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Cytotoxic effects of Ceiba pentandra L. mediated silver nanoparticles on HCT-116 colon cancer cell lines through ROS generation and cell membrane damage

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ABSTRACT

In this study, we assessed the biological effect of *Ceiba pentandra* bark silver nanoparticles (CP-AgNPs) on HCT-116 cancer cells with an emphasis on cell cytotoxicity, quantification of ROS and determination of mitochondria membrane potential. The synthesized *Ceiba pentandra* bark silver nanoparticles were characterized by UV-vis spectrometer, High-resolution transmission electron microscope (HR-TEM), X-ray diffraction (XRD) and Selected area electron diffraction (SAED). The synthesized silver nanoparticle cytotoxic and apoptotic effects were studied on colorectal cancer cells (HCT-116). The nanoparticles exhibited an inhibitory concentration (IC_{50}) at (60μg/mL). CP-AgNPs significantly inhibited cell viability and changed the morphology of HCT-116 colon cancer cells. Moreover, CP-AgNPs increased the level of reactive oxygen species, induced cell apoptosis in HCT-116 cell lines through the interference of the mitochondrial membrane potential. These results indicated that *Ceiba pentandra* bark silver nanoparticles (CP-AgNPs) might act as prospective anticancer agents in colon cancer cells.

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INTRODUCTION

In the 21st century, cancer is the leading cause of death in humanity globally and second only after cardiovascular diseases (Ferlay *et al.*, 2015). Worldwide colorectal cancer (CRC) is the third most commonly diagnosed malignancy, and the fourth leading cause of cancer-related deaths in the world and its burden is expected to increase by 60% to more than 2.2 million new cases and 1.1 million cancer deaths by 2030 (Bopanna *et al.*, 2017). According to the statistical data in the year 2015 in India concerning colon cancer, 12,483 males and 15,205 females were diagnosed. There is a prediction that by the year 2020, the numbers would skyrocket to 13,420 and 19,013 cases, respectively (Takiar *et al.*, 2010). Cancer date rate is still on the rise, and there is no significant improvement in the past years even though there are advanced high- tech solutions towards the combating the disease. For instance, there are new techniques that have the capability to understand the molecular mechanisms of cancer in addition to the identification of several cancer biomarkers (Desai *et al.*, 2008).

Cancer treatment and management are being faced up with many hindrances, for instance, multiple drug resistance, less drug reaching the tumor site, nonspecific systemic distribution of antitumor agents and intolerable cytoxicity (Jeyaraj *et al.*, 2013). Therefore, this implies that great efforts are required in order to overcome cancer and increase the patient survival, for instance through

the improvement and designing of new strategies, tools and drugs (Misra *et al.*, 2010).

Most of the modern medicines in third world countries, 50% are of natural product origins (Huang *et al.*, 2009). The phytochemicals derived from the plants are believed to posses anticancer agents that have the capabilities of regulations of genes involved in the inhibition of apoptosis and ant proliferative activity in cancer cells, and on top of that the plant-derived anticancer compounds are said to be less toxic towards the normal cell lines, greater pharmacological and biological activities, and this, therefore, showcase the importance of natural medicines over chemical chemotherapeutic drugs (Xu *et al.*, 2015; Vemuri *et al.*, 2017). However, most of the herbal origin drugs possess insoluble character leading to low bioavailability, lack of targeting ability and increased systemic clearance or higher dose makes the drug as a poor candidate for therapeutic use and hence majority of researches are focusing on phyto-chemical in order to identify new anticancer agents (Ansari *et al.*, 2012).

The new modern field of science known as nanotechnology, it has diverse applications in the enhancement and growth of human being welfare. To be precise, nanotechnology appears to be a promising field in the area of nano-medicine, more so in cancer treatment through a new discipline of nano-oncology. Through the incorporation of nanoparticles and phytochemical agent, this provides a promising alternative for novel cancer treatment. The nanoparticles, due to their unique chemical and physical characteristics, can be developed as a delivery vehicle to carry diagnostic and therapeutic agents directly to the specific cell. This, therefore, is a major breakthrough in overcoming the major challenges being faced, when using herbal extracts in cancer treatment such as their bulk dosing and less absorption hence better efficacy will be achieved (Ding *et al.*, 2012). The other factor to take into consideration, previous studies on different cell lines proved that metal nanoparticles, have the capability to easily cross through the cellular barriers and cause DNA damage, chromosomal aberrations and finally cell cycle arrest (Asharani *et al.*, 2009).

The nanoparticles can be synthesized by different methods of physical, chemical, and biological method. The major setback for physical and chemical methods for nanoparticle synthesis is the use of hazardous toxic solvents and the need high temperatures, pressure and energy. At present, the biological synthesis of nanoparticles is on the upper hand due to factors such as environmentally feasible, low-cost, energy-efficient, and high-yield procedures (Mubarakali *et al.*, 2012). There are different biological approaches (fungi, bacteria, enzymes, fungi, algae and plant extract) for synthesizing nanoparticles. However the microbial mediated synthesis of nanoparticles is not industrially feasible as it requires expensive medium and maintenance of highly aseptic condition and hence; the use of plants mediated synthesized nanoparticles is more significant since its less expensive, eco-friendly and the plant extract can reduce the metal ions to nano-sized particles and also stabilizes the nanoparticles formed (Mubarakali *et al.*, 2011; Gopinath *et al.*, 2012; Sathishkumar *et al.*, 2012).

Ceiba pentandra L. Gaertn. of Bombacaceae family is also known as silk-cotton, are a tropical tree, and the capsules are known as Kapok (Alvarado *et al.*, 2002). Recent pharmacological studies revealed that solvent extracts of various parts of the plant have anticancer, hypolipidemic, hepatoprotective, anti-inflammatory, and anti-ulcerogenic effects (Kumar *et al.*, 2016; Aloke *et al.*, 2010; Bairwa *et al.*, 2010; Alagawadi and Shah, 2011; Fofie *et al.*, 2019).

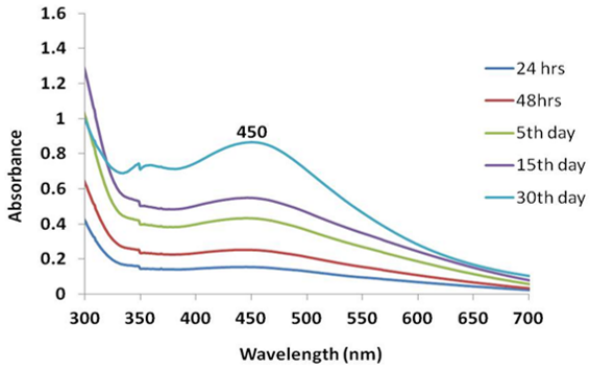

Figure 1: UV-visible spectroscopic analysis of synthesized CP-AgNPs

To the best of our knowledge, the present study is the first one that evaluates *Ceiba pentandra* bark silver nanoparticles (CP-AgNPs) on HCT-116 cancer cells. In this study, an attempt was made to the green synthesis of AgNPs from *C .pentandra* bark and was characterized. The same was also used to assess the in vitro anticancer activities of synthesized nanoparticles on Colon cancer cell line HCT-116 which were carried out using MTT assay, DCFH-DA staining and Rhodamine 123 staining and AO/Et-Br staining.

MATERIALS AND METHODS

Synthesis of CP-AgNPs

For the synthesis of CP-AgNPs, 5ml of *C.pentandra* ethanol bark extract was added into 45 ml of 1mM aqueous silver nitrate solution. The reaction process was incubated from day 1 to day 30 and peri-

odically monitored by the use of UV-vis spectrophotometer in the range of 300-700. The purification of the synthesized AgNPs was done via centrifugation at 10,000 rpm for 30 minutes as per our earlier report (Masese and Selvi, 2019).

Characterization of CP-AgNPs

Various techniques were used for the characterization of the synthesized CP-AgNPs. For instance, the determination of the reducing agents in CP-AgNPs was done by FTIR Spectrophotometer Shimadzu UV-1800 in a range of 4000-400 $cm^{-1.}$ The crystalline of the synthesized CP-AgNPs was evaluated using Selected area electron diffraction (SAED) while the dimension features of the CP-AgNPs were determined by XRD (PAN analytic). Transmission electron microscopy (TEM CM 200) was used to study morphology. Quantification of the synthesized AgNPs was studied using UV-vis spectra (JASCO V-650) in a wavelength range of 300-700 nm.

Anticancer activity of synthesized CP-AgNPs on HCT-116 cells

The estimation of the potential cytotoxicity effect of the synthesized CP-AgNPs on cancerous HCT-116 cell lines was done by MTT assay as described by (Mosmann, 1983). HCT-116 cells (1×10^{4}) were seeded in a 96-well plate. In an increasing concentration of (5-100 μg/ml), the cells were treated with CP-AgNPs and followed by incubation for 24 h at 37ºC in the presence of 5% CO_2 in an incubator. MTT solution (5 mg/ml) was added to the treated cell and further incubated at 37º C for 4 h after which the formazan crystals were dissolved by adding 200 μl of DMSO. The viability of the cells was carried out by using a scanning multi-well microplate reader at 570 nm.

HCT-116 morphological observation

The inverted phase microscope (Nikon, Japan), was used to determine the morphological alterations on the HCT-116 after treatment and incubation of the cells with CP-AgNPs at different concentration for 24 h in comparison to the control (untreated cells).

Detection of ROS in CP-AgNPs

For the determination of the cellular ROS generated in the CP-AgNPs treated cells, 2, 7, dichlorodihydofluorescein (DCFHDA) was used (Rastogi *et al.*, 2010). For the assay, 10μM DCFHDA was added to the wells for 30 min at 37ºC in darkness. The cells were then washed twice with PBS, and then after observed under a fluorescent microscope.

Determination of mitochondria membrane potential

The mitochondrial membrane potential (MMP) was determined by the use of Rhodamine -123 dye (Dash *et al.*, 2013). For the assay in a 6 well plate, the cells were cultured, treated and incubated for 24 h. This is then followed by incubation of the treated cells with Rhodamine -123 dye for 30 min. Finally, the cells were washed twice with PBS and analyzed by fluorescence microscope.

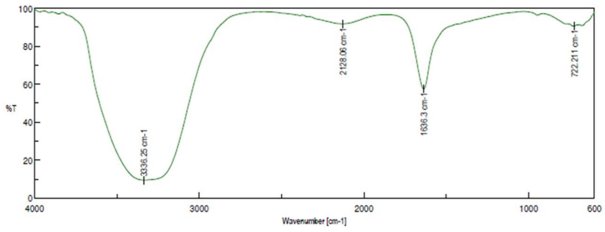

Figure 2: FTIR spectra of the synthesized CP-AgNPs

Apoptotic evaluations

To determine whether the cell viability in HCT-116 cells was either through apoptosis or necrosis after treatment with CP-AgNPs, Acridine orange/ ethidium bromide (EO/EB) was used (Darzynkiewicz *et al.*, 1994). The CP-AgNPs treated cell were stained with AO/EB in a ratio of 1:1 in PBS and incubated for 30 min. This was followed by washing of the unbounded dye by PBS, and the stained cells were visualized under UV-illumination using the 40$\times$ objective to identify whether the cells undergo apoptosis.

RESULTS AND DISCUSSION

Synthesis and characterization of CP-AgNPs

In the present study, we managed to synthesis silver nanoparticles from ethanol *C.pentandra* bark extract. The preliminary indication of synthesis of silver nanoparticles from silver ions was through the color change due to surface plasmon resonance from light yellow to brown and finally to reddish-brown. The absorption spectra of CP-AgNPs, was analyzed at different time intervals 24 hours, 48 hours, 5 days, 15 days and 30 days by the use of UV-vis spectroscopy Greater absorption peak was observed at 450 nm after completion of the reaction on the 30^{th} day as shown in Figure 1. From the results, we can conclude that the increase of reaction time between the silver ions and biological reductant, leads to the synthesis of stable plasmonic peaks. Similar results were obtained by (Wang *et al.*, 2018).

Characterization of synthesized CP-AgNPs

The reducing and capping agents present in the *Ceiba pentandra* bark ethanol extract were identified by the FTIR. The CP-AgNPs FTIR spectra

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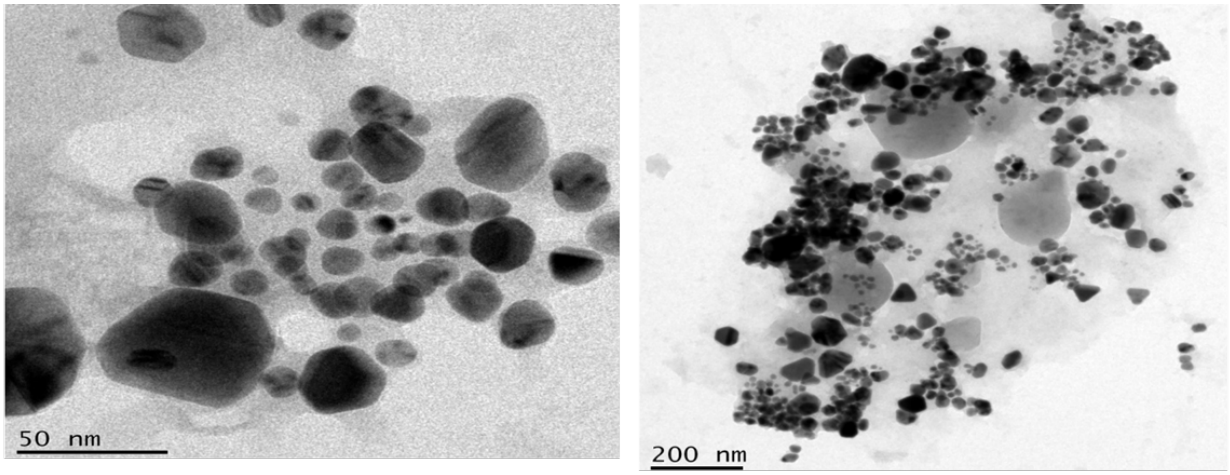

Figure 3: HR-TEM measurements of synthesized CP-AgNPs at 50 and 200nm

analysis, as shown in Figure 2 displayed absorption bands at different wavelength such as 3336, 2128, 1636 and 722 cm^{-1}. The AgNPs the bands around 700–750 cm−1may be attributed to stretching vibrations of C–Cl alkyl halides (Sarah, 2017). The bands around 1600–1650 may correspond to stretching vibrations of primary and secondary amines (Sivakumar *et al.*, 2012; Shanmugavadivu *et al.*, 2014; Dakal *et al.*, 2016). The bands around 3100–3400 cm−1 may be due to C O stretch of carboxylic acids (Sarah, 2017). The high-resolution transmission electron microscopy (HR-TEM) Figure 3 revealed the morphological shape of the synthesized CP-AgNPs to be spherical in shape, and some had irregular circles without uniform edges, with a diameter ranging around 5 and 50nm.

Surface electron diffraction (SAED), Figure 4 showed a bright circular pattern rigs indicating that the synthesized CP-AgNPs was crystalline in nature similar to the study conducted previously by (Srikar *et al.*, 2016). XRD patterns showed the diffraction ring inner to outer which were obtained at (111), (200), and (220), (311) Braggs reflections with peaks corresponding to 38.41○, 44.40○, and 67.57○, respectively hence confirming the crystalline nature of the biosynthesized AgNPs Figure 5 (Venugopal *et al.*, 2017).

In vitro anti-cancerous efficacy of synthesized CP-AgNPs

The anticancer activity of the synthesized CP-AgNPs was found to be dose-dependent against the HCT-116 as shown by MTT assay. There were variations in the percentage of cell viability between the control and different dosage of CP-AgNPs (5-100 μg/ml) treated HCT-116 cells. The IC50 inhibitory concentration was found to be 60μg/ml, as shown in Figure 6 For this study, 40μg/ml and 60μg/ml concentrations were selected for the optimal treatment

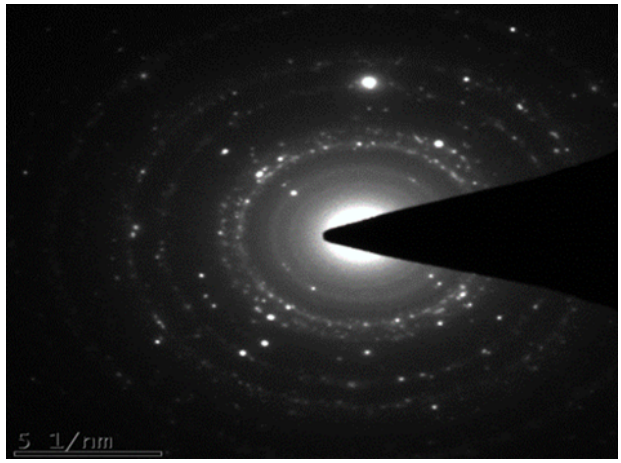

Figure 4: SAED pattern of synthesized CP-AgNPs

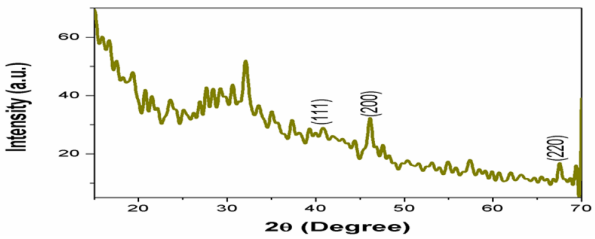

Figure 5: XRD analysis of CP-AgNPs

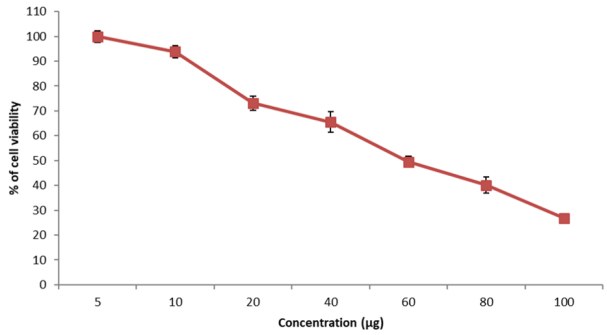

Figure 6: Cytotoxic potential of CP-AgNPs on HCT-116 cell lines

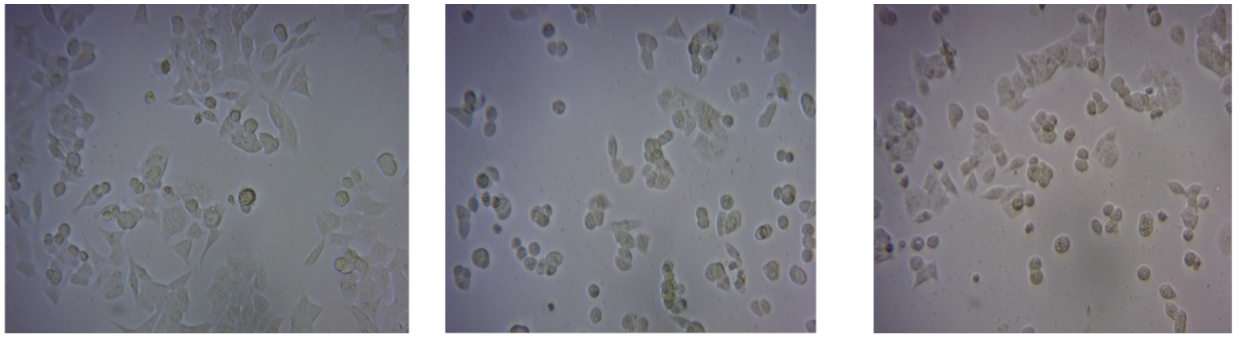

Figure 7: In vitro anticancer activity of CP-AgNPs against HCT-116 cells

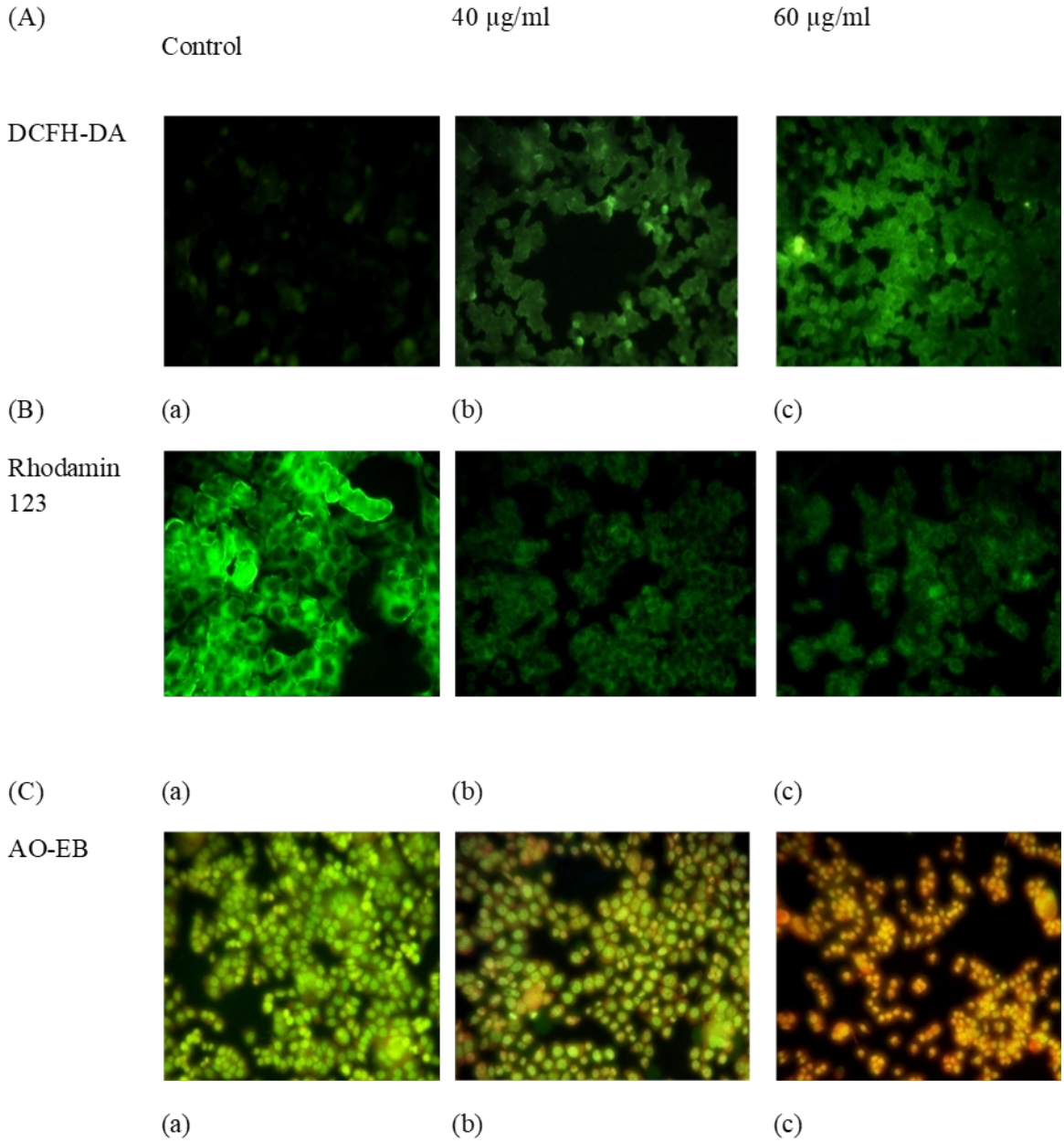

Figure 8: In vitro anticancer activity of CP-AgNPs against HCT-116 cells (A) DCFH-DA method (B) AO-EB staining (C) Rhodamin-123. (a) Control (b) 40 μg/ml (c) 60 μg/ml

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for the other anticancer studies. Similar results were obtained using various plant extracts and cell lines (Vivek *et al.*, 2012; Jacob *et al.*, 2012; Suman *et al.*, 2013) The morphological changes observed on the HCT-116 cells treated with CP-AgNPs due to the cytotoxic effects, it's due to the ability of the synthesized nanoparticle to induce cell death (Farah *et al.*, 2016).

Phase-contrast microscopy was used to analyze the morphological changes on the cells after treatment with CP-AgNPs. HCT-116 cells were exposed to CP-AgNPs at 5–100 μg/ml for 24 h are presented in Figure 7. There was no significant change observed in the morphology of control HCT-116 cells. The control cells appeared in normal shape and were attached to the surface. However, the HCT-116 cells exposed to CP-AgNPs showed morphological changes, including cell shrinkage and formation of apoptotic bodies due to CP-AgNPs toxicity.

ROS generation by CP-AgNPs

At the beginning of apoptosis, ROS is usually formed. In our study, CP-AgNPs induced the generation of ROS on the HCT-116 cells, as shown in Figure 8 (a). By the use of DCFH-DA method, the HCT-116 cells were treated with 40 and 60 μg/ml of CP-AgNPs. In order to determine the production of ROS, a high tremendous green fluorescence was observed on the treated cells, at 40 and 60 μg/ml, when compared to the control. More ever, the highest intensity was observed at 60 μg/ml, reflecting that the production of ROS increased with increased concentration of CP-AgNPs. The ROS are usually believed to be involved in the regulating of the intracellular signaling pathways such as apoptotic cell death mainly due to oxidative stress (Stroh *et al.*, 2004). For instance, (Jeyaraj *et al.*, 2013) also reported that AgNPs and Ag^{+}induced cell death in Hela cells through ROS- mediated apoptotic process.

Mitochondria membrane potential

In order to determine the loss of mitochondrion membrane potential, the HCT-116 cells were tainted with Rhodamine -123 dye, as shown inFigure 8 (b). The treatment of the HCT-116 cells with the synthesized CP-AgNPs drastically decreased the mitochondria membrane potential. The control cells emitted a high intensity of green fluorescence, indicating the mitochondrial membrane dysfunction. Meanwhile, the HCT-116 cells treated with 40 and 60 μg/ml of CP-AgNPs, showed diminished green fluorescence. Previously it has been reported that the production of ROS could be the major reason for the cellular damage leading to mitochondria membrane damage hence inducing cytotoxicity (Dwivedi *et al.*, 2014; Nicoletti *et al.*, 1991).

CP-AgNPs apoptotic studies

Apoptotic morphological studies were investigated through microscopic images observation on CP-AgNPs treated HCT-116 cells stained with Et/Br as shown in Figure 8 (c). Apoptotic characteristics such as morphological changes and cell condensation were observed after treatment of the cells with CP-AgNPs then followed by staining with Et/Br. The control cells the untreated emitted green fluorescence with no apoptosis features indicating that the cells were viable. Treated cells with 40 and 60μg/ml of CP-AgNPs, displayed bright green fluorescence indicating early apoptosis while the bright orange fluorescence indicated late apoptosis. Our study, the results are in agreement with the recent reports that have shown apoptosis cell death due to the contact with nanoparticles (Pan *et al.*, 2007). These results indicate that either the mitochondrial pathway or apoptotic pathway might be involved in the CP-AgNPs induced HCT-116 cell death. The reason here is that high levels of ROS leads to apoptotic, necrotic cell death and DNA damage (Foldbjerg *et al.*, 2009; Hsin *et al.*, 2008). Studies from other researchers have stated out that AgNPs has the capability to induce cytotoxicity through mitochondria-mediated cell death (Jeyaraj *et al.*, 2013).

CONCLUSION

From our results, we can conclude that the synthesized CP-AgNPs produced a significant cytotoxity effect in an increase dosage-dependent manner against HCT-116 cell lines, whereby the results were evident by MTT assay. On the other end, CP-AgNPs induced the production of ROS, which later on leads to cell membrane damage and finally apoptosis on the HCT-116 cancer cell lines. Further studies are necessary in order to investigate the anticancer agent present in *C.pentandra* bark and the exact mechanism involved.

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