Supporting Information

Green chemistry approach for the synthesis of gold nanoconjugates that induces the inhibition of cancer cells proliferation through induction of oxidative stress and their *in vivo* toxicity study

Sudip Mukherjee[a,#], Mamatha Dasari[a], Sumahitha Priyamvada[a], Rajesh Kotcherlakota[a], Vishnu Sravan Bollu[a], Chitta Ranjan Patra*[a,#]

[a]Biomaterials Group, CSIR-Indian Institute of Chemical Technology, Uppal Road, Tarnaka, Hyderabad - 500007, Telangana State, India
[#]Academy of Scientific and Innovative Research (AcSIR), 2 Rafi Marg, New Delhi, India

***To whom correspondence should be addressed**
Chitta Ranjan Patra, Ph.D
[a]Biomaterials Group, CSIR-Indian Institute of Chemical Technology,
Uppal Road, Tarnaka, Hyderabad - 500007, India
Tel: +91-9666204040 (Mobile), +91-40-27191480 (O)
Fax: +91-40-27160387
E-mail: crpatra@iict.res.in; patra.chitta@gmail.com

Running title: Anticancer activity of biosynthesized gold nanoconjugates

Key words: Green synthesis, Gold nanoconjugates, *Lantana montevidensis*, Anti-cancer activity, Reactive oxygen species (ROS), Oxidative stress

1. EXPERIMENTAL PROCEDURES

1.1. Cell viability test using MTT reagent

The MTT assay (3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide), has been used to determine the cell viability in presence of any cytotoxic potential medicinal agents/nanomaterials. Viability of CHO, A549, MCF7 B16 cells were tested by cell viability assay using MTT reagents by incubation of cells with (i) LM, (ii) b-Au-LM-1, (iii) c-Au-PEG, in a dose dependent manner for 48 h. 100 µL of this MTT solution (0.5 mg/mL) was added to each well by replacing the media and allowed to incubate for 4 h. Finally, the MTT-media was replaced by 100 µL of DMSO-Methanol mixture (1:1 volume ratio) and the absorbance of the mixture was measured at 570 nm using a microplate reader (Varioskan Flash). All the experiments were carried out in triplicate and the results are expressed as normalized viability= $\{1/Abs_{\lambda=570}$ (untreated cells-blank)$\}\times\{Abs_{\lambda=570}$ (treated cells-blank)$\}$.

1.2. Cell cycle assay (FACS)

A549 and MCF-7 cells were cultured in 60 mm dishes and incubated for 24 hrs in DMEM (Dulbecco's modified Eagle's medium) at 37°c 5% CO_2. The next day media was replaced with fresh media, cells were treated with LM-2.5µL, b-Au-LM-2.5µL, c-Au-PEG-2.5µL kept in incubator for 48 hours. Cells were washed with 2mL of DPBS for 2 times, followed by trypsinization with Trypsin-EDTA (1ml) for 2-3 mins. The cells were collected the by washing with DPBS (2ml) and DMEM (500µL) media in 15 ml centrifuge tubes. Centrifuge the cell suspension at 2000 rpm for 2 min and decant the supernatant. The cell pellet was washed with DPBS for 2 times. To the cell pellet 500 µl of 70 % Ethanol was added and kept in -20^0c for 1 day. The cell mixture was resuspended and centrifuge at 4 ^{0}C, 12,000 rpm for 2mins. Decant the supernatant, 2-3 ml of DPBS was added and resuspended the cell pellet and centrifuge at 4^0c,

1000 rpm for 2 mins for 2 times. 500 µL of staining mixture [from 6.5 ml of PI + 325 µl triton-X + 32.5 µl of RNase] was added to each centrifuge tubes and incubate at 37 ^{0}C for 30 mins. Samples were analyzed using FACS.

1.3. Oxidative Stress: In vitro GSH Oxidation: Ellman's Assay

The concentration of thiols in GSH was quantified by the Ellman's assay. b-Au-LM-1 treated cell lysates (225 µL) in different concentrations in 50 mM bicarbonate buffer (pH 8.6) were added into 225 µL of GSH (0.8 mM in the bicarbonate buffer) to initiate oxidation. GSH solution without b-Au-LM-1 was used as a negative control and GSH (0.4 mM) oxidization by H_2O_2 (1mM) was used as a positive control. The b-Au-LM-1 treated cell mixtures were transferred into a 24-well plate covered with alumina foil to prevent illumination, placed in a shaker with a speed of 150 rpm at room temperature for 2 h. After incubation, 785 µL of 0.05 M Tris-HCl and 15 µL of DTNB (Ellman's reagent, 5, 50-dithio-bis-(2-nitrobenzoic acid), Sigma-Aldrich) were added into the mixtures to yield a yellow precipitate. The solutions have been filtered and 250 µL of filtered solutions (0.22 µM PVDF, Merck Millipore) from each sample was then placed in a 96-well plate and absorbance was measured by using a microplate reader (Varioskan Flash) at 570 nm. The loss of GSH was calculated by the following formula: loss of GSH % = (absorbance of negative control - absorbance of sample)/absorbance of negative control×100.

1.4. Cellular uptake of gold nanoparticles by ICP-OES

A549 cells treated with b-Au-LM-1 (1 ppm) and c-Au-PEG (1 ppm) were washed after 4 hour of incubation by DPBS (3-4 times). Finally, cells were trypsinized and to the cell pellet 500 µL of concentrated nitric acid was added and kept for 15 minutes in 40 ºC. Finally the digested solutions were diluted in Milli-Q water and the concentration of gold was measured by plasma optical emission spectrometer (ICP-OES, IRIS intrepid II XDL, Thermo Jarrel Ash).

2. CHARACTERIZATION TECHNIQUES

The b-AuNPs-LM was thoroughly characterized by various physico-chemical methods:

2.1. UV-VIS spectroscopy

The absorption of various bio-synthesized b-AuNPs (synthesized by different volumes of LM extract) was observed by UV–vis spectroscopy (JASCO dual-beam spectrophotometer (*Model V-570*)) by using a quartz cuvette from 800 to 200 nm with a resolution of 1 nm.

2.2. X-ray diffraction (XRD)

Initially, 50 mL of as-synthesized b-Au-LM-1 were centrifuged for 40 minutes at 14,000 rpm at 15 ºC using Thermo scientific, Sorvall-WX ultra 100 centrifuge. A glass slide was coated with b-Au-LM-1 pellet and submitted for (X-ray diffraction) XRD analysis. The structure and phase purity of b-Au-LM-1 was determined by XRD analysis by using a Bruker AXS D8 Advance Powder X-ray diffractometer (using CuKαλ=1.5406 Å radiation).

2.3. Transmission electron microscopy (TEM)

The size, shape and morphology of b-Au-LM-1 were monitored on a FEI Tecnai F12 (Philips Electron Optics, Holland) instrument operated at 100 kV with the selected area electron diffraction (SAED) patterns. Sample i.e. b-Au-LM-1 for TEM analysis were prepared by placing a drop of the diluted loose pellet of b-Au-LM-1 on carbon-coated copper grid and allowed the grid to evaporate the solvent.

2.4. Inductively coupled plasma optical emission spectrometry (ICP-OES)

To determine the gold concentrations in b-Au-LM-1 and c-Au-PEG an inductively coupled plasma optical emission spectrometer (ICP-OES, IRIS intrepid II XDL, Thermo Jarrel Ash) was

used. A series of standard $HAuCl_4$ solution (concentration range of 5-100 ppm) was kept as gold standard for the preparation of standard curve.

2.5. Fourier transformed infrared spectroscopy (FTIR)

Initially, 50 mL of as-synthesized b-Au-LM-1 were centrifuged for 40 minutes at 14,000 rpm at 15 ºC using Thermo scientific, Sorvall-WX ultra 100 centrifuge. A glass slide was coated with this loose b-Au-LM-1 pellet and submitted for (Fourier transformed infrared spectroscopy) FTIR analysis. The FTIR spectra were recorded by thermo Nicolet Nexus 670 spectrometer in the diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets.

2.6. Dynamic light scattering (DLS)

The hydrodynamic radius and zeta potential of b-Au-LM-1 was measured by a Zetasizer Ver. 6.20 (Malvern- MAL1004428). All the samples are diluted (1:10) by water and then injected for the measurement of hydrodynamic size and surface charge.

2.7. Gel electrophoresis (SDS PAGE)

In order to find out the specific proteins present in the *Lantana montevidensis* (LM) leaf 12% SDS-PAGE was carried out. The concentrations of proteins had been measured by Bradford assay and used as samples. LM extract, supernatant of b-Au-LM-1, LM extract, supernatant of b-Au-LM-1 were mixed with one third of 4x laemmli sample loading buffer with 2-mercaptoethanol (Sigma) and were heated at 100 ºC for 5 mins before loading and same amount of proteins (60-70 μg) has been loaded in 12% SDS-PAGE. A standard protein molecular weight marker (BIORAD) was loaded in separate well. Finally, the gel was stained with silver nitrate according to standard protocol and scanned with a HP scanner.

2.8. Thin layer chromatography:

The confirmation of presence of several compounds in LM extract and the absence of those

compounds in the b-Au-LM-1 supernatant was performed using thin later chromatography (TLC) on silica coated glass plates, Millipore in 40% (EtOAC: Hexane) mobile phase and images are taken under UV exposure.

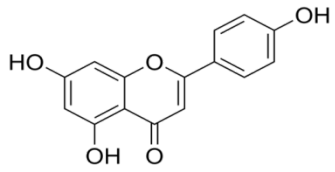
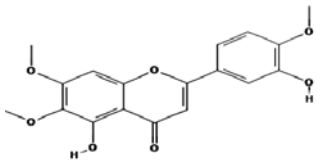
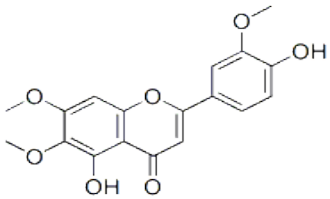
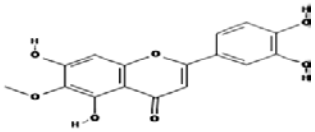
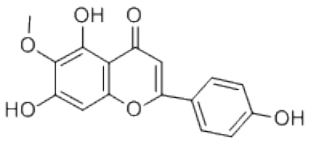
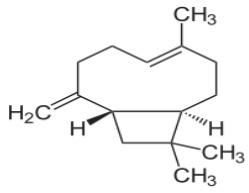
2.9. Mass spectrometry analysis:

The mass spectral data of LM extract, b-Au-LM-1 and b-Au-LM-1 supernatant obtained by the centrifugation of b-Au-LM-1 NPs (synthesized by interaction of $HAuCl_4$ and LM extract) was determined using a LCQ ion trap mass spectrometer (ThermoFinnigan, SanJose, CA, USA) which is equipped with an ESI source or micromass Quatro LC triple quadrapole mass spectrometer for ESI analysis.

Table S1: Stability studies of b-Au-LM-1 by DLS (hydrodynamic diameter) in different buffers

Sample Name	Hydrodynamic diameter (nm) Day 0	Hydrodynamic diameter (nm) Day 7	Hydrodynamic diameter (nm) Day 14
b-Au-LM-1	70.1	71.4	72.2
DPBS	71.6	72.2	72.4
FBS	86.5	87.9	88
pH=5	75.2	76.1	76.5
pH=7	70.5	74.1	74.7
pH=9	84.2	86.7	88.1
10 % NaCl	80.1	81.4	82.1

Table S2: Probable anticancer compounds present in the *Lantana montevidensis* (LM) leaf

Structure	Name	Molecular weight	Activity	Ref
	Apigenin	270.24	Autophagia (a kind of cellular dormancy)	1-2
	Eupatorine	344.3	Anti-Proliferative	3-5
	Cirsinileol	330.28	Anti-proliferative (induces apoptosis in Caov-cells)	5
	Eupafolin	316.26	Apoptotic by DNA Fragmentation	6
	Hispidulin	300.27	Anti-inflammatory & Anti-oxidant	7
	β-Caryophyllene	204.35	Anti-oxidant	

REFERENCES

1. R. R. Ruela-de-Sousa, G. M. Fuhler, N. Blom, C. V. Ferreira, H. Aoyama, M. P. Peppelenbosch*, Cell Death Dis*., 2010; **1**: e19.
2. M. Zhao, J. Ma, H. Y. Zhu, X. H. Zhang, Z. Y. Du, Y. J. Xu, X. D. Yu*, Mol. Cancer,* 2011, **10**, 104.
3. I. Dolečková, L. Rárová, J. Grúz, M. Vondrusová, M. Strnad, V. Kryštof. *Fitoterapia*, 2012, **83**, 1000-1007.
4. V. Androutsopoulos, R. R. Arroo, J. F Hall, S. Surichan and G. A Potter, *Breast Cancer Res*., 2008, **10**, R39.
5. V. P. Androutsopoulos, N. Li, R. R. Arroo. *J. Nat. Prod.*, 2009, **72**, 1390-1394.
6. K. Liu, C. Park, H. Chen, J, Hwang, N. R. Thimmegowda, E. Y. Bae, K. W. Lee, H. G. Kim, H. Liu, N. K. Soung, C. Peng, J. H. Jang, K. E. Kim, J. S. Ahn, A. M. Bode, Z. Dong, B. Y. Kim, Z. Dong, *Mol Carcinog.* 2014, DOI: 10.1002/mc.22139.
7. L. He, Y. Wu, L. Lin, J. Wang, Y. Wu, Y. Chen, Z. Yi, M. Liu, X. Pang. *Cancer Sci.*, 2011, **102**, 219-225.