, B- Au MNPs and C-Ag-Au BNPs

$$D = \frac{0.9\,\lambda}{\beta \cos\theta} \qquad (2)$$

Where
D – Crystallite size,
λ - Wavelength of the incident x-ray beam,
Θ - Bragg's diffraction angle,
β - Full width at half maxima (FWHM) of the MNPs and BNPs peak.[22]

The XRD patterns of synthesized Au nanoparticles showed the distinct diffraction peaks with 2θ values at 38.13°, 44.30°, 64.50° and 77.51° related to (111), (200), (220) and (311) planes, respectively. These planes are well matched with JCPDS file no.04-0784 which indicates the face centered cubic (FCC) of Au nanoparticles.[23] In Ag nanoparticles, the similar characteristics peaks were obtained at 2θ values at 32.14˚, 46.12˚, 64.36˚ and 76.68˚ indexed to (111), (200), (220) and (311) planes, respectively. These planes were corresponds to FCC of Ag nanoparticles as per JCPDS file no.04-0783[24]. The XRD patterns for Ag-Au bimetallic nanoparticles showed broader peaks with 2θ values at 38.04˚, 46.21˚, 64.77˚ and 77.87˚ related to (111), (200), (220) and (311) planes, respectively. These results suggests the homogenous preparation of Ag-Au bimetallic nanoparticles, which is also indicates the same lattice structure and planes of individual Ag and Au monometallic nanoparticles.[12] Apart from that, the rest of diffraction peaks of Ag and Ag-Au BNPs show 2θ values at 27.73, 54.74 and 57.37 corresponds to (111), (311) and (222) planes were accountable for phytochemical constituents of C.A. leaf extract. These planes may also be due to presence of AgCl, which is attributed to the Ag nanoparticles also these planes were coexisting with Ag in Ag-Au BNPs.[25] The estimated average crystallite size of Ag, Au MNPs and Ag-Au BNPs were 26.72, 29.54 and 15.5 nm, respectively. The size of bimetallic nanoparticles was comparatively lower than monometallic nanoparticles.

High-resolution transmission electron microscopy analysis

The morphological characteristics, crystallinities and particles size of synthesized nanoparticles were studied through the high-resolution transmission electron microscopy (HR-TEM) analysis. Fig. 4. A-F represents the HR-TEM images and SEAD patterns of C.A-Ag NPs, Au MNPs and Ag-Au BNPs. The HR-TEM images confirm the formation of Ag, Au MNPs and Ag-Au BNPs. Ag nanoparticles were spherical like morphology. The Au nanoparticles were polydispersed consisting of rod, spherical with irregular contours. In the case of C.A-Ag-Au BNPs showed spherical shapes and some level of agglomeration was observed. The size ranges of up 10 to 30 nm.[26,27] The SEAD patterns of the C.A-Ag, Au MNPs and Ag-Au BNPs confirm that the particles are highly crystalline in nature. Further, the existence of FCC phase of Ag, Au MNPs and Ag-Au BNPs with (111), (200), (220) and (311) planes are proved by characteristics diffraction ring patterns.[28]

SEM and EDX analysis

The elemental analysis and surface morphology of biosynthesized MNPs and BNPs were studied through employing EDX and SEM techniques. Fig. 5. (A-F) depicts SEM micrographs and EDX spectra of Ag, Au MNPs and Ag-Au BNPs. The surface morphology of synthesized Ag nanoparticles showed tiny spherical shape. A shiny agglomerated nanoparticle indicates the morphology of Au nanoparticles. The morphological feature of bio mediated Ag-Au bimetallic nanoparticles were showed cubic shape and seem to be agglomerated.[21] The EDX analysis illustrates the elemental composition of synthesized MNPs and BNPs. These spectra show the peaks at 3, 2.3 KeV suggest the presence of Ag and Au monometallic nanoparticles. The Ag-Au bimetallic nanoparticles show

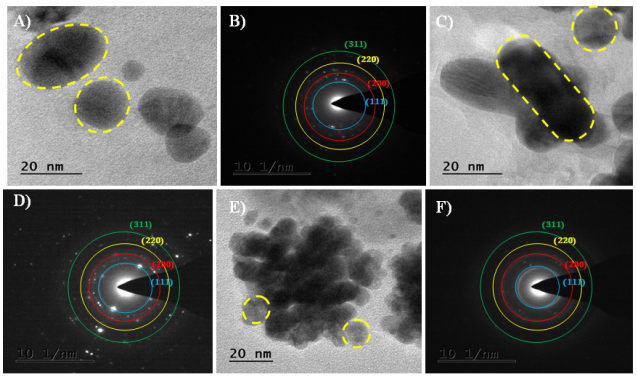

Fig. 4: HR-TEM images and SEAD patterns of A, B -Ag MNPs C, D- Au MNPs E, F-Ag-Au BNPs

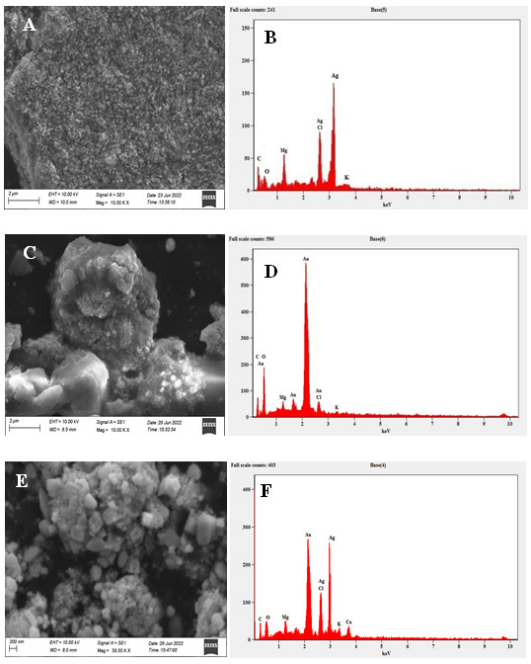

Fig. 5: SEM micrographs and EDX spectra of A-Ag MNPs B- Au MNPs C-Ag-Au BNPs

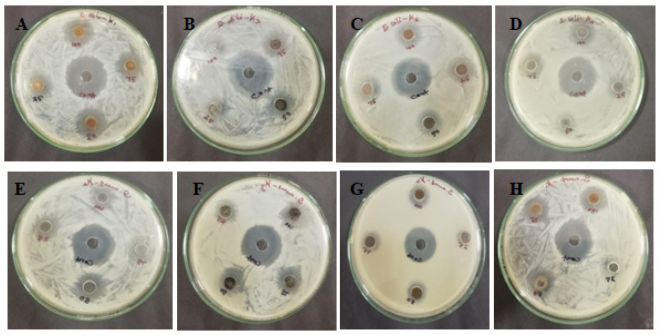

Fig. 6: a: Inhibition zone of *Escherichia coli* (G -ve) b: Inhibition zone of *Staphylococcus aureus* (G +ve)

the same peak at 3 KeV, 2.3 KeV. The significant peak at 0.2 KeV and 0.5 KeV indicates the presence of C and O while the additional peaks were noted for the presence of Mg, K and Cl. These peaks were indicates the elements which are present in plant extract. Therefore, the EDX spectrum confirms the synthesized metal nanoparticles were fabricated by C.A. leaf extract.[8]

In-vitro Biological Activities

Antibacterial activity

The antibacterial activity of biosynthesized mono and bimetallic nanoparticles were investigated against *Escherichia coli* gram-ve (ATCC10536) and *Staphylococcus aureus* gram +ve (ATCC33591) using well diffusion method. The sample dosage range is 25, 50, 75 and 100 µL. Chloramphenicol used as a control. The observed antibacterial activity of C.A. leaf extract, MNPs and BNPs were given in Table 2 and the zone of inhibitions was showed in experimental photographs (Fig. 6 a and b). The bio mediated mono and bimetallic nanoparticles showed excellent antibacterial activity against both categories of bacterial strains. The C.A aqueous leaf extract shows moderate activity towards the bacterial strains. The Ag nanoparticles exhibit strong efficacy against both bacterial strains. The Ag^+ metal cations possess to form an electrostatic bond with gram –ve bacterial cell membrane. Due to its negatively charged cell constituents could easily attract the positively charged metal cations. This interaction disrupts the bacterial cell membrane and arrest the function of mitochondria leads to the production of reactive oxygen species (ROS). These ROS causes the death of bacterial cells.[29] In Au nanoparticles cause the lower toxicity towards the both bacterial cell membrane so that lower inhibition zone was observed than Ag nanoparticles. The Ag-Au bimetallic nanoparticles exhibited strong efficacy against human pathogens. Due to the synergistic action of both metal cations shows the significant effect and produce good inhibition zone against both bacterial strains. The Ag-Au bimetal nanoparticles has a high synergistic effect than Ag and Au mono metal nanoparticles this may induce the death of bacterial cells.

Table 2: Antibacterial activity of C.A leaf extract, Ag, Au MNPs and Ag-Au BNPs against bacterial strains

Strain	*Sample*	*Zone of Inhibition (mm)*				
		Control	*Concentration level (µL)*			
			25	*50*	*75*	*100*
E. coli	C.A. Leaf Extract	27	12	14	13	15
	Ag NPs	27	14	16	15	17
	Au NPs	27	15	17	16	18
	Ag-Au NPs	26	16	18	17	20
S. aureus	Extract	26	12	14	13	15
	Ag NPs	26	13	15	14	16
	Au NPs	25	14	16	15	17
	Ag-Au NPs	24	15	17	16	18

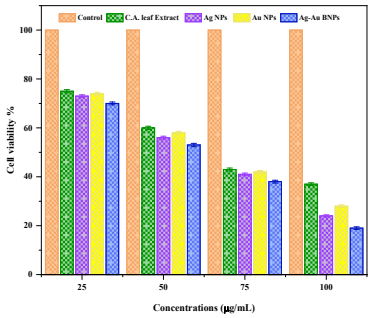

Fig. 7: MTT assay for C.A. Leaf extract, Ag, Au MNPs and Ag-Au BNPs treated A549 human lung cancer cell lines. Control without addition of C.A. Leaves extract, Ag, Au MNPs and Ag-Au BNPs

Therefore, the bimetallic nanoparticles exhibit strong antibacterial efficacy than mono metallic nanoparticles.[30]

Anti-cancer MTT assay

The anticancer activity of synthesized MNPs and BNPs were evaluated of against human lung cancer cells (A549) by using MTT assay. This assay used to determine the cell viability. From this experimental result, the synthesized nanoparticles have more potent to kill the cancer cells at various concentrations. The concentrations range from 25 to 100 µg/ mL. Table. 3 shows the minimum inhibitory concentration (IC_{50}) values of the samples while the plot was representation of percentage of cell viability to concentration of samples (Figs 7 and 8). depicts the cell morphology at IC_{50} concentration of C.A. leaf extract, Ag, Au MNPs and Ag-Au BNPs. From the results, the

Table 3: IC_{50} Value of CA leaf extract, Ag, Au MNPs and Ag-Au BNPs

IC_{50} (µg/mL) ± SD values	
Tested samples	A549 (lung cancer)
C.A. leaf extract	85.45 ± 0.05
Ag MNPs	64.05 ± 0.05
Au MNPs	79.75 ± 0.05
Ag-Au BNPs	57.35 ± 0.05

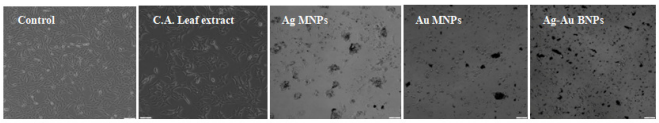

Fig. 8: Cell morphological analysis of A549 cancer cell lines control and exposed with C.A. leaf extract, Ag, Au MNPs and Ag-Au BNPs

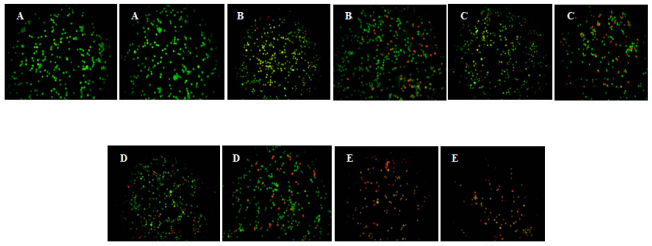

Fig. 9: Apoptosis assessment in cancer cells by dual (AO/EB) fluorescent staining of A549 cells control (A) after treatment with aqueous C.A. leaf extract (B), Ag (C), Au (D) MNPs and Ag-Au BNPs (E) (IC_{50} µM) for 24 hours

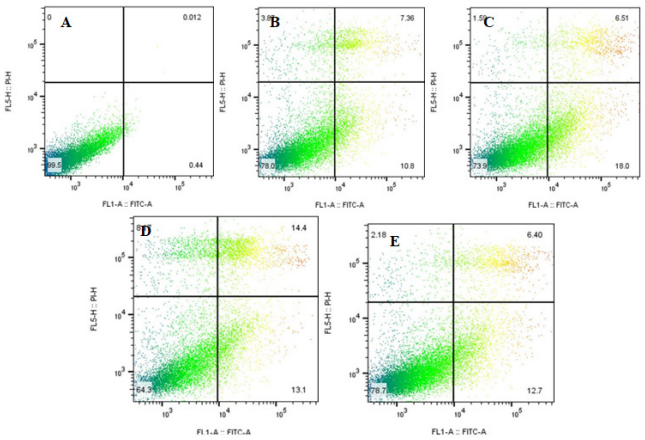

Fig. 10: Annexin V-FITC/PI double staining analysis of apoptosis in the cells after C.A. aqueous leaf extract, MNPs and BNPs were treated with concentration (24 hours) A- Control, B- C.A aqueous leaf extract C- Ag NPs D- Au NPs E- Ag-Au BNPs

percentage of cell viability was decreases with increasing the concentration. Initially the cell viability (%) was recorded as 78, 73, 76, and 69 at 25 µg/mL^{-1}. When the concentration was increasing at a 100 µg/mL^{-1} the cell viability was decreased as 37, 26, 35 and 22. The recorded IC_{50} values are 85.45 ± 0.05 µM, 64.05 ± 0.05 µM, 79.75 ± 0.05 µM and 57.35 ± 0.05 µM for C.A. leaf extract, Ag, Au and Ag-Au BNPs, respectively. Based on cell viability and IC_{50} values, the Ag-Au bimetallic nanoparticles exhibited more anticancer activity than Ag, Au MNPs. The increasing order of anticancer activity is Ag-Au BNPs > Ag MNPs >Au MNPs> *C. annuum* leaf extract. This result proves increasing the sample concentration leads to decrease the cell viability.[25]

Assessment of Cell Apoptosis

Fluorescent staining method

The cell apoptosis and necrotic were quantified by dual (AO/EB) fluorescence staining method. When the staining are intercalate with nucleic acid and it emit respective fluorescence colors which used to differentiate cellular organelles.[31] The apoptotic morphological observations were shown in Fig. 9. The green color (AO dye) indicates the apoptotic cells with morphological changes such as cell blebbing and the formation of apoptotic bodies. The untreated cells are also shown in green color with typical morphology but the necrotic cells are shown in red/ orange (EB) fluorescence with fragmented chromatin and apoptotic bodies. This result indicates the IC_{50} values are predominantly induced the cell apoptosis and necrotic in addition the nanoparticles show higher cytotoxic potency than the control.[32]

Flow cytometry analysis

The flow cytometry analysis is used to detect and quantify the level of cell apoptosis/necrotic in C.A- Ag Au BNPs treated lung cancer cells. To quantify cell apoptosis, the cells were exposed to extract and nanoparticles at different concentration were stained using Annexin V-FITC/propidium iodide double-staining and analyzed by flow cytometry. During the cell apoptosis, plasma membrane loses their symmetry so that phospholipids leave phosphatidylserine behind on the outer leaflet of the plasma membrane. Annexin V is a Ca^{2+} dependent phospholipid binding protein which has high affinity for phosphatidylserine. Hence, it can be used as a sensitive probe for the presence of phosphatidylserine on the cell membrane and hence as a marker of apoptosis. Propidium iodide is a counter strain which is binds to double stranded DNA. It is used for assessing cell viability as well as is excluded by the plasma membrane of living cells, and thus can be used to distinguish necrotic cells from apoptotic and living cells by supravital staining without prior permeabilization. The results were shown in Fig. 10. The C.A. aqueous extract, BNPs and MNPs were corresponding to the representative dot plots of Annexin V propidium iodide staining in the percentages of apoptotic cells.[33] The top right quadrant, dead cells in the late stage of apoptosis;

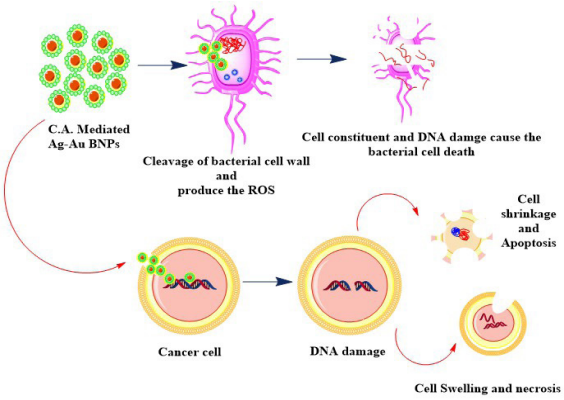

Fig. 11: Schematic illustration of possible mechanism for anti bacterial and anticancer activity of bio-mediated MNPs and BNPs

bottom right quadrant, cells undergoing apoptosis; bottom left quadrant, viable cells. Therefore, the biosynthesized mono and bimetallic nanoparticles were exhibited higher cytotoxic potential than the control which induced the cell apoptosis. The possible mechanism of anticancer activity of the nanoparticles was shown in Fig. 11. When the nanoparticles can cleave the cell wall membrane it cause the cellular fragmentation or leakage of cell contents, membrane blebs as well as they can damage the DNA which is leads to cell necrotic and apoptosis.[31]

Conclusion

In conclusion, the noble mono metallic and bimetallic nanoparticles were modified by using *C. annuum* leaf extract. This simple green method is well suits to produce stable, eco-friendly, nontoxic and biocompatible nanoparticles. The formation and structural conformation of synthesized nanoparticles were analyzed by using spectroscopic and morphological techniques. The UV-vis spectroscopy analysis shows the surface plasmon resonance band at 543 nm confirmed the formation of homogenous bimetallic nanoparticles. The phytochemical fabrications of nanoparticles were confirmed by FTIR analysis. The crystalline nature, size, purity and cubic structure of mono metallic and bimetallic nanoparticles were confirmed by XRD techniques. The average crystallite size is 26.72, 29.54 and 15.5 nm for Ag, Au MNPs and Ag-Au BNPs, respectively. Besides, HR-TEM images clearly indicate the mixture of shape in metal nanoparticles and the spherical shape were predominant with sizes ranges up to 10 to 25 nm. The elemental composition and surface morphology of nanoparticles were reported by SEM and EDX techniques. Due to the synergistic action of bimetallic nanoparticles, they showed highest antibacterial activity against both bacterial strains than the monometallic nanoparticles. The anticancer activity of the mono and bimetallic nanoparticles were evaluated against lung cancer cells by using MTT assay. They exhibited highest cytotoxic potential than control. The fluorescent staining and flow cytometry analysis exhibit the IC_{50} values were predominantly induced the cell apoptosis and necrotic. This study suggests the bio mediated Ag, Au MNPs and Ag-Au BNPs can be used as an antibacterial and anticancer agent in future.

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