Phytochemical Synthesis and Characterization of Bioactive Gold Nanoparticles using Hypaphorine and their Cytotoxic Activity against HCT 116 Human Colorectal Cancer Cells

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Abstract— Green synthesised gold nanoparticles (AuNPs) have received intense interest owing to their remarkable biomedical applications, particularly in cancer therapy. The plant mediated synthesis of AuNPs is of interest to biologists and chemists due to non-toxic, rapid, and convenient benefits over chemically and physically synthesised counterparts. However, the fabrication methods of AuNPs using pure phytochemicals and their application in cancer therapy are sparse. In the present study we used hypaphorine to successfully reduce gold salt to corresponding AuNPs in a single step; a process that fulfils all criteria of green nanotechnology. Characterization of hypaphorine capped AuNPs by UV-Vis spectroscopy; Fourier transform infrared spectroscopy; Dynamic light scattering and Transmission electron microscopy revealed that the synthesised AuNPs were spherically shaped with size range of 20-60 nm and polydispersed. The synthesised hypaphorine-capped AuNPs showed excellent *in vitro* cytotoxic activity against HCT 116 colorectal cancer cells and little or no toxicity to normal BHK 21 cells. The results of this study demonstrate the feasibility of using hypaphorine for the synthesis of AuNPs to fulfil the growing demand for rapid, convenient and non-hazardous AuNPs. Also the study provides guidance towards the development of alternative treatment of cancer therapy using biologically synthesised AuNPs. However, there is a need for further clinical studies to ascertain their potential as highly active anticancer agents. To the best of our knowledge, there is no report available on the synthesis of AuNPs using pure hypaphorine and no such articles is available to describe their cytotoxic effect in HCT 116 human colorectal cancer cells.

Index words— colorectal cancer cell line, cytotoxic activity, gold nanoparticles, green synthesis, hypaphorine.

I. INTRODUCTION

Nanotechnology has broad application prospects in the treatment of cancers and has opened new avenues for its chemotherapy[1]. There is a significant interest towards the use of biologically synthesised and medical applications of metallic nanoparticles as they provide completely new and improved properties which are based on size and shape [2,3]. Amongst metallic nanoparticles, AuNPs possesses remarkably unique physicochemical properties, tunable optical properties, good

biocompatibility, low toxicity and ease of surface functionalization, as well as antibacterial and anticancer activities as compared to other metal nanoparticles [4,5]. Furthermore, AuNPs are known to enhance the cytotoxic activity of their capping agent [1,6].

Over the past few years, a variety of physical and chemical methods have been employed for the synthesis of AuNPs [7,8]. However, these methods are quite expensive, energy intensive and they employ hazardous chemicals and perilous non-polar solvents, thus preventing their application in the biomedical field because of biological risks [7]. For this reason, it is desirable to devise alternative biological methods of AuNPs synthesis that are eco-friendly, rapid, convenient, compatible for clinical application, easily scaled up for large production and cost effective [9]. Green chemistry approach, also termed green nanotechnology of nanoparticles synthesis employs environmentally benign materials such as plant extracts, plant products, algae, bacteria and fungi among others for the synthesis of nanoparticles [5]. Many researchers have recently reported the biosynthesis of AuNPs using phytochemicals, which have become an interesting area of research in the biomedical field by producing safe nanoparticles that can be used in medical applications. A plethora of research studies in which AuNPs are synthesized using plant extracts and pure phytochemicals have been reported, with a few of them being mulberry leaf extract [3], *Achyranthes asparalin* seed extract [10], *Solanum nigrum* leaf extract [5], *Ananas comosus* fruit extract [11], *Elettaria cardamomum* aqueous extract [12], *Stevia rebaudiana* leaf extract [13], *Buta monosperma* leaf extract [14], gallic acid [15] Epigallocatechin-3-gallate (EGCG) [16] and Hesperidin [17]. Although there are many reports, the fabrication methods of AuNPs using phytochemicals and their application in cancer therapy are still sparse.

Over the past decades, plants, either as pure compounds or as standardized extracts, have been providing the most novel and wide range of biological activities for use in medicine as they contain numerous phytochemicals that have different biological functions [18–20]. Alkaloids are an example of phytochemicals that particularly have anticancer activity against various cancers [18]. These types of phytochemicals are

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nitrogen containing heterocycles that are a very important class of organic compound in virtue of their potent bioactivity with regards to therapeutic activities [21,22]. Phytochemicals have been reported to act as reducing and capping agents in the synthesis of metallic nanoparticles even though their biochemical mechanisms are yet to be revealed or elucidated [20,23]. In this study, pure alkaloid: hypaphorine was used for the synthesis of AuNPs. The medicinal value, natural availability, non-toxic nature and low cost of hypaphorine have intrigued us to use it for the synthesis and capping of AuNPs and to further evaluate their cytotoxic activity in colorectal cancer cells.

Hypaphorine (also known as lenticin), is an indole alkaloid (fig. 1). It was first isolated and characterized from *Erythrina hypaphorus* in 1911 [24], and has since been found in a large number of different organisms, from plants to fungi, sponges and mammals. Hypaphorine has never been tested on its own for its anticancer properties. It is only the crude plant extracts that has been observed for antimicrobial properties.

To the best of our knowledge there is no report available on the synthesis of AuNPs using pure aqueous solution of hypaphorine and to describe their cytotoxic effect observed in HCT 116 colorectal cancer cells and BHK 21 normal kidney cells. The synthesised AuNPs were characterized using characterized by UV–vis spectroscopy, dynamic light scattering (DLS), transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR). The cytotoxicity potential against HCT 116 colorectal cancer cells were assessed on MTS and xCELLigence assays.
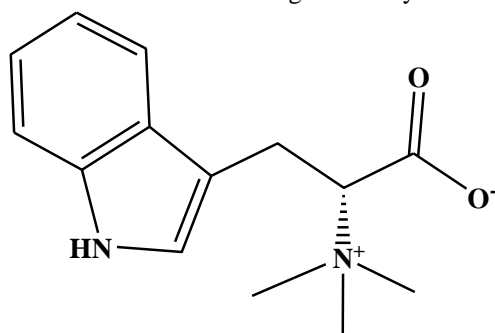

Fig. 1. Chemical structure of hypaphorine

II. MATERIALS AND METHODS

A. Reagents and chemicals

All the chemicals were of analytical grade and obtained from accredited suppliers, unless otherwise specified, and were used without any further treatment. Hydrogen tetrachloroauric hydrate ($HAuCl_4.3H_2O$), ≥99.9% was obtained from Sigma–Aldrich (St. Louis, MO, USA) and used as such. Freshly prepared double distilled water was used throughout the experimental works. Aqueous methanol (99.8%) (Sigma Aldrich, South Africa) was used to prepare hypaphorine solution. Hypaphorine was previously isolated in our lab from both *Erythrina mildbraedii* and *Erythrina addisioniea* and

used without further purification.

B. Preparation of 1 mM hypaphorine solution

Hypaphorine solution was prepared by solubilising in 100 ml aqueous methanol. Briefly, 0.02 g of hypaphorine was taken into an Erlenmeyer flask. Hundred (100) mL of methanol was then added and the mixture was stirred for 5 minutes using magnetic stirrer (MS200, China (Mainland)) in order to ensure that there is no particle matter left in the solution.

C. Preparation of 1 mM $HAuCl_4$ stock solution

A stock solution of $HAuCl_4$ salt was prepared by dissolving 0.02 g of $HAuCl_4$ in approximately 100 mL double distilled water. The mixture was stirred until completely dissolved using a magnetic stirrer.

D. Phytochemical synthesis of AuNPs

The synthesis of AuNPs was prepared by modified chemical reduction of salt solution of $HAuCl_4$ using hypaphorine dissolved in aqueous methanol and was achieved by slight modification of the method described by Stephen and Seethalakshmi [17]. A small quantity of methanol used to dissolve hypaphorine acted as a reducing agent and hypaphorine as a capping agent for the synthesis of AuNPs. Ten (10) mL of prepared hypaphorine solution was mixed with 90 mL of 1mM $HAuCl_4$ solution in a 250 mL Erlenmeyer flask. A solution of hypaphorine was added drop wise to a heated solution of $HAuCl_4$ under magnetic stirring condition until the $HAuCl_4$ solution turned into purple colour which indicated the formation of hypaphorine-capped AuNPs. These results are similar in term of colour change which confirms the formation of AuNPs to those reported by [25]. The whole experimental procedure was conducted in the dark and the solutions were also stored in brown vials to avoid photoactivation of Au salt. These were further confirmed by various characterization techniques (fig. 2).
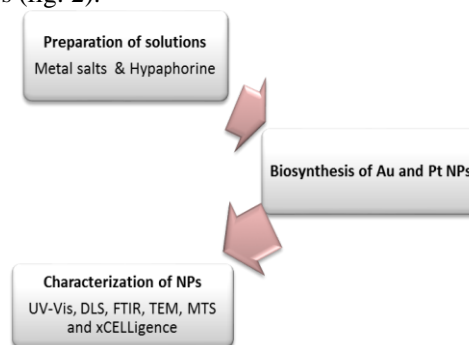

Fig. 1. Experimental procedure for synthesis of AuNPs using hypaphorine

E. Characterization of hypaphorine-capped AuNPs

UV-vis spectroscopy

The formation of AuNPs was monitored by UV–visible spectroscopy, recorded on a Shimadzu (UV-2450) UV–visible Spectrophotometer with samples in quartz cuvette in a wavelength range from 200 to 800 nm. Distilled water was

used as blank and sample preparation was done by diluting a small aliquot of the mixture solution in distilled water. The obtained spectral data was then recorded and plotted using Origin Pro software version 8.1.

FTIR

FTIR spectroscopy is used to identify the functional groups that are bound to the surface of NPs [3,26]. The identification of functional groups are important due to the fact that the surface chemistry of AuNPs affects their properties and applications [8]. The FTIR spectra of the AuNPs were recorded on FTIR spectrophotometer (PerkinElmer instrument) with the sample in solution form. The spectrum was scanned in the wavelength range of 500–4500 cm^{-1}.

TEM

TEM analysis was carried out to study morphological properties of AuNPs. The solution of AuNPs were prepared on carbon copper grid by putting a small drop of solution into carbon coated copper grid and were allowed to dry at room temperature for two hours attached to the sample holder. The measurements were taken on a JEOL model 3010 microscope (JEOL, Tokyo, Japan) operated at an accelerated voltage of 200 kV.

DLS

The average size, zeta potential and size distribution of hypaphorine capped AuNPs were determined by DLS using Malvern Zetasizer instrument (Zetasizer NanoZS, Malvern instruments). Zeta potential was done to develop information about the stability of the NPs and determine whether the particles within the solution sample will tend to agglomerate or not whereas the zeta sizer was done to measure the hydrodynamic size of the AuNPs [26]. The zeta sizer and zeta potential measurements of synthesised AuNPs were measured in a zeta dip cell and disposable cuvette at 25ºC, using Zetasizer software. All samples were diluted with distilled water and sonicated for 5 minutes in an ultrasonic bath before taking measurements.

F. In-vitro cytotoxicity

MTS Assay

An MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sul fophenyl)-2H-tetrazolium) assay was conducted to assess the cytotoxic effects of the hypaphorine-capped AuNPs and pure hypaphorine on HCT 116 and BHK 21 cells. The MTS compound (yellow) is metabolised by viable cells to form a dark purple coloured compound, visible through UV-Vis spectroscopy at 490 nm using Multiskan Go UV-Vis spectrophotometer (Thermo Scientific) [27]. The BHK 21 (6 x 10^4 cells/ml) and HCT 116 (2 x 10^5 cells/ml) cells were seeded in 96 well tissue culture plates at 37°C overnight, according to the method by [28], with slight modifications. Various concentration ranges of hypaphorine capped AuNPs and hypaphorine (100, 50.0, 25.0, 12.5, 6.25, 3.13 and 0.00 μM) were tested to investigate the toxicity of the hypaphorine and

AuNPs capped with hypaphorine. In all cases, the cells were left to incubate for 4 days after compound addition, whereupon MTS (5 μl) was added to the cells. The absorbance values were measured at 490 nm after 1h, 2h and 4 hours incubation periods.

xCELLigence assay

The xCELLigence is a Real-Time Cell Analysis (RTCA) system that was used for comparative cytotoxicity analysis (ACEA Biosciences) [29] . The xCELLigence was performed according to the method described by [29]. Briefly, the BHK 21 (6 x 10^4 cells/ml) and HCT 116 (2 x 10^5 cells/ml) cells were incubated at 37°C overnight, in an Au electrode coated 96 well E-plate, with the subsequent addition of the AuNPs sample, in concentrations of (100, 50.0, 25.0, 12.5, 6.25, 3.13 and 0.00 μM). The cells were left to incubate for a minimum of 4 days, with impedance measurements taken at various time points during the course of the incubation period. The data was retrieved and a graphic representation of the toxicity constructed.

III. RESULTS AND DISCUSSION

CHARACTERIZATION OF HYPAPHORINE-CAPPED AuNPs

Physical observation revealed the change in colour of the solution from light yellow to dark purple within few minutes of adding the hypaphorine solution to the aqueous metal salts solutions (Fig. 3). This change in colour was observed due to the excitation of surface plasmon resonance (SPR) of AuNPs [30]. Similar changes in colour have also been observed in previous studies [30–34]. This intense colour change confirmed the completion of reaction and the formation of AuNPs. The resulting solution of the AuNPs was further examined by the UV-Vis spectroscopy**.**

Fig. 3. Physical observation revealing the change in colour of AuNPs

Formation of hypaphorine capped AuNPs was confirmed by the UV-visible spectrum of solution after completion of the reaction. It has been established that the optical properties of AuNPs depend on the size and shape of the particles [26]. In the UV-vis spectrum, the reaction solution of hypaphorine showed the absorption peak at 550 nm, indicating the purity of AuNPs (fig. 4). This strong peak is characteristic of the SPR of the AuNPs in the UV-vis spectrum [26].
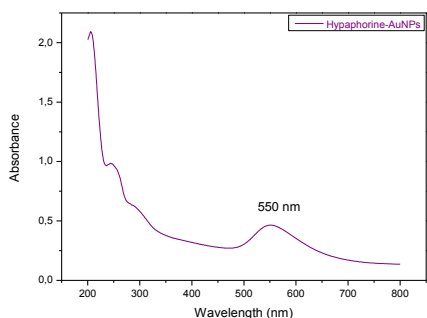
Fig. 4. The UV-Vis spectrum of AuNPs prepared at 5 ml of hypaphorine solution

The FTIR spectra were obtained from the aqueous solutions of the hypaphorine and the colloidal AuNPs solution. The main goal of the FTIR measurements was to identify the presence of possible functional groups in the reaction mixture and predict their involvement in AuNPs synthesis. The FTIR spectra of the pure hypaphorine and the corresponding hypaphorine capped AuNPs is shown in Fig. 5 respectively. Intense absorption peaks were observed at 3338.45 and 1636.87 cm^{-1} representing the O-H and C=O stretching's. The hypaphorine solution had peaks at 3354.48 cm^{-1}, 1726.90 cm^{-1} these peak values correspond to functional groups present on the surface of the AuNPs. This indicates that hypaphorine has been effectively involved in capping of the AuNPs. Therefore, it is possible that functional groups present in the hypaphorine played a role in the reduction of gold metal ions. Despite the similarities between the FTIR spectrums of pure hypaphorine to its derived AuNPs, some absorption peaks exhibited shifts in their position. These shifted peaks are assumed to be associated with the functional groups around the AuNPs. This indicates the molecular association of pure hypaphorine with the surface of the AuNP through -OH and C=O groups (Fig. 6). Our results are in agreement with the report of [20] on the synthesis of silver nanoparticles using flavonoids. FTIR thus proved hypaphorine capping in hypaphorine-capped AuNPs.
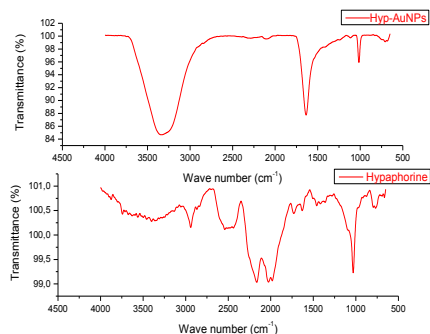

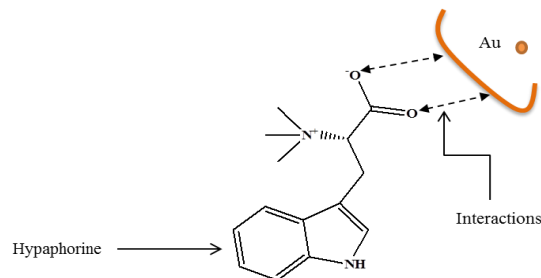Fig. 5. FTIR spectra of pure hypaphorine (lower) and AuNPs (upper)

Fig. 6. Schematic representation of possible interactions between hypaphorine and the surface of AuNPs

The average hydrodynamic size and zeta potential of the hypaphorine capped AuNPs was determined by the DLS as shown in Fig. 7 and 8. The average size of the AuNPs was found to be 141 nm with polydispersity index (PDI) of 0.182, respectively. The zeta potential of the hypaphorine capped AuNPs had a positive zeta potential value of +13.6 mV indicating stability at room temperature. From Table 1, it was clear that the synthesised AuNPs exhibited a lower zeta potential, indicating less stability and thus tendency to agglomerate and form large particles. However, this is typical for green synthesis of metallic nanoparticles to have a lower zeta potential which gives stability for few weeks to months.
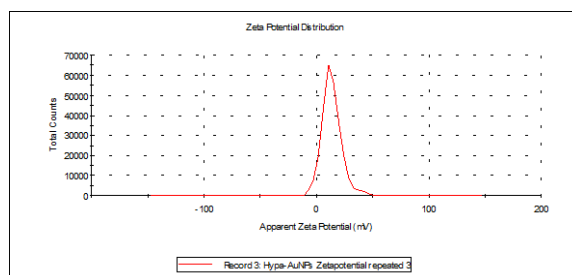

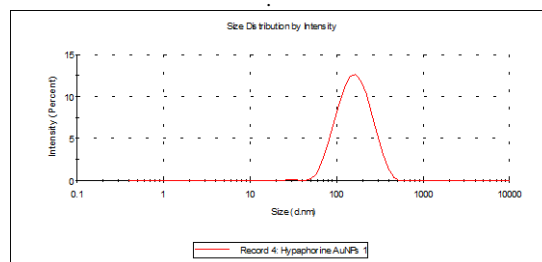Fig. 7. Zeta potential graph of hypaphorine capped AuNPs

Fig. 8. DLS graph of hypaphorine capped AuNPs

Transmission electron microscopy was used to for accurate size and morphology determination of the synthesised AuNPs. The TEM images shown in Fig. 9 at different magnifications (100 and 200 nm) clearly provided the size and shape on the AuNPs. The shapes of hypaphorine capped AuNPs were spherical and polydispersed. The size of the AuNPs was found in the range of 25 to 60 nm, respectively.
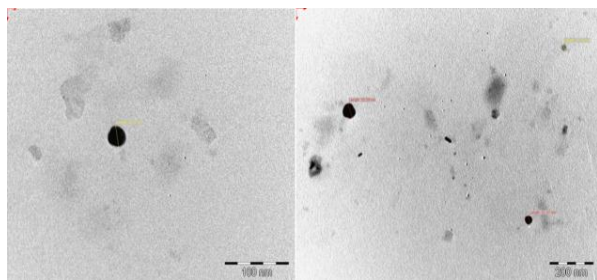
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Fig. 9. TEM images of AuNPs taken at (a) 100 nm and (b) 200 nm

CYTOTOXICITY OF HYPAPHORINE-CAPPED AuNPs

The cytotoxic effects of the hypaphorine capped AuNPs and the pure hypaphorine was investigated in HCT 116 colorectal cancer cells by MTS and xCELLigence assay at different concentrations. The results indicated that the hypaphorine capped AuNPs showed concentration dependent cytotoxicity in the HCT 116 cells and little or no toxicity in BHK 21 cells. The size, shape and agglomeration state of the AuNPs may be the reason for the lower toxicity [20].

The MTS assay results showing the toxicity of the tested hypaphorine and AuNPs to HCT 116 and normal BHK cells at various concentrations (0, 3.13, 6.25, 12.5, 25, 50, and 100 μM) are illustrated in Fig. 10 to 12. Fig. 10 illustrates hypaphorine toxicity between the two different cell lines. It is clear from the graphs that hypaphorine seemed to be more toxic to the cancerous HCT 116 cell line. An increase in cellular growth rate, as seen against the HCT 116 cell line at 100 μM, is also an indication of toxicity. Here the cells would try to compensate for the toxicity, or manage the toxicity, by increasing cellular growth rate, as can be seen below.
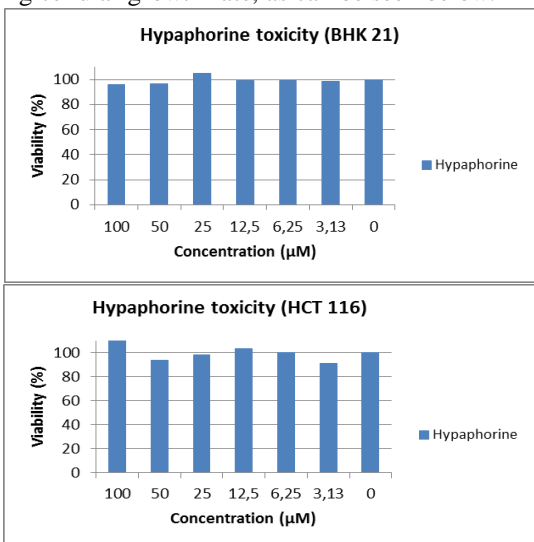

Fig. 10. MTS assay for the hypaphorine tested against normal BHK 21 and HCT 116 cells at various concentrations

In Fig. 11 and 12, little definitive toxicity is seen for both cell lines in all the NPs tested. Here, it showed that the hypaphorine AuNPs had the greatest toxicity to the HCT 116 cell line, with the least amount to toxicity seen against the normal BHK 21 cell line.
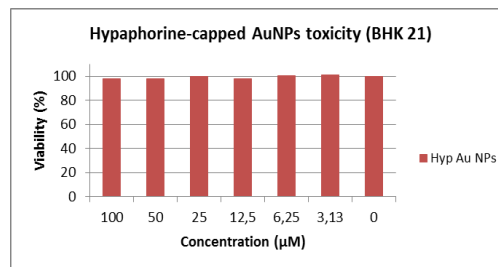

Fig. 11. MTS assay for the hypaphorine-capped AuNPs tested against normal BHK 21 cells at different concentrations.
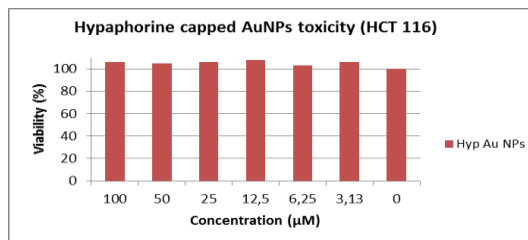

Fig. 12. MTS assay for the hypaphorine-capped AuNPs tested against HCT 116 cells at different concentrations

It is clear from the results obtained by MTS, that pure hypaphorine seemed to be more toxic to the cell lines than their respective nanoparticle counterparts. This is likely due to the functional groups of the hypaphorine, that most likely cause the toxicity, that bind to the surface of the produced AuNPs. The xCELLigence system is a real time cell based analysis system that uses impedance as a measure of cell viability [29]. The system uses Au electrode coated 96-well plates to measure current across the base of a single well. A living cell with active metabolic function affords resistance and so produces a visible graph. This growth pattern is specific to the type of cell line used. As long as the cells remain viable, there will be current impedance and a graph to match. When the cells are no longer viable, there will be no impedance and therefore no impedance graph. The normal cellular growth pattern is seen, in all cases, as the light grey-blue line, shown at a concentration of 0.00 μM (Fig. 13 and 14). This illustrates the unhindered cells and the "100%" viability curvature. As seen in the MTS assay, some toxicity was expected for the hypaphorine and AuNPs against BHK 21. Greater concentration dependent toxicity was seen for the hypaphorine however, with a great amount of toxicity observed for the hypaphorine-capped AuNPs at higher concentrations. Although some toxicity was observed for the AuNPs in the MTS assay, the amount of toxicity is greatly underestimated in the end point assay. The AuNPs showed an increase in cellular viability, an indication of toxicity through nuclear attack to the cells. Although AuNPs showed some concentration dependent toxicity, the effect of capping agent on cell line was minimal (Fig. 15 and 16).
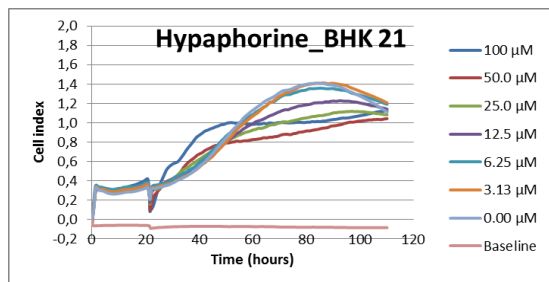
Fig. 13. xCELLigence results for the hypaphorine tested against normal BHK 21 cells at various concentrations
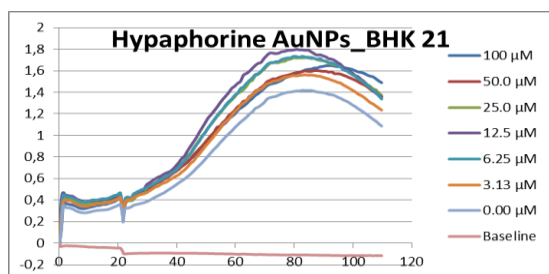

Fig. 14. xCELLigence results for the hypaphorine-capped AuNPs tested against normal BHK 21 cells at various concentrations.
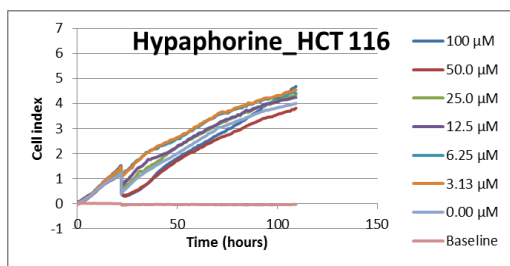

Fig. 15. xCELLigence results for the hypaphorine tested against HCT 116 cells at various concentration ranges
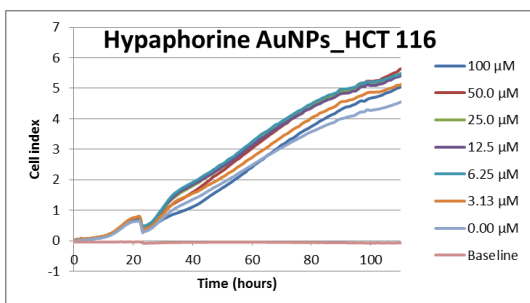

Fig. 16. xCELLigence results for the hypaphorine-capped AuNPs tested against HCT 116 cells at various concentrations

IV. CONCLUSION

In conclusion, pure alkaloid capped AuNPs were successfully synthesized by a feasible green method using hypaphorine in methanol. The UV-Vis analysis confirmed the formation of AuNPs and also showed that they were of pure Au. The TEM analysis established that the average diameter range of the core Au was 25-60 and spherically shaped. The FTIR analyses have shown that the capping agent fully capped the AuNPs and that possibly the hydroxyl groups of methanol and

hypaphorine were involved in the reaction for the bio-reduction of gold ions into AuNPs. The zeta analysis showed the hydrodynamic size of the synthesised AuNPs and found it to be 141 nm with a lower positive zeta potential charge of +13 mV. Generally, in the characterizations of AuNPs, these techniques have provided conclusive information on the nature of the synthesised hypaphorine-capped AuNPs and demonstrated the feasibility of using hypaphorine for the synthesis of AuNPs to fulfil the growing demand for rapid, convenient and non-hazardous NPs. The synthesised AuNPs exhibited potential cytotoxic effects against HCT 116 human colorectal cells and little or no toxicity against BHK 21 normal kidney fibroblast cells in vitro. The selective killing of cancer cells substantiates the potential of hypaphorine-capped AuNPs to be used as anti-cancer agents for cancer therapy. These preliminary *in vitro* cytotoxicity data elevates the promising therapeutic applications of AuNPs in future nanomedicine, which could be a viable starting point for development of future combinatorial therapeutic treatments against colorectal cancer and other cancers. However, there is a need for clinical studies to establish their potential as anti-cancer agents.

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