

Synthesis, Characterization and Anti-Cancer Therapeutic Potential of Withanolide-A with 20nm sAuNPs Conjugates Against SKBR3 Breast Cancer Cell Line

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Background: Nanotechnology is gaining emerging interest in advanced drug discovery therapeutics due to their tremendous properties including enhanced delivery of therapeutic payload, extensive surface to volume ratio, high permeability, retention behaviors, etc. The gold nanoparticles (AuNPs) are favored due to their advanced features, such as biogenic, tunable physiochemical response, ease in synthesis, and wide range of biomedical applications. The phytochemicals have been focused to design Au nano-carrier-based conjugation for active-targeting drug delivery due to their nano conjugation ability.

Aim: The present study describes the facile synthesis of 20nm spherical AuNPs and their conjugation with reported anti-cancer phytochemical Withanolide-A (1).

Methods: The 20nm sAuNPs were synthesized chemically and characterized their phytochemical gold nanoconjugates through UV-visible spectroscopy, dynamic light scattering (DLS) and transmission electron microscopy (TEM) imaging techniques. The anti-cancer therapeutic potentials were tested with both nanoconjugates and pure WithanolideA (1) by using SKBR3 breast cancer cells line.

Results: The synthesized sAuNPs showed significant conjugation with Withanolide-A and showed stability. Furthermore, these Au nanoconjugates with Withanolide-A (1) significantly induce blockage of SKBR3 cell growth at half maximal active concentration that compared to pure Withanolide-A (1).

Conclusion: Our findings provide a foundation to further progress how they can overcome cancer drug resistance by conjugating active drugs in combination with AuNPs through optimizing the effective drug concentration and removing the surface barrier.

Keywords: *Withania somnifera*, Withanolide-A, gold nanoparticle; AuNP, breast cancer, anti-cancer and incucyte

Introduction

Nanotechnology has received a lot of attention in recent years because of its enhanced payload to targeted therapeutics sites, modifying cellular permeability, uptake or pharmacokinetic profile.¹⁻³ Among the diverse range of nanoparticle carriers (iron oxide, silicone material and quantum dots) the AuNPs are favored due to their high biocompatibility, quenching efficiencies, facile synthesis, multiple functionality and tunable optical nature.⁴⁻⁷ Several studies⁸⁻¹¹ reported a mechanism of action of AuNPs as an active carrier of drug payload to targeted

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site. Their interaction modifies the cell and surface protein interaction kinetics profile; however, their action varies with size and charge factor. The previously reported study describes through ultra-high-resolution imaging atomic force revealed that AuNPs carrier have tendency to alter membrane roughness of cell, which enhanced their cellular uptakes.¹²

Breast cancer is frequently diagnosed among women worldwide, with the highest rate in Australian women, diagnosed more than other types of cancer.^{13,14} The accelerated rate of mortalities of around 73% in the metastatic disease is usually linked with various genetic and epigenetic factors.^{13,15,16} The conventional therapies have a number of flaws: poor accessibility to tumor tissues, non-selectivity, severe side-effects – hair loss, diarrhea, high dose, less efficacy, and safety profile concerns – which always demand novel and more effective treatment methods for this type of cancer. In this context, we prepared AuNPs combinational strategy with a reported anticancer compound (Withanolide-A) is being using to overcome disease burden and more effective potential.¹⁷

The phytochemical-based remedies have been reported over thousands of years ago to treat multiple disorders in traditional medicinal system and many of them approved by Food and Drug Administration (FDA) clinically.¹⁸ In previous years *Withania somnifera* extract passed clinical trial phase one (ID number: NCT00817752) against cancer disorders in 2010 and powder extracts passed phase and second clinical trial (ID number: NCT00689195) against osteosarcoma in 2011. The withanolide compounds from the medicinal plant *Withania somnifera* have been extracted and tested *in vitro* and *in vivo* against a number of disorders; including as an anticancer, neuromodulators, antidiabetic, adaptogenic, cardioprotective, and neuroprotective agent, etc.^{19–21}

However, there are numbers of issues raised related to the use of Withanolide-A (1), so previous raised issues should be addressed; either concern with their low solubility or lesser bioavailability (rapid passage to type I, II type metabolism) and improved pharmacokinetics profile with enhanced targeted cellular uptake and delivery, which may modify its unique functions. Figure 1 showed the structural formula of tested compound 1.

The application of nanobiotechnology along with the combination therapies is discussed in a number of studies, where nanoparticles conjugate with phytochemical for enhanced drug delivery mechanism researchers.^{22–25} The current study was designed to synthesize 20nm sAuNPs and their conjugation with the previously reported anticancer

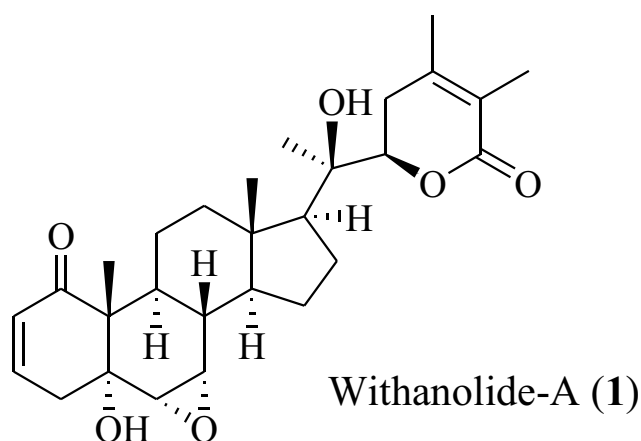


Figure 1 Structural formula of Withanolide-A (1). A phytochemical from *Withania somnifera*.

compound Withanolide-A (1),^{26,27} which was extracted from the *Withania somnifera* plant and purified to test cytotoxicity against the SK-BR3 breast cancer cells line. To the best of our knowledge only limited work has evaluated this before, so we believe this is first time there has been a comprehensive study of 20nm sAuNPs+1 conjugation and cytotoxicity study against the breast cancer SKBR3 cell line.

Experimental Design

Chemical Materials

The Withanolide-A (1) (CAS: 32911–62-9) was purchased from ChromaDex. $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (99.99%); tri-sodium citrate (Na_3 citrate), tannic acid and K_2CO_3 (Potassium carbonate) were purchased from Sigma-Aldrich (Australia). The phosphate buffer saline (PBS) was procured from Gynea, NSW, Australia. Formvar/carbon coated, square 200 mesh size copper grids and Millex-GV syringe filters (0.22 μm , PVDF, Cat. No. SLGV033RS) were purchased from Sigma-Aldrich (Australia). The RPMIM (Roswell Park Memorial Institute Medium), MTT reagent and DMSO (Me_2SO) were purchased from Life Sci. Technology, Australia. The deionized H_2O (electric resistivity >18 $\text{M}\Omega\cdot\text{cm}$) was supplied by Milli-Q.

Cell Culture

The SKBR-3 cell line was a kind gift from Dr. Thomas Grewal at the University of Sydney. The cell line procured from the American Type Culture Collection (ATCC). The Cell line was authenticated via short tandem repeat (STR) polymorphism, fingerprint analyses and single nucleotide

polymorphism (SNP), passaged for less than 6 months, and cultured as formerly described.²⁸

Instrument Used

The UV-Vis spectrophotometer (Shimadzu 2600, Japan), Ultracentrifugation Sorvall™ WX Floor (Thermo scientific USA), DLS (Nano ZS, Malvern Instruments, UK), transmission electron microscopy (TEM; JEOL-JEM-1400, Tokyo, Japan), Incucyte, Multi-plate reader (Perkin-Elmer victor X4, US), Incucyte (Incucyte® ZOOM Live-cell Analysis System ESSEN, UK) were used during study.

Synthesis of Multifunctional and Spherical 20nm Au Nanoparticles (sAuNPs) and Conjugation with Withanolide-A (I)

Phytochemical

Preparation of Different Working Solutions

The AuCl₃ solution (12.5 mM or 0.05%) was prepared by the addition of 49.2 mg of HAuCl₄.3H₂O in sterile Milli-Q H₂O (10 mL). The Na₃ citrate (1% TSC) solution was prepared by dissolving 100 mg of Na₃ citrate in sterile Milli-Q H₂O (10 mL). A 2.5 mM tannic acid (TA) solution was prepared by dissolving 21.2 mg of it in 1% TSC solution (5 mL). The aq. K₂CO₃ solution (150 mM) was prepared by dissolving of 103.6 mg anhydrous K₂CO₃ in sterile Milli-Q H₂O (5 mL). A 10 mM or 1 × PBS storage buffer (pH 7.4) was prepared by dissolving 2 tablets in 200 mL of sterile Milli-Q H₂O and was filtered through 0.22 μm filter paper to obtain a 10 mM or 1× PBS phosphate buffer solution (pH 7.4 at 25 °C).

Colloidal sAuNPs Synthesis and Conjugation with Withanolide-A (I)

The synthesis of 20nm colloidal mono-dispersed sAuNPs solution was prepared following a protocol reported by Turkevich et al (2006), Frens et al (1973)^{29,30} and Jordi Piella et al (2016).³¹ The 12.5 mM (0.05%) AuCl₃ solution in Milli-Q H₂O, 1% TSC in Milli-Q H₂O, 2.5 mM TA dissolved in 1% TSC and 150 mM aq. K₂CO₃ in Milli-Q water were prepared. The 0.05% AuCl₃ solution (0.6 mL) was diluted to 25 mL with Milli-Q H₂O and stirred for two minutes. Then, 1% TSC (150 μL), 2.5 mM TA (40 μL) and 150 mM aq. K₂CO₃ (40 μL) were added into a previously mixed solution and stirred slowly for 5 minutes at room temperature. The color of the mixed solution changed from light yellow to ash black. The wrapped glass container was charged with magnetic bar

at 60 °C for 5 minutes without stirring. The mixture changed the color from dark to wine red and finally purple within 1–2 minutes due to presence of high reacting citrate-tannic acid complex. The heated Au nanoparticles colloidal solution was kept at 40 °C for 10 minutes followed by cooling to ambient temperature and finally stored at 4 °C. After cooling, the prepared sample was diluted around 1.0 OD (Optical Density) value.³² For conjugation, 10 μg of Withanolide-A (I) was dissolved in 1 mL of sAuNPs solution followed by overnight incubation in cold room with gentle shaking. For stability analysis, 10μL of 10% NaCl solution was added into conjugated and control samples for 15 minutes to check the color change as well as UV/Vis, TEM and Zetasizer measurements.³³

Conjugation Purification

The number of moles in 1 mL gold solution was calculated and various molar excess compounds were tried to cover the gold particle surface. Interestingly, we found a nice surface covering with 10 μg/mL compound concentration that was checked with UV shift. There was no absorption of compound detected in supernatant. All the compounds might be bound to nanoparticles according to reports by Farooq et al (2018).¹ Abs of conjugated compound-unconjugated compound in supernatant/Abs conjugated compound*100.

Characterization of Bare sAuNPs and Phyto-Conjugates

UV-Vis Spectroscopy

To obtain absorption spectra phyto-conjugate (70 μL of bare sAuNPs) solution and Withanolide-A (I), the solution was measured from 200 to 800 nm with blank subtraction of media and distil H₂O.

Dynamic Light Scattering (DLS)

Certain physical parameters of conjugated nanoparticle such as polydispersity index (PDI), average particle hydrodynamic diameter and surface charge were measured with DLS. The sample was diluted to 1:10 and 1:2000 with Milli-Q H₂O for size and zeta-potential analysis respectively. All the measurements were done in triplicate and reported as average of ± standard deviation (SD).

Transmission Electron Microscopy (TEM)

To check the particle core image before and after conjugation, TEM analysis was performed by using small metal

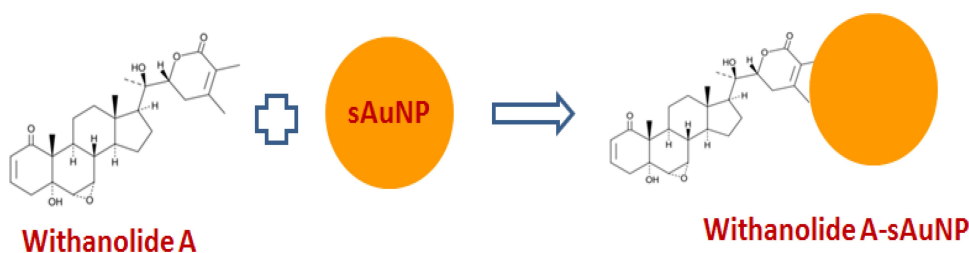


Figure 2 Conjugation of Withanolide-A with 20nm sAuNPs.

grid. Briefly, 10 μL of 1:4 diluted sample solution with and without conjugation were applied on Cu grid having C coating. Next, the grid was air dried overnight and analysis was performed at 120 kv.

Cell Culture and Cytotoxic Evaluation

The SKBR3 breast cancer cells were grown into RPMI media that was supplemented with 10% fetal bovine serum (FBS) and 1% suspension of penicillin-streptomycin. The flask mixture could grow under standard condition in incubator, which was humidified with 5.0% CO_2 level at 37 $^\circ\text{C}$. To check the Au-phytoconjugates cytotoxicity, 5000 cells/well were seeded for 24 hours. The next day's cells were treated into collagen-coated Thermanox plastic well with corresponding concentrations of the tested sample 10, 20, 40 $\mu\text{g}/\text{mL}$ and replenished with fresh media containing negative DMSO 1% and AuNPs 40 $\mu\text{L}/\text{mL}$ with positive control 10 $\mu\text{g}/\text{mL}$ (Cisplatin drug) in triplicates. After specific time intervals (0, 24, 48, and 72 h) image collection of the well was done with 10 \times magnification using Incucyte zoom. After 72 hours, the percentage of confluence/time was obtained by using v2016A incucyte software to generate cell growth curves over time. The cell viability assay was performed by using MTT reagent (20 $\mu\text{L}/100$ mL) and cell viability count was done according to following equation:

$$\% \text{ cell viability} = \left(\frac{\text{Average of treated cell absorbance-BLANK}}{\text{Average of non-treated cell absorbance-BLANK}} \right) \times 100$$

Where, blank is MTT reagent and non-treated cells are negative control such as media and AuNPs correspond concentration in media.³⁴

Statistical Analysis

All the experiment was performed in triplicate to obtained the mean value and SD. For data processing SPSS software 19 and Microsoft Excel were used. The difference in activities of various group in term of mean was checked by Tukey post-hoc analysis where $p < 0.05$ is an indication of statistically significant.³

Results and Discussion

Synthesis of Au-Phyto-Nanconjugate and Characterization

The AuNPs can be synthesized by various *in vitro* and *in situ* methods but uniform spherical particle only acquired by limited strategies. For ease of method working procedure and apparatus used chemical reduction considered as one of favorite method for synthesis of these spherical AuNPs synthesis, which involves reduction and stabilization. In this study, chemical synthesis method was used to prepare spherical AuNPs. It is already known that phytochemical withanolides possess strong hydrophobic hydrogen and electrostatic interactions. Herein, we conjugate Withanolide-A (1) 10 $\mu\text{g}/\text{mL}$ with spherical AuNP solution according to the same strategy as outlined in²⁶ Figure 2.

After conjugation the phytonanoconjugate of sAuNP+1 subjected to ultracentrifugation to obtained purified red

Table 1 Phyto-Nanoconjugate Size, Zeta Potential and UV-Absorption Maximum and TEM Characterization

Sample	Z-Avg Size (nm)	Polydispersity Index (PDI)	Zeta Potential (mV)	λ_{max} (nm)	TEM (nm)
Bare sAuNP	25.35 \pm 0.610	0.285 \pm 0.020	-44.3 \pm 0.860	523.5	20
Phyto-nanoconjugate AuNPs	29.73 \pm 0.650	0.3 \pm 0.009	-20 \pm 0.100	525.5	20

Note: Values are means \pm SD (n=3) of three separate experiments.

Abbreviations: TEM, transmission electron microscopy; UV, ultraviolet.

loose pellets for 40min at 17,000 rpm at 10°C. The pellet was used to perform further analysis.

UV-Vis

The UV–Vis spectroscopy is a sensitive method for detecting colloidal metal, due to exhibiting a characteristic absorption peak at about 490–600 nm, which is attributed

to surface plasmon excitation. The absorption band maximum for bare sAuNPs and phytoconjugates were observed at λ_{max} 523.5 and 525.5 nm respectively (a little bit shift is mentioned in Table 1 and shown in Figure 3) (supplemental Figure 1).

These shifts indicate the formation of adsorbed layers around AuNPs. Aggregation of AuNPs is accompanied

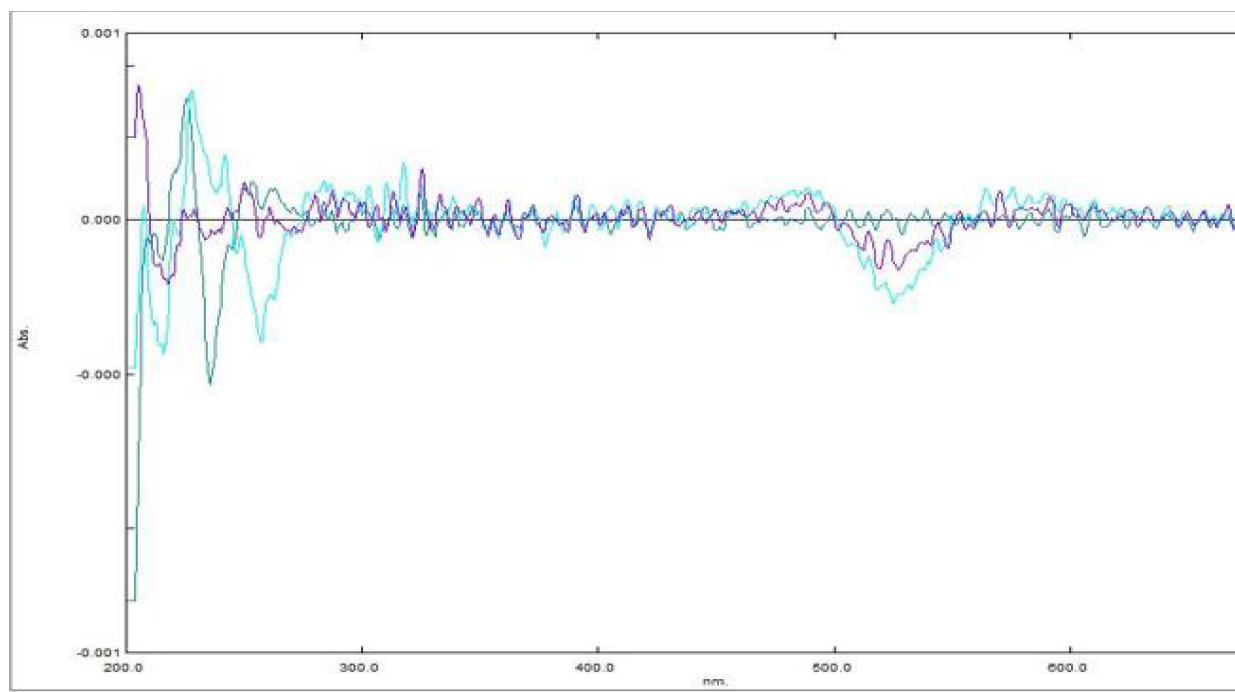
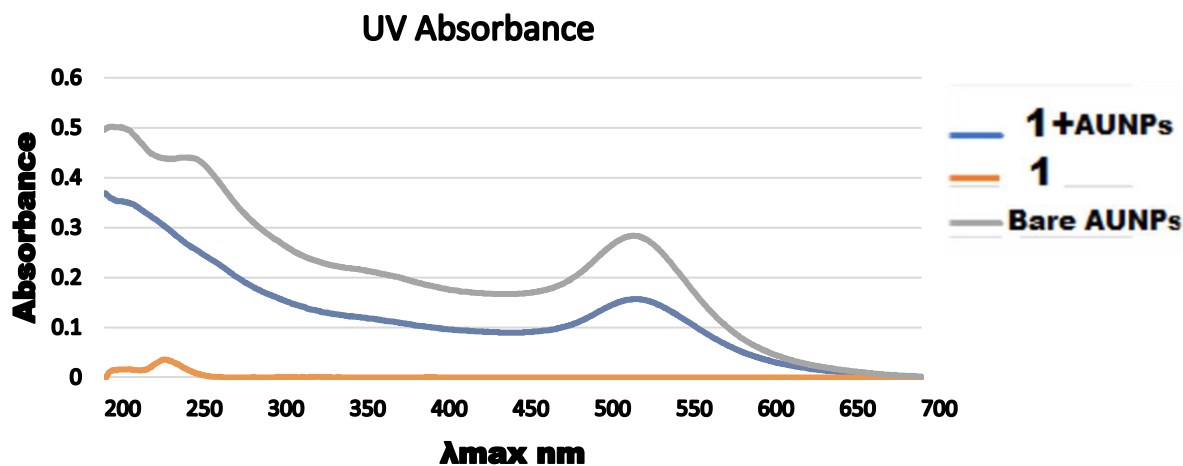


Figure 3 Image (A) UV absorbance of 20 nm Bare spherical Au nanoparticles (sAuNPs), Withanolide-A (I) and I+sAuNPs. Image (B). Secondary derivative green line corresponds to compound I absorbance followed by blue line Au nanoparticles and purple I +sAuNPs absorbance.

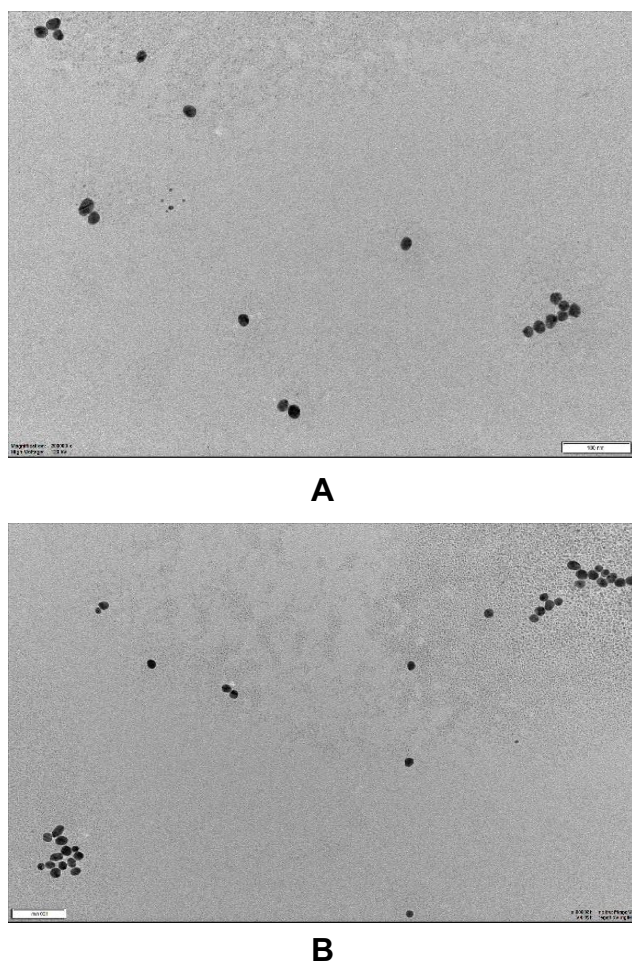


Figure 4 Image (A) is for Bare spherical Au nanoparticles and image (B) is for spherical Au nanoparticles + Withanolide-A at 100nm scale bar.

with a change of color: optical absorption spectroscopy quantifies this process. We did not noticed any color change in our tested samples before and after conjugation, which is similar reliability parameter as reported in previous finding.^{35,36} These sAuNPs showed λ_{\max} at 523.5 nm, which is quite close in agreement with Shafiq et al (2018)³⁷ work who reported that 20nm particle is for λ_{\max} 522 nm. Furthermore, secondary derivatives were recorded which ensure us confidence on binding between two moieties with stability [Figure 3](#).

Dynamic Laser Scattering DLS

Characteristic features, such as hydrodynamic size; PDI and surface charge were measured with DLS before and after conjugation with average corresponding value ([Table 1](#)). Before and after conjugation, Zeta potential value showed from -44.3 ± 0.86 mV to -20 ± 0.1 mV, which was a significant difference due to the presence of a slightly positively charged hydrogen atom of 1 on the Au surface. The PDI changed a little bit from 0.285 ± 0.02 to 0.3 ± 0.009 , which was less than 0.3. It indicates that the particles were not aggregated and homogenous in size hydrodynamic size was less increased from 25.35 ± 0.61 nm to 29.73 ± 0.65 nm and authenticates that 1 molecule attached on Au surface.¹³ The results are presented in [Table 1](#), before and after conjugation showed stability of this nanoconjugate. (See detail [Supplemental Figure 2](#)).

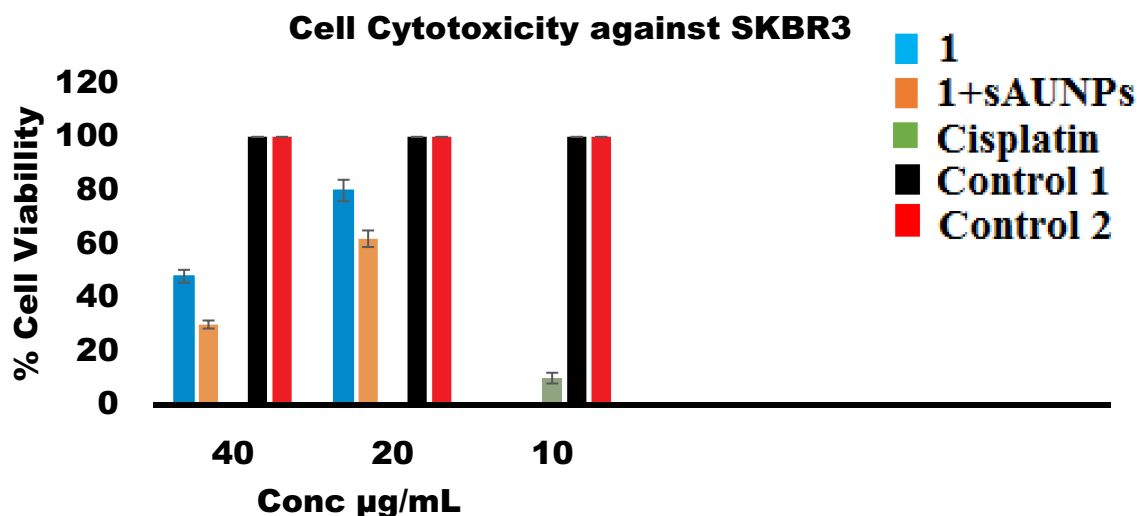


Figure 5 Anticancer activity of Withanolide-A, Phyto-nanoconjugate at different concentration against SKBR3 Cell line. While control 1 and 2 correspond to AuNPs and media with corresponding concentration of DMSO (1% used to prepared sample Withanolide-A). Values indicate mean \pm SD of three independent experiment. While, activity-based differences among various concentration found significant having $p < 0.05$.

Transmission Electron Microscopy (TEM)

Characterization of bare and conjugated nano formulation through TEM displayed a uniform structural morphology and distribution. The size and size distribution of synthesized AuNPs were determined using TEM [Figure 4](#). The average size 20 nm was recorded on micrograph reported in ([Table 1](#)) before and after conjugation (see more images in [supplemental Figure 3](#)).

Determination of Cytotoxicity

We compared free compound 1 anticancer activity and phytonanconjugate with 20 nm sAuNPs against SKBR3 breast cancer cell lines. The standard MTT assay³⁸ were used to determine the level of cell viability coupled with % confluence analysis of incucyte 10× magnification images results software generated growth curve/time.³⁹ The anticancer compound 1 with its varying concentration 40, 20, 10 µg/mL along with the same nanoconjugate concentration applied as triplicate.

In the MTT assay results a dose dependent cytotoxicity response was observed. Interestingly, we found that our phytonanconjugate shows higher antiproliferative effects and showed cell viability of 30% compared to 1 alone of 45% at the concentration of 40µg/mL followed by 60% and 80% at 20µg/mL, which might be due to higher uptake of complex mixture in nucleus or cytoplasm functionalized Au nanoparticle carrier. The results followed the same behavior towards cell viability a we observed in Vijayakumar (2019)⁴⁰ studies, the Au nanoconjugate showed a higher antiproliferative profile than with conjugation.

As has already been proven in several studies (see, for example, Agarwalla et al, 2016;⁴¹ Wing Lee et al, 2016;¹³

and Lara et al, 2019),⁸ Au nanoparticle conjugated with phytochemical enhanced their payload and cellular uptake. However, in our findings we noticed slightly higher cell growth inhibition activity at concentration 40 µg/mL with 1 and 20 µg/mL in Au nanoconjugate sAuNP+1 compared to previous literature as shown in [Figure 5](#) ([supplemental Figure 4](#)). There was no cell growth inhibition observed with corresponding negative control, while standard cisplatin drug behaves better than 1 and phytonanconjugate.

A similar trend has been seen with incucyte studies result where a minimum percentage of live cell confluency (number of cells) observed with Cisplatin (10 µg/mL) > (40 µg/mL 1 + 20 nm sAuNPs) > (40 µg/mL 1) > (20 µg/mL 1 + 20 nm sAuNPs) > (20 µg/mL 1) > (10 µg/mL 1 + 20 nm sAuNPs) > (10 µg/mL 1) > Media > AuNPs ([Figures 6 and 7 \(A, B\)](#)).

Due to the higher uptake and active carrier delivery through AuNPs based targeted drug delivery it is possible to destroy the cancer cells by combining phytochemical with minimal effective cytotoxic concentration due to higher safety profile. Therefore, it is worthwhile exploring the biosynthesized nanoparticles as a possible source of novel anticancer drugs.

Conclusion

In conclusion this study has reported the effective synthesis of 20 nm sAuNPs and their conjugation with phytochemical anticancer compound 1, which was previously reported safe to normal cell line. Later characterization was done with UV-Vis, DLS and TEM techniques. Three important points observed as valuable findings. The synthesized phytonanconjugate proved as a stable moiety,

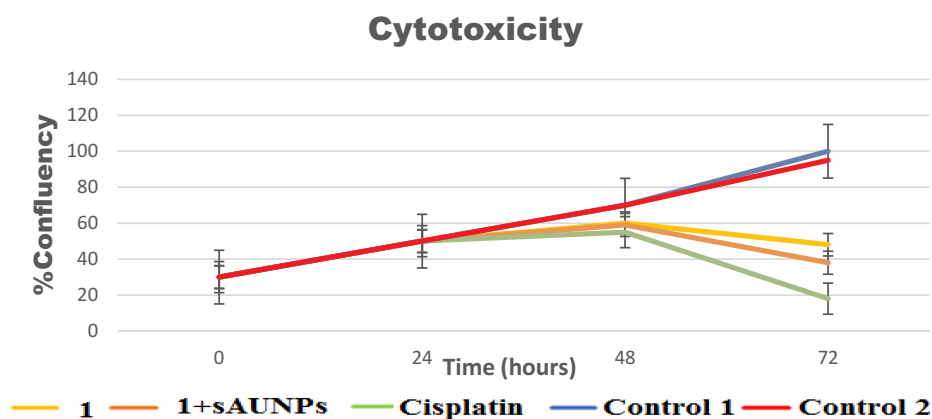
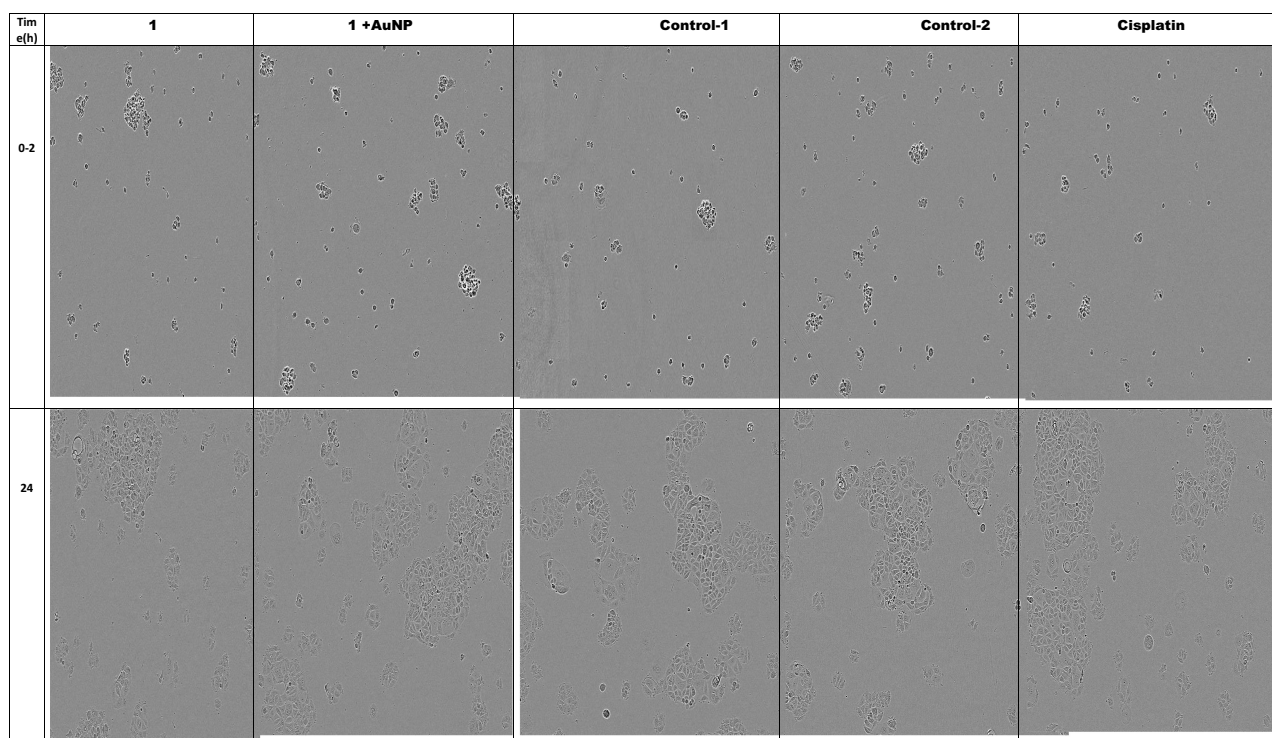
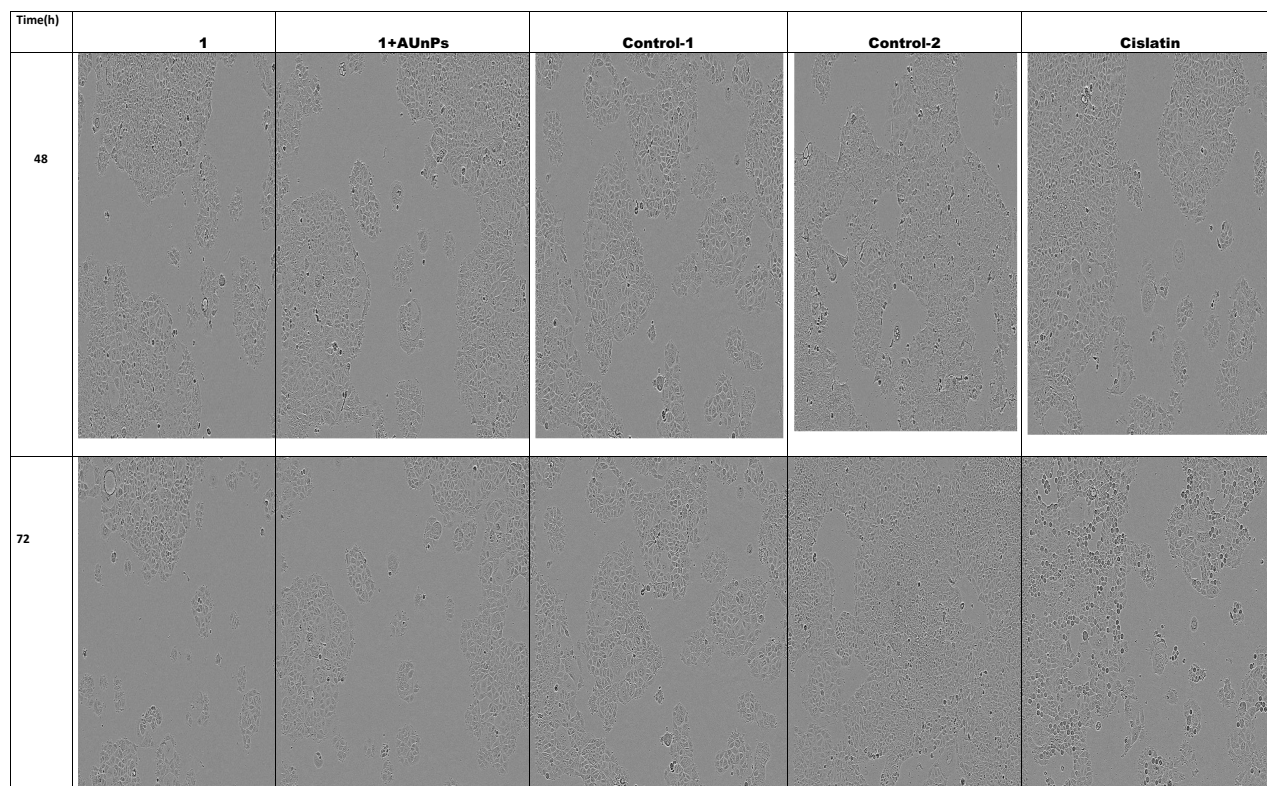


Figure 6 Percentage of Confluency (number of cell) growth curve/time against SKBR3 breast cancer cell line. There was significant difference found with specific interval of time having $p < 0.05$.



A



B

Figure 7 Image collection of cell growth inhibition assay after specific interval of time. **(A)** Images at time 0–2 (h). **(B)** Images at time 48,72(h).

tested nanoconjugate showed higher toxicity than normal compound and values of cytotoxic concentration seem to be higher. However, the stability and better performance of these phytonanconjugate towards *in vitro* SKBR3 cells make it an active target to explore for other applications in future.

Abbreviations

Withanolide-A, ATCC, American Type Culture Collection; Au, gold; AuNPs, gold nanoparticles; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; HAuCl₄, chlorauric acid or tetrachloroauric acid; nm, nano meter; PBS, phosphate buffer saline; PDI, polydispersity index; PVDF, polyvinylidene fluoride; sAuNPs, spherical Au nanoparticles; SKBR3, Sloan Kettering-breast cancer; STR, short tandem repeat; TA, tannic acid; TEM, transmission electron microscope; TEM, transmission electron microscopy.

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Disclosure

The authors report no conflicts of interest in this work.

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