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Supporting Information

Bioconjugated gold nanoparticles accelerate the growth of new blood vessels through redox signaling

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Running title: Pro-angiogenic nature of gold nanoconjugates

2. EXPERIMENTAL SECTION:

2.1. Materials:

Tetrachloroauric acid ($HAuCl_4$), propidium iodide, dihydroethidium (DHE), 2′,7′-dichlorofluorescin diacetate (DCFDA), polyethylene glycol, sodium borohydride, NBT/BCIP and apigenin were purchased from Sigma-Aldrich, St. Louis MO, USA. MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was received from Calbiochem. Anti-Akt and anti-phospho Akt (tyr460) antibodies were obtained from Millipore whereas goat anti-rabbit and anti-mouse IgG-ALP secondary antibodies were purchased from Genei. Anti-NF-kappa B (p65) and Anti-α/β tubulin primary antibodies were procured from Cell Signaling Technology. All the chemicals purchased were used without any further purification. The leaves of *Hamelia patens* plant were collected from the Horticulture Department of CSIR-IICT, Hyderabad.

Hamelia patens (HP):

Hameliapatens (HP) is a medicinal shrub usually distributed in tropical areas of the American continent (Table-S1). It is commonly known as the firebush, which belongs to the order gentianales and family rubiaceae of the plant kingdom. HP consists of a new mono terpenoids oxindole alkaloid, named (-)-hameline along with other eight known alkaloids such as tetrahydroalstonine, pteropodine, aricine, uncarine F, isopteropodine, palmirine, rumberine and speciophylline.[1] It has been used for treatment of wound healing, menstrual disorders etc. It has also been used as a folk medicine since ancient times for skin eruptions and dysentery. Constituents of HP extract helps to relax the high KCl-induced contraction in rat myometriumin a concentration-dependent manner.[2]

Cell culture reagents:

The human umbilical vein endothelial cells (HUVEC) and corresponding endothelial growth media supplemented with various growth factors and antibiotics were purchased from Lonza. ECV-304 (eNOS-GFP transfected) cells were a kind gift from Dr. V. Shah, Chair, Gastroenterology and Hepatology Department, Mayo Clinic, Rochester, MN, USA.[3] The murine melanoma B16 F10 and ovarian carcinoma SKOV3 cell lines were obtained from ATCC, USA. The Dulbecco's modified eagle`s medium (DMEM) and the supporting antibiotics penicillin, streptomycin, kanamycin and fetal bovine serum were purchased from Sigma-Aldrich.

2.2. Preparation of aqueous leaf extract of Hamelia patens:

The fresh leaves of the plant HP were weighed (35 g) and washed thoroughly with autoclaved Millipore water, sterilized by soaking in diluted ethanol and washed repeatedly with water again. The leaves were triturated in a mortar using a pestle to make a fine paste of the leaves. The mixture was then boiled in a domestic microwave oven (Samsung 800 W, DE68-03844L) followed by continuous stirring on a magnetic stirrer overnight. The extract was subsequently centrifuged at 5000 rpm to obtain the pale yellow colored supernatant. This supernatant was further used for the synthesis of gold nanoparticles (b-Au-HP).

2.3. Chemical synthesis of gold nanoconjugates:

The chemical synthesis of colloidal gold nanconjugates (c-Au-PEG) was carried out by using borohydride method according to our previous reports. [4] Briefly, 2 ml of autoclaved water was taken in a beaker containing 200 µl of 10^{-2}(M) $HAuCl_4$ and 2.8 ml of sodium borohydride (0.05 mg/ml) was added drop-wisely to that reaction mixture. The solution was stirred for 30 min and then 15 µl of poly ethylene glycol

(1%) was added to that colloidal solution with continuous stirring for 4-5 h. The resulting colloidal c-Au-PEG was used for further experiments.

2.4. Biosynthesis of gold nanoparticles (b-Au-HP):

In typical synthesis, 10^{-2}(M) $HAuCl_4$ was used as a precursor for synthesis of AuNPs while HP leaf extract was used as a reducing and stabilizing agent with water as a solvent. A series of reactions was carried out using different volumes of HP extract (200-1000 µl) to reduce $HAuCl_4$ [10^{-2}(M) 200 µl] in water medium keeping the volume of the reaction mixture fixed at 5 ml as shown in Table-S2. All the as-synthesized AuNPs were characterized by UV-visible spectroscopy. Among the different sets of AuNPs, b-Au-HP 800 (synthesized by using 800 µl -HP extract) was found to be more stable compared to other samples. Therefore, b-Au-HP 800 was further characterized by several physico-chemical techniques and analyzed by various *in vitro* and *in vivo* biological assays.

2.5. Cell culture:

The HUVECs were grown in endothelial basal media (EBM) supplemented with 5% FBS and were serum starved in EBM-starving media with 0.2% FBS for overnight before incubating with different treatments. The ECV-304, B16 and SKOV3 cells were cultured in DMEM containing 10% FBS. All the cell lines were maintained in a humidified 37^0C incubator with 5% CO_2.

2.6. MTT assay:

Briefly, the cells (HUVEC, ECV-304, B16 and SKOV3) were seeded in 96 well cell culture plates at a density of around 10^4 cells per well for 24 h. Next day, the cells were incubated with HP, b-Au-HP, c-Au-PEG (5-20 µg/ml) and VEGF (40 ng/ml) for 24 h. Then the media in each well of the plate was replaced with 0.5 mg/ml MTT solution and incubated for 4 h. Finally the MTT solution was replaced with DMSO:

Methanol (1:1; v/v) to dissolve the purple formazan products and absorbance of each sample was measured at 570 nm using a BioTek multimode plate reader.

2.7. Egg yolk angiogenesis assay:

The fertilized eggs of brown leghorn were obtained from the local government poultry farm and incubated at 37 ^{0}C and 60% RH for 4 days. After that, those eggs of fourth day incubation were broken in petri dishes. The treatments (HP: 5-10 µg/ml, b-Au-HP: 5-10 µg/ml, c-Au-PEG: 5-10 µl and VEGF: 40 ng/ml) were soaked with filter paper discs and placed on the exposed blood vessels of the egg yolk for 4 h. The formation of new blood vessels was imaged by using a stereo microscope at regular time-points up to 4 h to observe the effect of the treatments on the growth of new blood vessels. The results were further quantified using the AngioQuant software.[5]

2.8. Wound healing (scratch) assay:

The HUVECs were seeded in a 24 well cell culture plate and grown to confluence. A wound was created in the middle of each well by scratching the cells using a sterile autoclaved tip. Then the cells were washed with DPBS and incubated with the treatments (HP: 5-10 µg/ml, b-Au-HP: 5-10 µg/ml, c-Au-PEG: 5-10 µl and VEGF: 40 ng/ml) up to 8 h and images of the wound area were captured using a Nikon microscope at regular time intervals to observe the closure of the wound due to the migration of the cells. The results were quantified using the Image J software and the histogram was plotted.[5]

2.9. Estimation of reactive oxygen species (ROS):

HUVECs and ECV-304 cells were seeded in 24 well plates at 40–50% confluency. The cells were incubated with treatments (HP: 5-20 µg/ml, b-Au-HP: 5-20 µg/ml, c-Au-PEG: 5-20 µl and VEGF: 40 ng/ml) for 24 h. The cells were thoroughly washed with DPBS followed by incubation with 10 µM DCFDA (HUVECs) or 10 µM DHE

(HUVECs and ECV-304 cells) for 30 min. The cells were again washed with DPBS for several times. The fluorescence images of both treated and untreated cells were taken in HBSS using Nikon fluorescence microscope.[6]

2.10. Cell cycle analysis:

ECV-304 cells were seeded in 6 well cell culture plate and grown to confluence. The cells were incubated with different treatments (b-Au-HP: 5-20 µg/ml, c-Au-PEG: 5-20 µl and VEGF: 40 ng/ml) for 24 h. The cells were then trypsinized, washed with DPBS and fixed in 70% ethanol overnight. Next day, the cells were again washed with DPBS, stained using propidium iodide and further processed for cell cycle analysis using BD Canto fluorescence activated cell sorting.

2.11. Immunoblot analysis (western blot):

The HUVECs were incubated with HP (20 µg/ml), b-Au-HP (20 µg/ml) and VEGF (40 ng/ml) for 30 min and processed for immunoblot analysis to determine the expression of NF-kappa B and phosphorylation status of Akt. Cell lysates were prepared using RIPA buffer and Bradford assay was carried out as per our previous reports. Briefly, the cells were scrapped using RIPA mixed with protease inhibitor cocktail (1%) and centrifuged at 10000 rpm for 15 min to obtain the supernatant cell lysates. The protein concentration of cell lysates was measured by Bradford assay. The samples were loaded equally (50 µg), separated on a SDS-PAGE and then transferred to a nitrocellulose membrane (Thermo Scientific). The membrane was then blocked in 5% non-fat dry milk prepared in Tris-buffered saline Tween-20 (TBS-T; 10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20). After blocking, the membrane was washed with TBS-T and incubated with primary antibodies (anti-phospho-Akt and anti-Akt/PKB; Anti-NF kappa B (p65) and Anti-α/β tubulin) for overnight at 4^0C. Next day, the membrane was again washed with TBS-T and

incubated with corresponding goat anti-rabbit IgG-ALP and/or anti-mouse IgG-ALP secondary antibodies for 1 h. Finally, the membrane was washed and the blot was developed using BCIP/NBT solution.

2.CHARACTERIZATION TECHNIQUES

2.1. Characterization methods:

The as-synthesized b-Au-HP using the HP leaf extract was centrifuged at 4000 rpm for 30 min and the pellet was collected and used for further characterizations.

1.1.1 UV-VIS spectroscopy:

The spectroscopic absorption of all as-synthesized b-Au-HP was measured by UV–Vis spectroscopy (JASCO dual-beam spectrophotometer (Model V-570) using quartz cuvette in a range from 800 to 200 nm with a resolution of 1 nm.

1.1.2. X-ray diffraction (XRD):

The crystallinity and phase purity of the as-synthesized b-Au-HP was determined by X-ray diffraction (XRD) analysis using a Bruker AXS D8 Advance Powder X-ray diffractometer (using CuKαλ=1.5406 Å radiation).

1.1.3. Transmission electron microscopy (TEM):

The TEM is done for morphological analysis like size and shape of the b-Au-HP examined on a FEI Tecnai F12 (Philips Electron Optics, Holland) instrument operated at 100 kV. Selected area electron diffraction (SAED) patterns were also taken using this instrument.

1.1.4. Fourier transformed infrared spectroscopy (FTIR):

FTIR spectral analysis helps in the identification of functional groups present in a chemical compound on the basis of their vibrational energy upon IR irradiation. The FTIR spectra of the HP leaf extract and as-synthesized Au NPs was recorded using

thermo Nicolet Nexus 670 spectrometer in the diffuse reflectance mode at a resolution of 4 cm-1 in KBr pellets. Their difference in the FTIR analysis gives information of the possible functional moieties of the extract responsible for the synthesis of gold nanoparticles.

1.1.5. Zeta potential analysis:

The charge of the biosynthesized b-Au-HP was determined using the Zeta potential analyzer (Malvern) by diluting the sample with deionized water.

1.1.6. Thin layer chromatography:

The confirmation of presence of apigenin in HP extract and its absence in b-Au-HP supernatant was performed using thin later chromatography (TLC) on silica coated glass plates, Millipore in 10% (methanol: chloroform) mobile phase and charred in an iodine chamber.

1.1.7. Mass spectrometry analysis:

The mass spectral data of HP extract and supernatant obtained by the centrifugation of b-Au-HP NPs (synthesized by interaction of $HAuCl_4$ and HP 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.

3. DISCUSSION

We have extensively searched the published literature that demonstrates the presence of various phytochemicals such as isopteropodine, rumberine, palmirine, maruquine and alkaloids, apigenin, ephedrine, flavanones, isomaruquine, narirutins, pteropodine, rosmarinic acid, narirutin, seneciophylline, speciophylline, tannin etc., in Hamelia patens (HP) plant extract.[7-11] Some major constituents have been listed in Table-S1

(Supporting Information). Structures of these phytochemicals present in HP extract are already well established by published reports.[7-12]

Again, the literature of HP plant extract shows that apigenin (4',5,7-trihydroxyflavone; Mol.wt. 270) is one of the major constituents of HP extract and plays an important role for wound healing.[7,12] In this context, we have purchased pure apigenin from Sigma-Aldrich, which was used to identify apigenin in HP extract and for *in vitro* angiogenesis assay. Comparing the results of TLC (thin layer chromatography) of HP extract and pure apigenin (Sigma), clearly suggests the presence of apigenin in the HP leaf extract which matches with the pure apigenin (Sigma) spot [Fig.S11 (a)]. However, the spot of apigenin could not be visible in supernatant of b-Au-HP nanoparticles (Fig.S11 (b), obtained by the centrifugation of b-Au-HP NPs that was synthesized by the interaction of $HAuCl_4$ and HP leaf extract. These results indicate the possible role of apigenin along with other phytochemicals in HP extract for the formation, stabilization of b-Au-HP nanoparticles and its conjugation with nanoparticles. The presence of apigenin in HP extract was further supported by mass spectroscopy analysis, which exhibited peaks at $[m+H]^{+\cdot}$ = 353/393, suggesting the possibility of presence of di-potassium apigenin & tri-potassium apigenin in HP leaf extract which diminishes in the supernatant of b-Au-HP nanoparticles, may be due to its involvement in formation, stabilization of b-Au-HP nanoparticles and its bio-conjugation. (Fig.S12).

HP is a medicinal plant and its different parts have been extensively used for the treatment various diseases related to the central nervous system, cardiovascular system, wound healing, etc.[7] However, the pharmacological activities of individual components of HP extract are not fully established. On the other hand, Lopez-Jornet et al has reported that potassium salts of apigenin (AP) and other flavonoids present in

verbena extract show the anti-inflammatory/wound healing properties in mouse model.[12] Considering the wound healing properties of apigenin in verbena extract, we believe that apigenin of HP leaf extract might be responsible for pro-angiogenic properties.[12] Hence, we have performed the endothelial cell proliferation assay (a key step for angiogenesis) using apigenin (purchased from Sigma) in order to check its pro-angiogenic activity and compare the results with HP extract. The MTT assay of HUVEC indicates that free apigenin (AP) and apigenin in Au-AP (incubation of AuNPs with AP for 1h) show the endothelial cell proliferation, pro-angiogenic activity like HP leaf extract and b-Au-HP. The new results are presented in Fig.S13, altogether which indicate that AP, a major component of HP extract might be responsible for stabilization of b-Au-HP and its angiogenic activity. Again, the MTT results clearly demonstrate that HP and b-Au-HP exhibit more pro-angiogenic properties compared to AP and Au-AP (Fig.S13). Hence the results indicate that apigenin along with other phytochemicals is responsible for pro-angiogenic activity of HP extract. Therefore, we prefer to use the entire crude extract of HP for the synthesis, stabilization and pro-angiogenic activity studies because individual component of HP extract can't help to synthesize and stabilize gold nanoparticles and does not exhibit significant pro-angiogenic activity. Thus utilizing the 'green chemistry' principles, it is easy to synthesize pro-angiogenic gold nanoparticles (b-Au-HP) in very short time through economically cheap and eco-friendly method.

The nuclear factor- kappa beta (NF-κB) regulates the cellular immune and inflammatory responses and plays a prominent role in the pathogenesis of cancer. [13-14] Analysis of NF-κB activation in response to various external stimuli (cytokines, drugs, nanomaterials etc.) can establish to validate their ability to induce cellular inflammatory responses.[14] In order to validate the effect of b-Au-HP on NF-κB

activation, we have performed the western blot analysis of HUVEC incubated with biosynthesized nanoparticles (b-Au-HP) (Fig.5.b). The results demonstrate no change of NF-κB expression in HUVEC treated with b-Au-HP compared to untreated HUVEC, suggesting high biocompatibility of b-Au-HP without exhibiting any inflammatory and carcinogenic effects.

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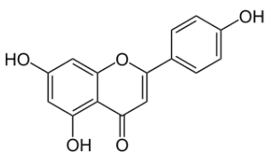
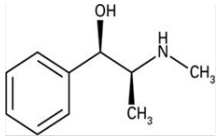
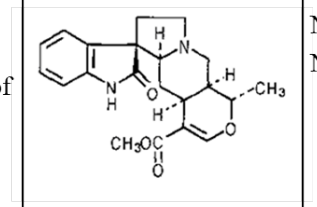
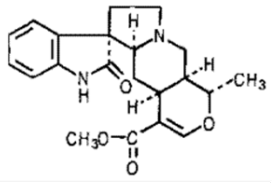
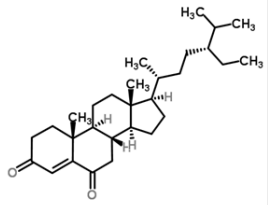
SNo	Chemical Constituent	Pharmacological activity	Structure	Reference
1.	Apigenin	Wound healing		Ahmad.A etal., Pharmacognosy Journal, 2012 June; 29(4): 1-4. Lopez-Jornet et al., Int Wound J. 2012 Nov 9.
2.	Ephedrine	Sympathomimetic Nasal decongestant.		Chaudhuri, P.K. et al., Planta Med. 1991 Apr;57(2):199.
3.	Pteropodine	Anti-Oxidant, Potent inhibitor of TNF-alpha,		Paniagua-Vega, D et al., Nat Prod Commun. 2012 Nov;7(11):1441-4.
4.	Iso-pteropodine	Antimicrobial		Ripperger, H. Pharmazie. 1977 Jul 7;32(7):415-6.
5.	stigmast-4-ene-3,6-dione	Antimicrobial		Ripperger, H., Pharmazie. 1978 Jan;33(1):82-3.

Table No. S1: Description of pharmacological activity and structure of the various known chemical constituents in the plant *Hamelia patens*.

S No.	Sample name	Volume of HP extract (µl)	Volume of $HAuCl_4$ (10^{-2} M) (µl)	Volume of water (ml)	Total reaction volume (ml)
1.	b-Au-HP 200	200	200	4.6	5
2.	b-Au-HP 400	400	200	4.4	5
3.	b-Au-HP 600	600	200	4.2	5
4.	b-Au-HP 800	800	200	4	5
5.	b-Au-HP 1000	1000	200	3.8	5

Table No. S2: Various reaction conditions for the bio-synthesis of gold nanoparticles using constant volume of $HAuCl_4$ and different volumes of HP leaf extract under ambient conditions.

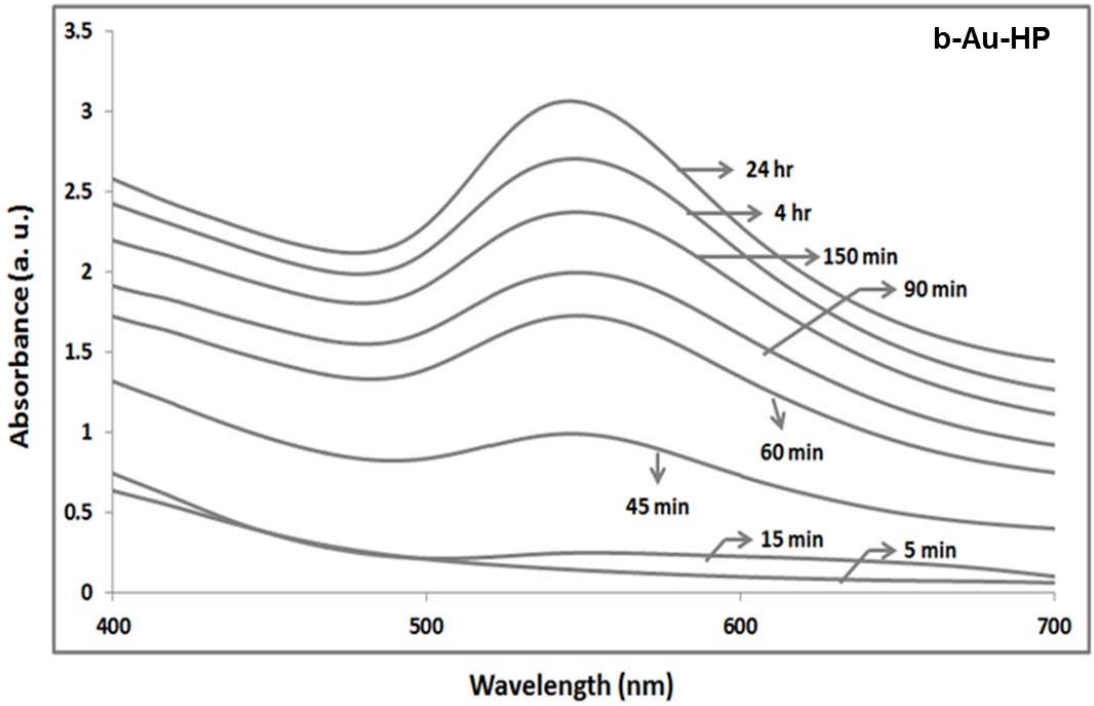


Fig.S1: UV visible spectra of gold nanoparticle synthesized by HP at different time intervals. Change of absorbance of bio-synthesized b-Au-HP NPs with time (5 min to 24 h) indicates the increase of absorption intensity with time suggesting the formation of more b-Au-HP NPs.

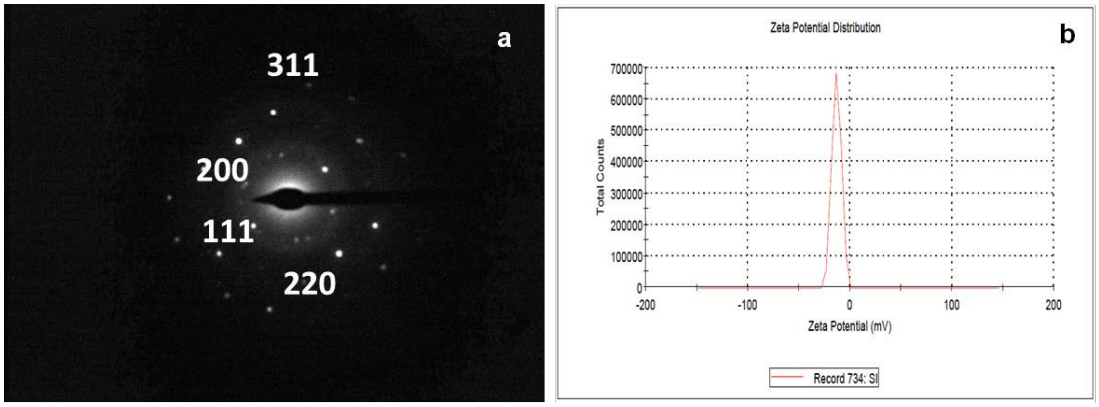


Fig.S2: (a) SAED pattern of the biosynthesized b-Au-HP. **(b)** Charge of the biosynthesized b-Au-HP NPs measured by DLS is found to be -13.3 mV.

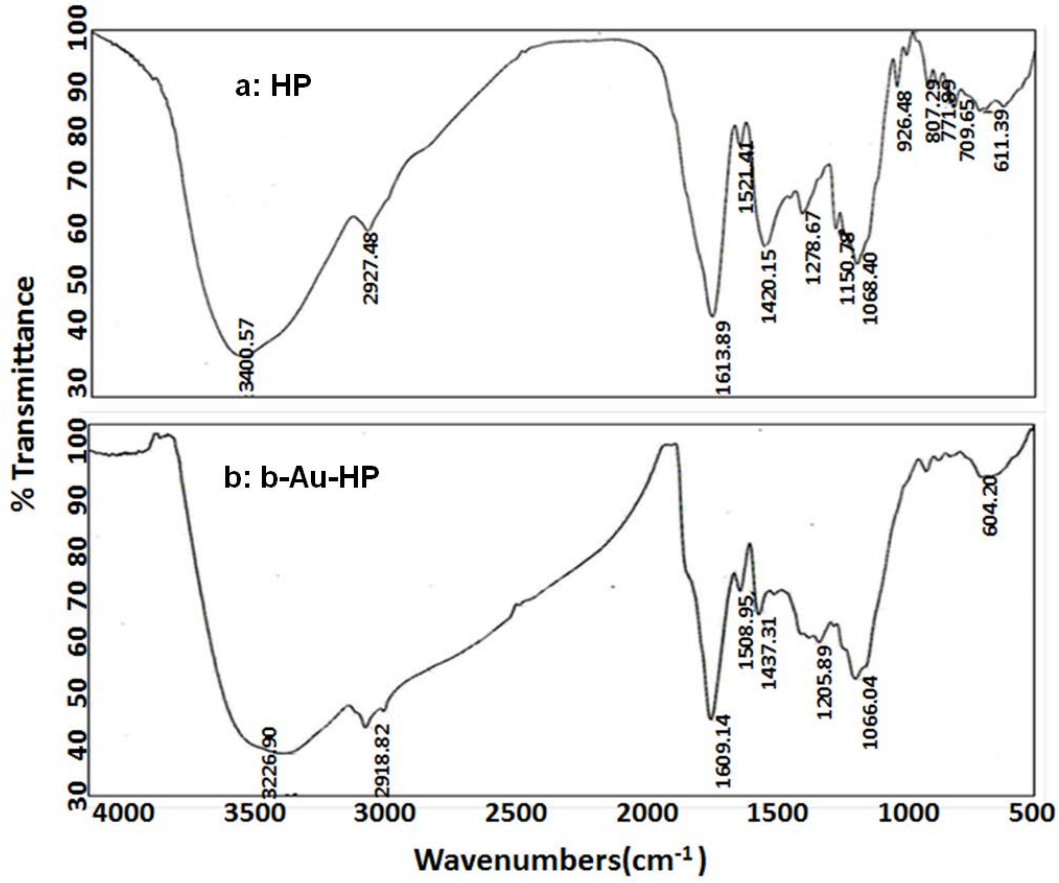


Fig.S3: FT-IR spectra of (a) HP leaf extract & (b) b-Au-HP NPs. The decrease in stretching frequency from 3400.57 cm^{-1} (HP) to 3226.90 cm^{-1} (b-Au-HP) suggests the reduction of $HAuCl_4$ and formation of b-Au-HP.

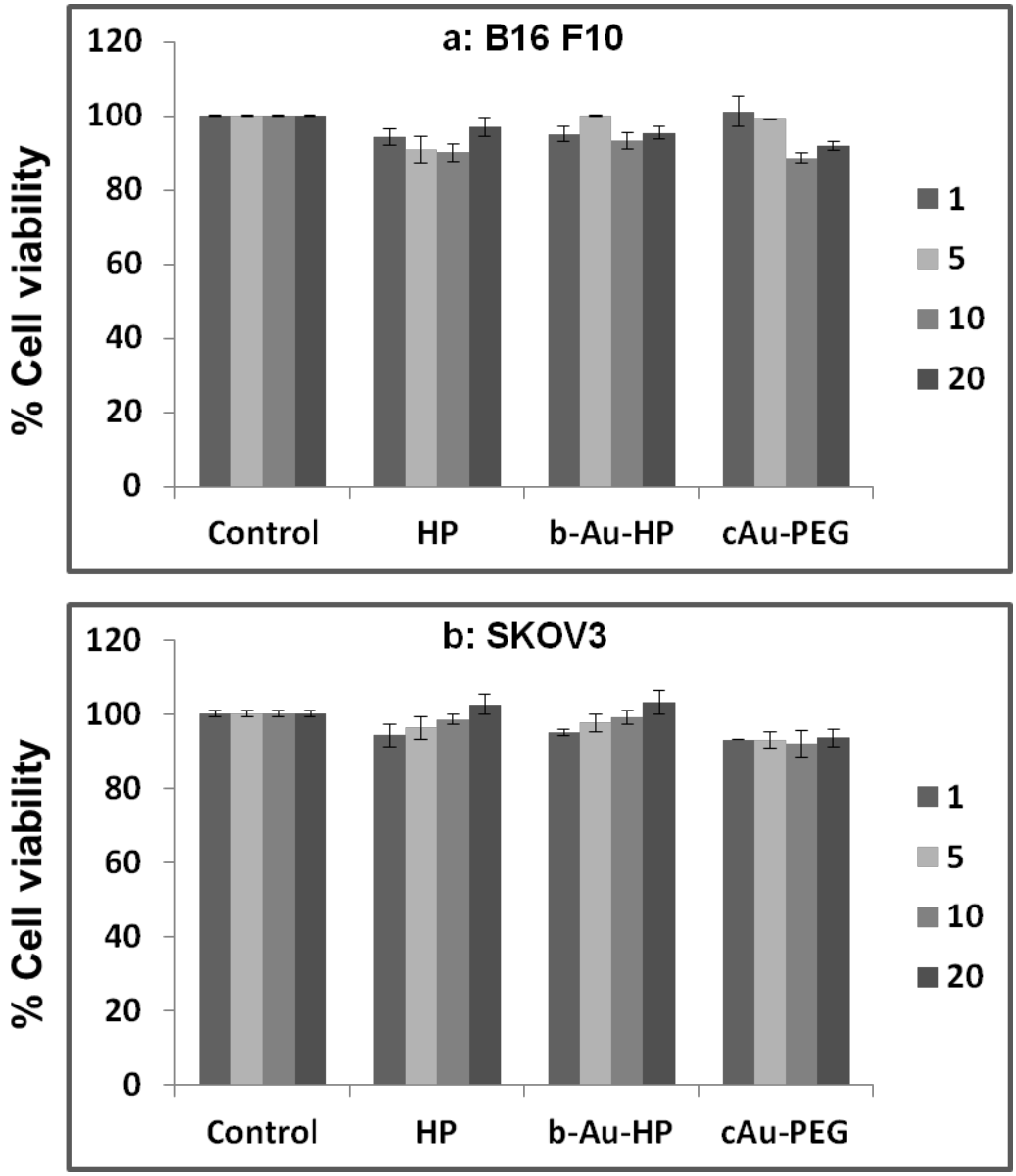


Fig.S4 (a-b): Cell viability in (a) B16 F10 murine melanoma and (b) SKOV3 ovarian carcinoma cells in response to HP, b-Au-HP and c-Au-PEG treatments with varied concentration (1-20 µg/ml) by MTT assay.

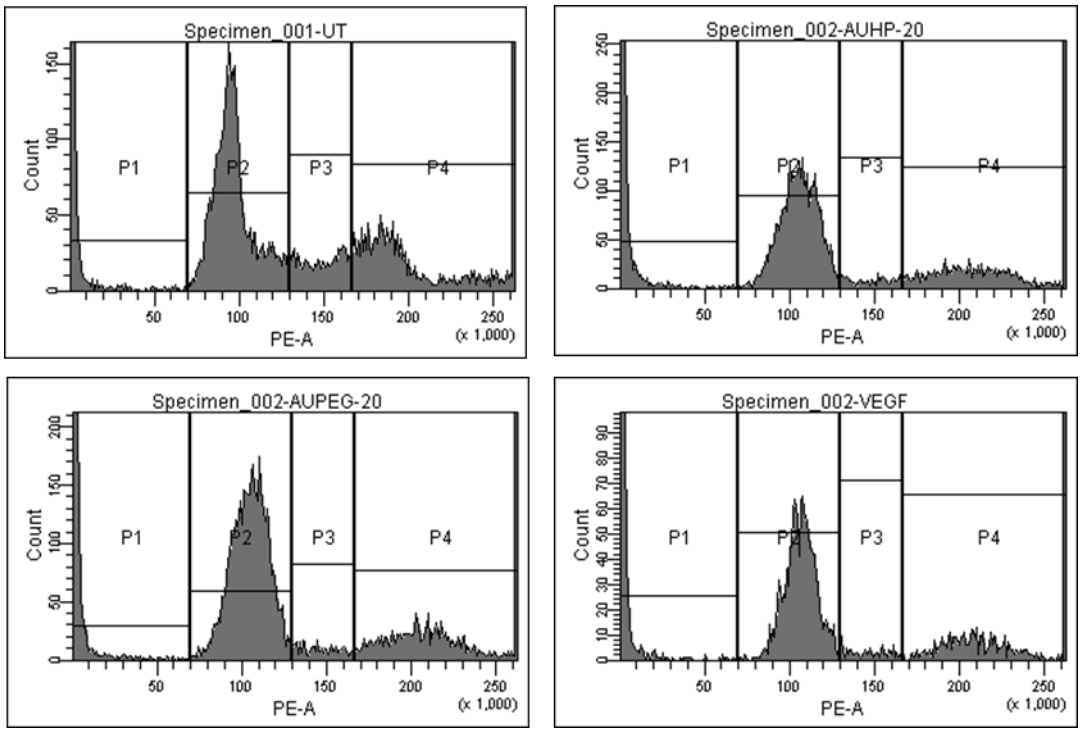


Fig.S5. Cell cycle analysis of ECV- 304 cells in response to treatment with b-Au-HP & c-Au-PEG nanoparticles (20 µg) using flow cytometry.

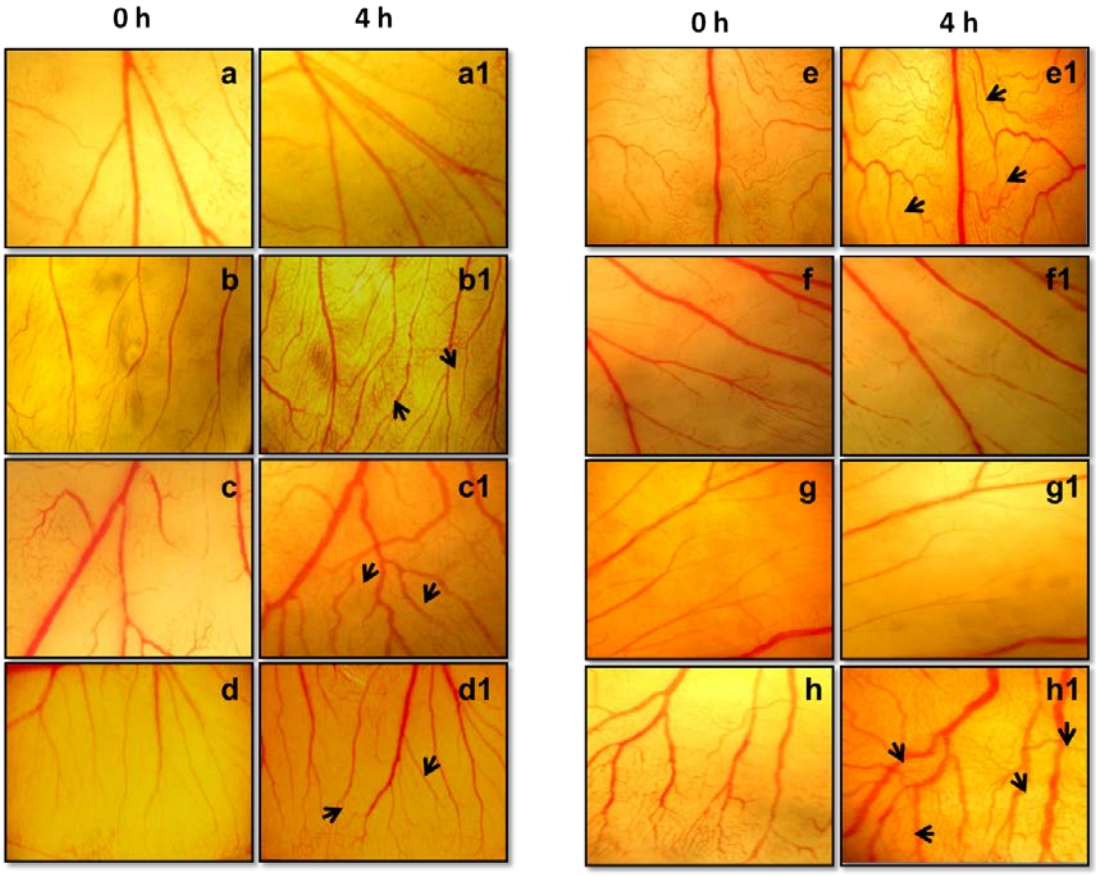


Fig.S6 (a-h): Chick embryogenesis angiogenesis assay (CEA) to access the *in vivo* angiogenesis. (a-a1): Control, (b-b1): HP 5 µg, (c-c1): HP 10 µg, (d-d1): b-Au-HP 5 µg, (e-e1): b-Au-HP 10 µg, (f-f1): c-Au-PEG 5 µl, (g-g1): c-Au-PEG 10 µl & (h-h1): VEGF 10 ng. VEGF is used here as positive control experiment.

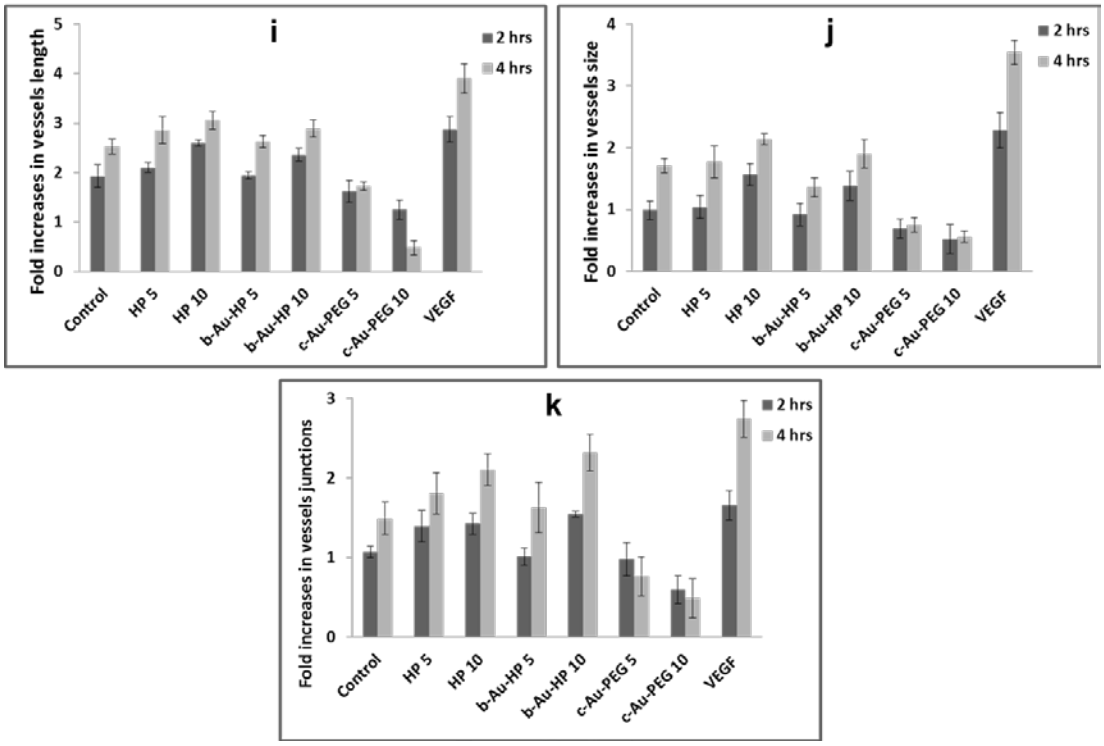


Fig.S6 (i-k): Quantification of the three parameters vessel length (i), size (j) and junctions (k) of the CEA by using AngioQuant software.

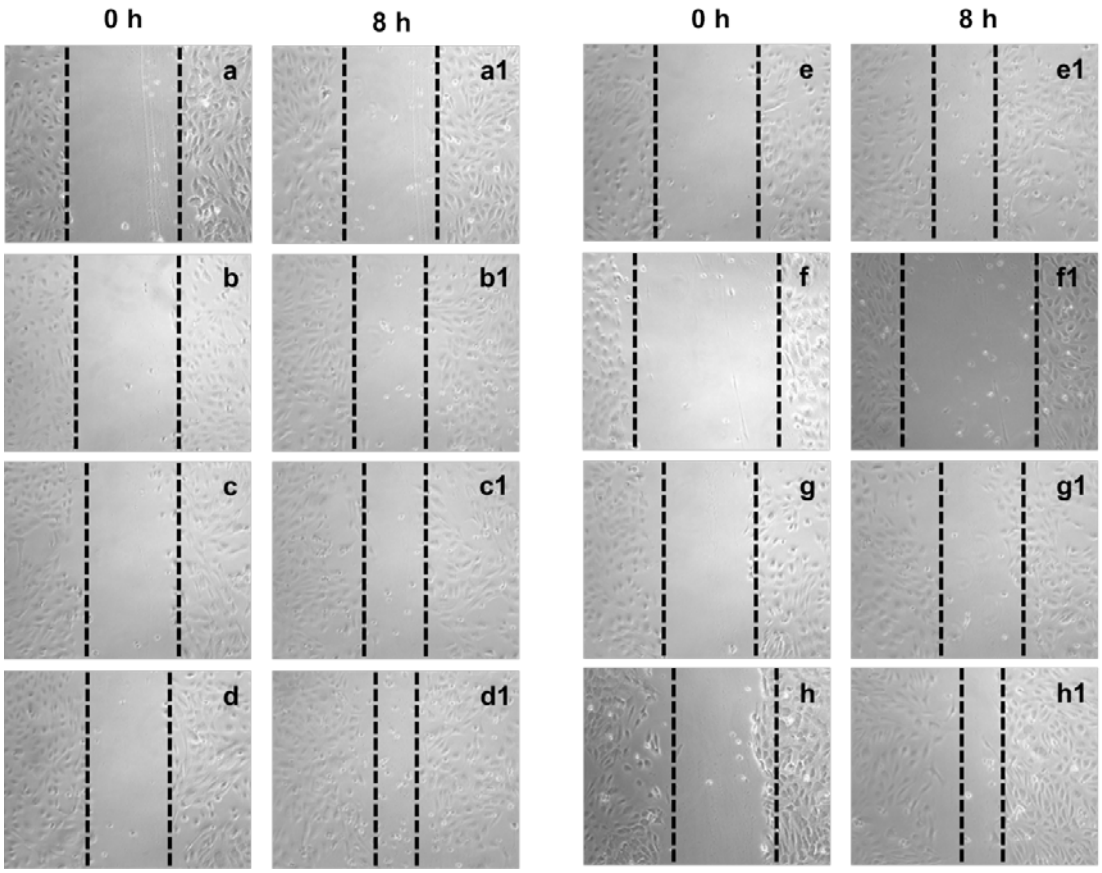


Fig.S7 (a-h): Wound healing assay to investigate HUVECs migration. (a-a1): Control,, (b-b1): HP 5 µg, (c-c1): HP 10 µg, (d-d1): b-Au-HP 5 µg, (e-e1): b-Au-HP 10 µg, (f-f1): c-Au-PEG 5 µL, (g-g1): c-Au-PEG 10 µL & (h-h1): VEGF 10 ng. VEGF is used here as positive control experiment.

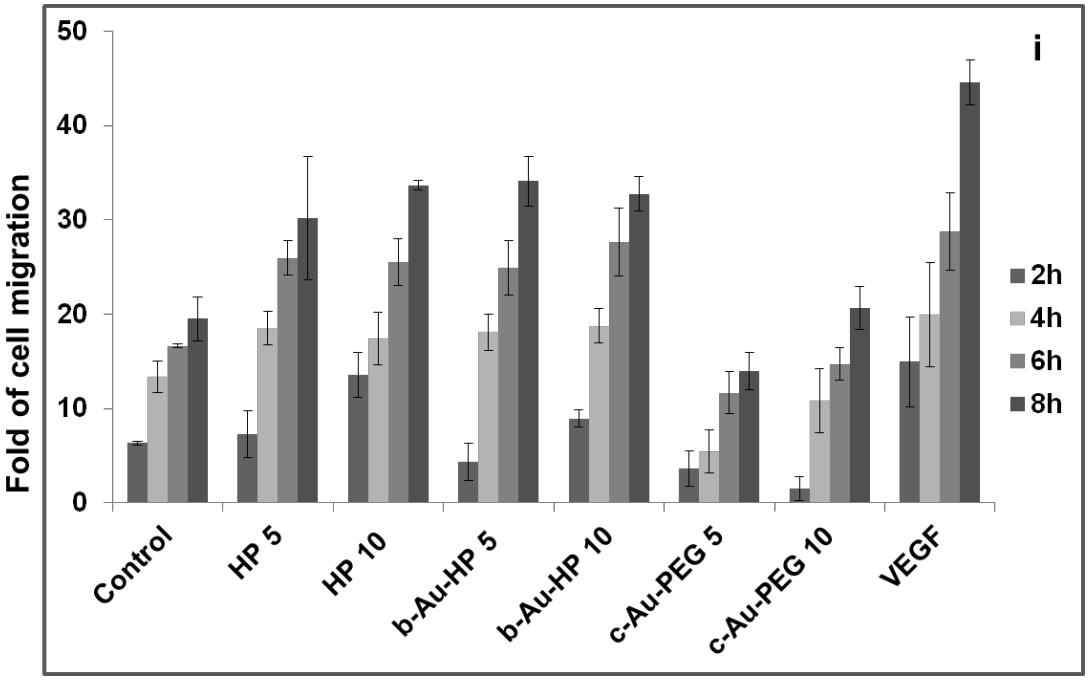


Fig.S7(i): Quantification of wound healing (scratch) assay through histogram.

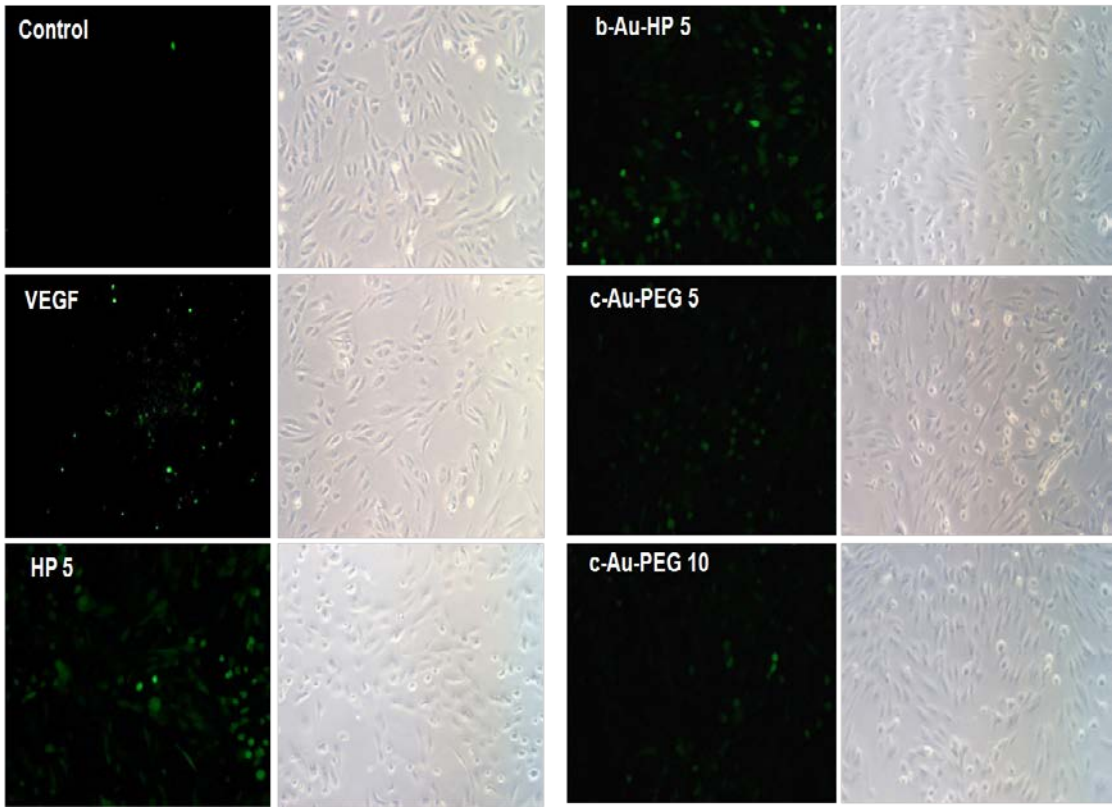


Fig. S8(a): Imaging of hydrogen peroxide (ROS) by DCFDA staining in HUVECs incubated with HP (5 µg/ml), b-Au-HP (5 µg/ml) & c-Au-PEG (5-10 µl) using fluorescence microscopy.

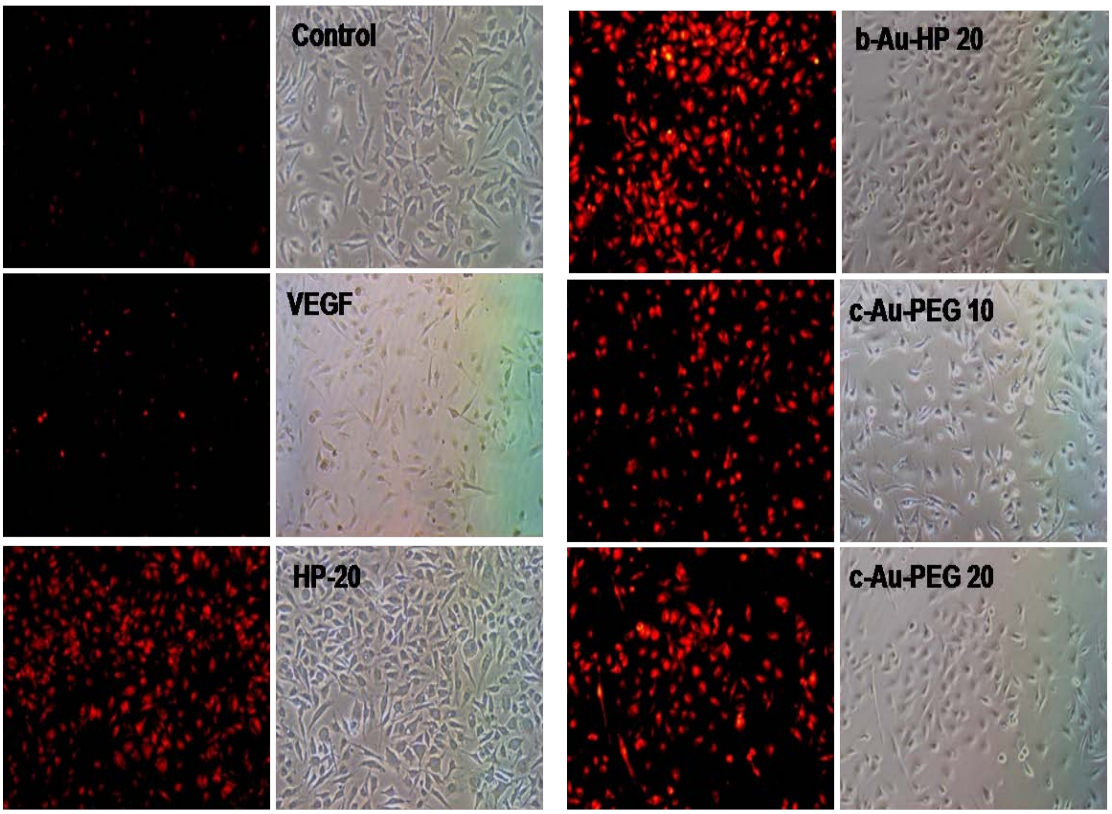


Fig. S8(b): Imaging of superoxide anion (ROS) by DHE staining in ECV-304 cells incubated with HP (20 µg/ml), b-Au-HP (20 µg/ml) & c-Au-PEG (10-20 µl) using fluorescence microscopy.

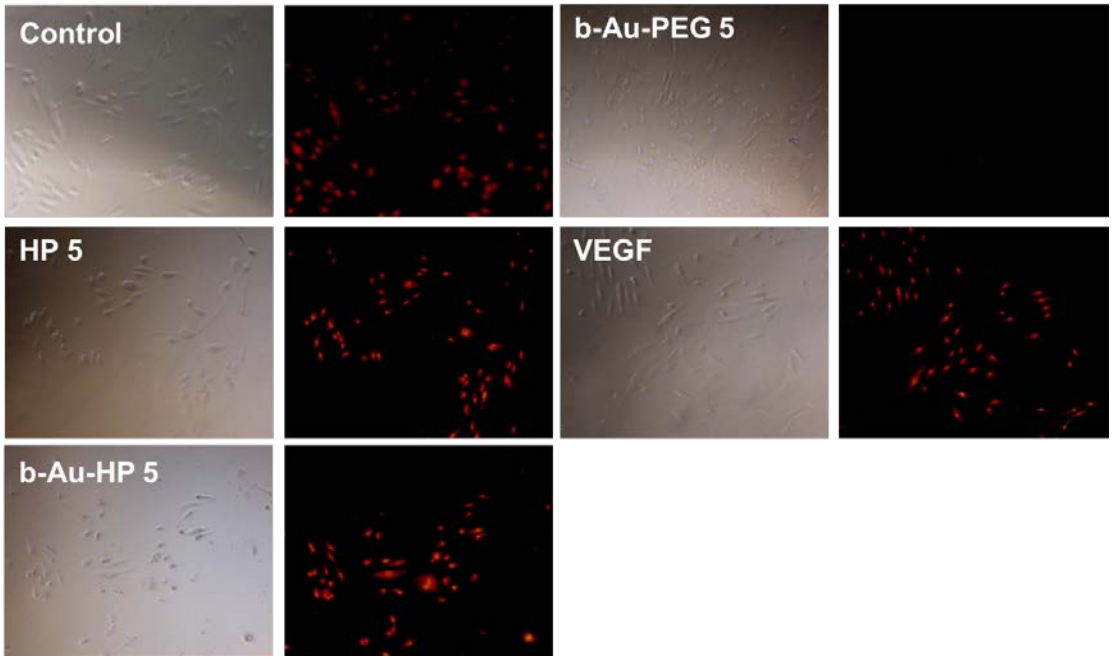


Fig.S9: Investigation of superoxide anion by DHE staining in HUVECs treated with HP (5 µg/ml), b-Au-HP (5 µg/ml) & c-Au-PEG (5 µl) using fluorescence microscopy.

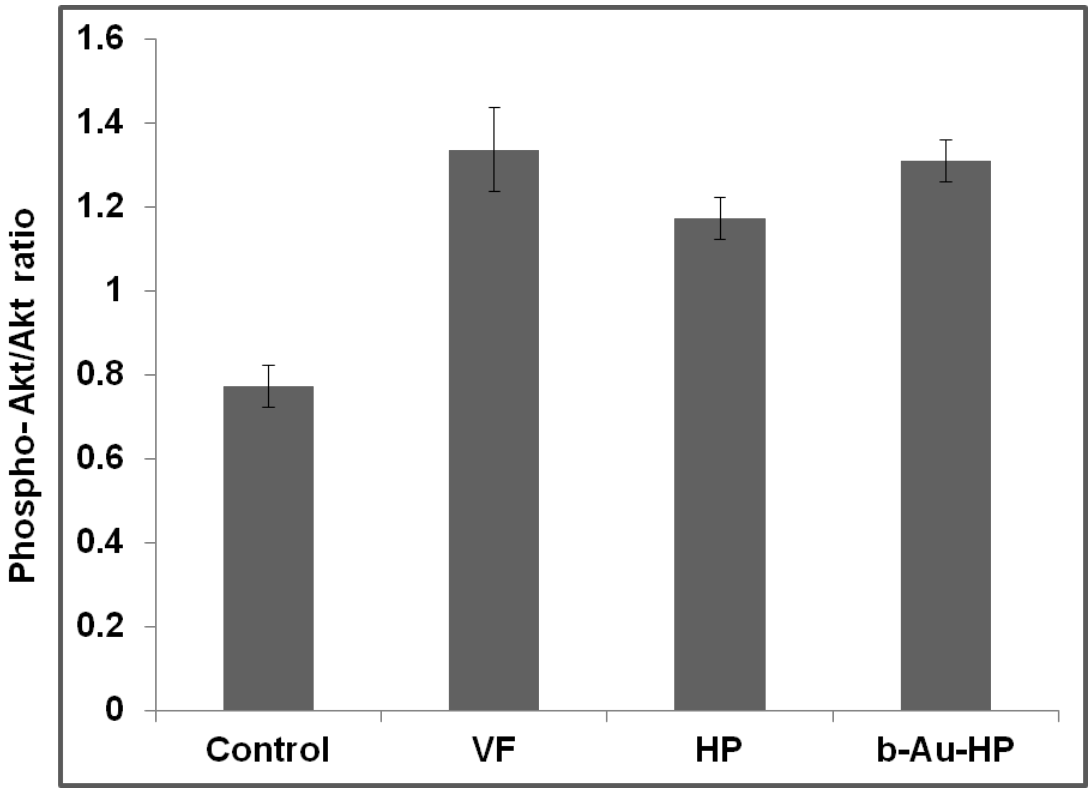


Fig. S10: Densitometric analysis of western blot demonstrating Akt phosphorylation in HUVECs treated with biosynthesized b-Au-HP NPs.

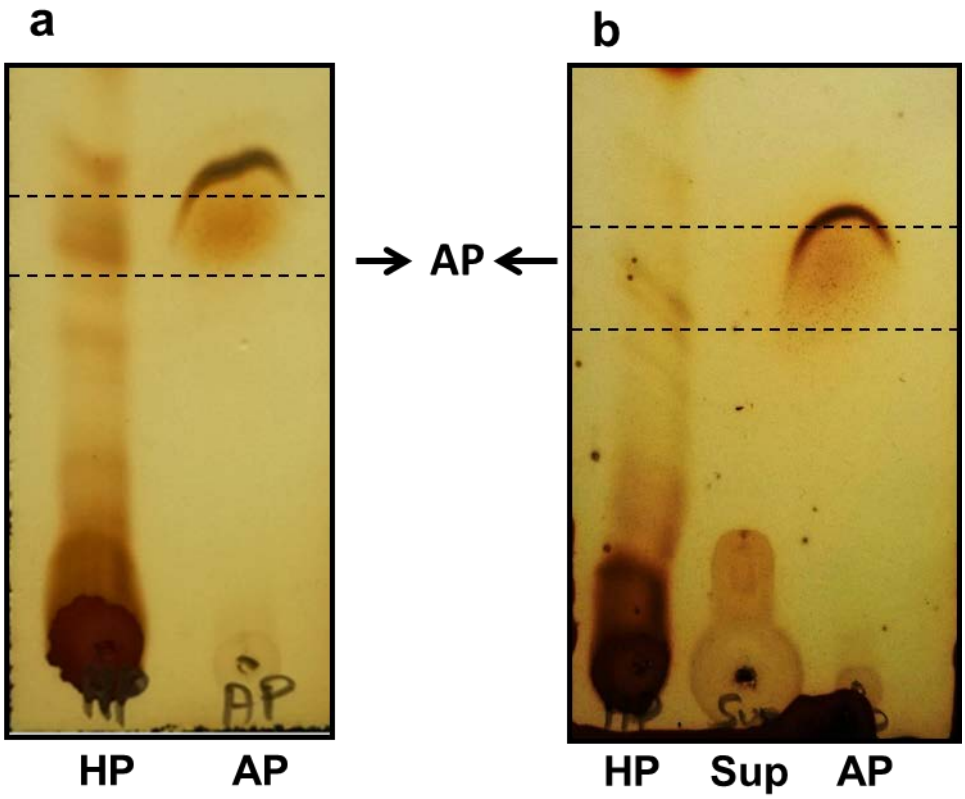


Fig.S11(a-b): Identification of apigenin as the chief molecule responsible for the formation of b-Au-HP NPs using thin layer chromatography (TLC) technique. (HP: HP extract, AP: Apigenin, Sup: b-Au HP NPs centrifuge supernatant).

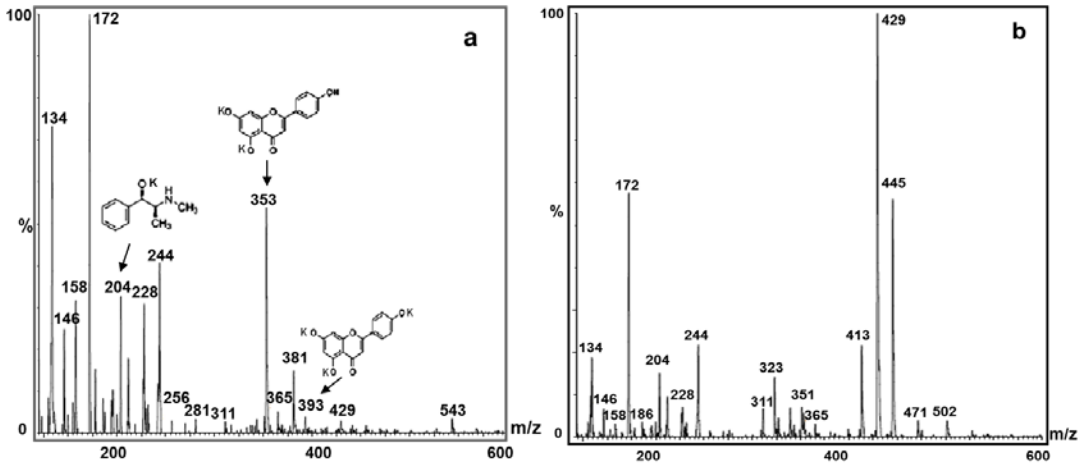


Fig.S12: Identification of the major chemical constituents responsible for the formation of b-Au-HP NPs using mass spectroscopic analysis of (a) HP extract and (b) b-Au-HP NPs centrifuge supernatant respectively.

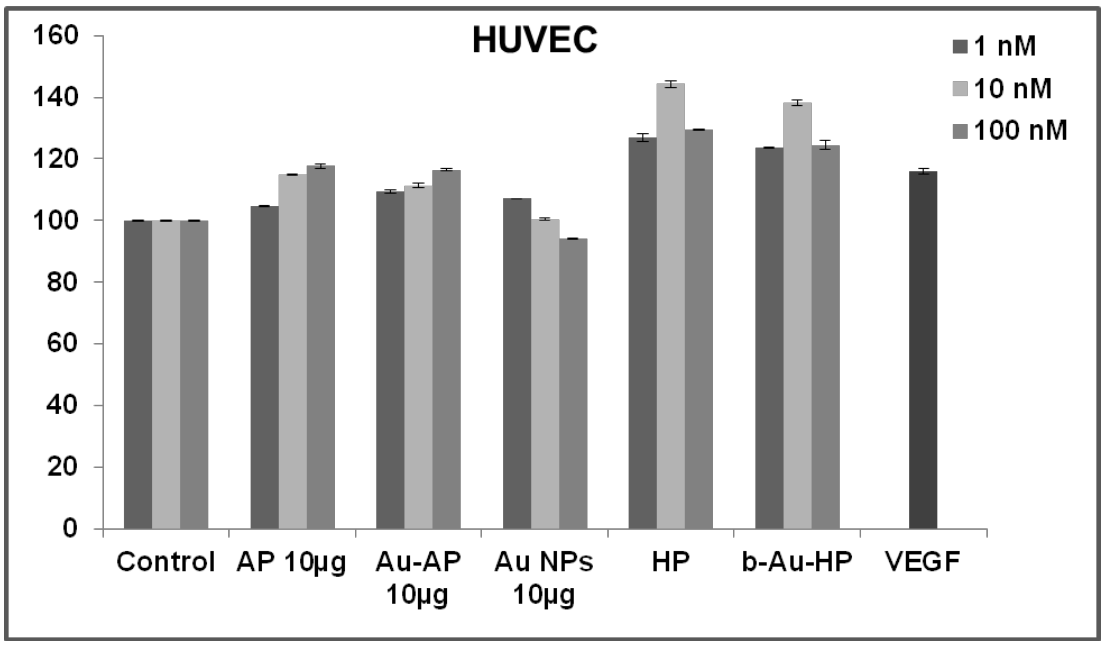


Fig.S13: Estimation of cell proliferation and viability of HUVECs incubated with Apigenin (AP), AP conjugated with Au NPs (Au-AP), Au NPs, HP (5, 10 & 20 µg) and b-Au-HP (5, 10 & 20 µg) respectively. VEGF is used as a positive control experiment.