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Research Article

Cytotoxic Potential of Humic Acid Synthesized Silver Nano Plates Against Dalton's Lymphoma Ascites

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ABSTRACT

This is a first report on the cytotoxic activity of humic acid synthesized silver nanoplates (HAgNpl) against Dalton's lymphoma ascites (DLA). Humic acid, a supramolecule, has been shown to possess various biological activities. Humic acid used in the present study for the synthesis of silver nanoplates is isolated from mangrove sediments. Chemical composition of the humic acid was analysed using solid state C-13 NMR spectroscopy. Morphology of nanoplates were investigated using SEM. Trypan blue viability assay was performed on Dalton's Lymphoma Ascites (DLA cell lines) to evaluate the cytotoxic potential of the HAgNpl. Humic acid synthesized silver nanoplates proved to be effective against these malignant cells. The HAgNpl showed a dose dependent activity against DLA cells. This result highlights the potential role of HAgNpl in developing safe medicines against malignant lymphoma.

Keywords: Humic Acid, Silver Nanoplates, Dalton's Lymphoma Ascites.

INTRODUCTION

Nanobiotechnology, a rapidly emerging field of nanoscience, is a promising area of research for developing treatment method to cure cancer and its diagnosis[1]. Nano-materials are potential alternative for conventional treatment methods due to their unique biological properties[2]. In this regard, drugs developed using silver nanoparticles are exceptional due to their typical morphologies and properties[3,4]. Considering the wide range of nanotechnology-based clinical applications possessed by silver nanoparticles, pharmaceutical companies are particularly focused on synthesizing silver nanoparticles with distinctive properties and morphologies. In this context humic acid, due to their unique supramolecualr nature and reduction potential[5,6], is an apt choice for synthesising silver nanoparticles with unique morphologies and biological properties. Humic acids have gained increasing interest in the field of pharmacology as they do not possess teratogenic, mutagenic, embryotoxic and carcinogenic properties and can be easily removed from the body[7]. Humic acid directly influence the biochemical activities by their stimulating effects, membrane activity, the transport of electrons in the electron transport chain of the mitochondria, activation of oxidative phosphorylation, ion exchange, complexation and sorption capacity[7].Various products of humic acids are used in veterinary and human medicines. In herbal medicines humic acid are used for treating fractured bones, dislocation, skin diseases, and peripheral nerve disorders[8-10]. Accelerated calcium deposition and expression of calcium phosphate, osteocalcin, core binding factor and suppressed osteoclasto-genesis were observed in human bone marrow mesenchymal stem cells and human fetal osteoblasts treated with humic acid[8]. Calus formation of fractured bone and phosphorus uptake is accelerated by 20-25% using humic acid[11,12]. Ability of humic acid to form film in the mucosa of the gastrointestinal tract enhances the defence mechanism against infections and toxins. Humic acid are good blood coagulating agent[13]. Mast cell protection potential by humic acid has made them an effective antiallergic[14,15]. The biological prospective of humic acid along with their potential to synthesis nanoparticles have prompted the study to understand the effect of humic acid synthesized silver nanoparticles on ascitic tumour. Aim of the present study was to characterise the silver nanoparticles synthesized using humic acid isolated from mangrove ecosystem and to determine their effect on Dalton's Lymphoma Ascites.

MATERIALS AND METHOD

Isolation and Characterization of Humic acid

Humic acid used for the synthesis of silver nanoplates were isolated and purified from sediments sampled from mangrove ecosystem in Cochin, southwest coast of India, following the protocol set by IHSS[16]. Purified humic acids were characterized using C-13 solid state NMR analysis. The 13C-NMR spectrum of the freeze dried humic acid sample was recorded on a Bruker AVANCE 300, with a working frequency of 75.475 MHz using the CP-MAS procedure. Sample (100–150 mg) were filled in 4 mm zirconia rotors with Kel-F caps and spun at 10 kHz. Cross

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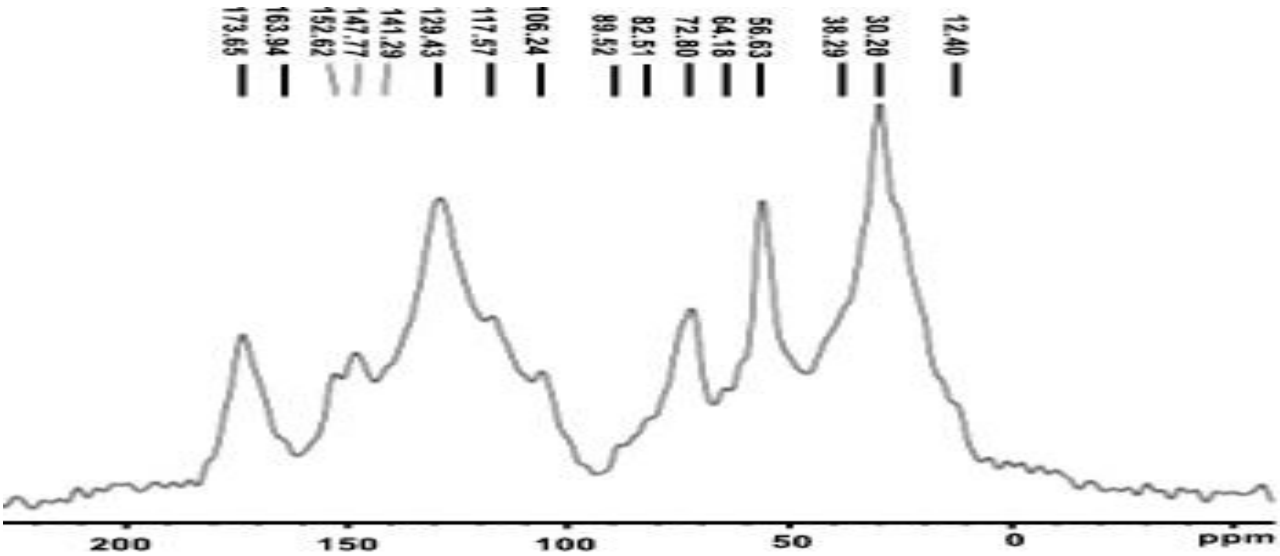

Figure 1: C-13 CPMAS solid state NMR spectra of humic acid molecule extracted from mangrove sediments, Cochin, south west coast of India.

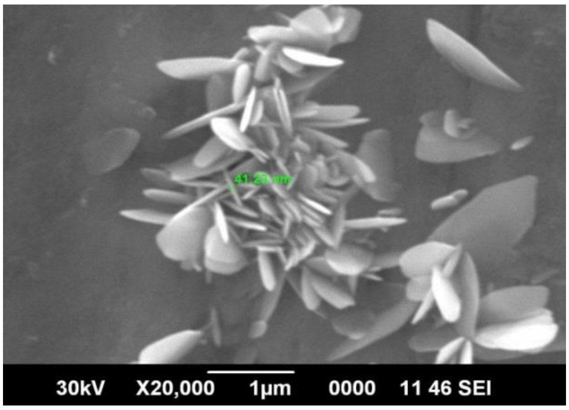

Figure 2: SEM images representing the silver nanoplates synthesized at 0.01 mol l^{-1} concentration of silver nitrate using humic acids.

polarization was done at a contact time of 1 ms, over an acquisition time of 36 ms and a recycle delay of 2.50 s. The quantitative processing was performed by numerical integration in the regions corresponding to the functional groups and molecular fragments with preliminary automatic correction of the phase and the baseline using Bruker Topspin 1.3 software. Groups of carbon species have been identified by the intervals of chemical shift (Table 1).

Synthesis and Characterization of HAgNpl

Humic-silver nanoparticles were prepared by reducing 10 mL 0.01M $AgNO_3$ aqueous solution with 1 mL 10 ppm solution of humic acid at 10 pH adjusted with 0.001 M NaOH. Silver reduction process was enhanced by heating[17] the homogenous solutions in an oven at 75 °C for an hour. The resulting silver nanoparticles were then characterized by SEM (JEOL Model JSM - 6390LV).

Cytotoxic activity of HAgNpl

In order to investigate the cytotoxic activity of HAgNpl against blood cancer, Dalton's Lymphoma Ascites (DLA) cells were used. These malignant cell lines were maintained at Amala Cancer Research Centre, Amala Nagar, and Thrissur, India. Trypan blue exclusion method was used to calculate the cytotoxicity of HAgNpl. This analysis differentiates intact live cells cell membranes from the dead cells using a light microscope. Latter, which due to the retention of dye in the cytoplasam are seen as blue coloured. All the chemicals used for the assay were purchased from HIMEDIA. Prior to the viability assay, DLA cells aspirated from the intra peritoneal cavity of mice with tumour were washed thrice using PBS. Different concentration of humic acid silver nanoplates (1 nM, 5 nM, 10 nM, 15 nM and 20 nM) uniformly dispersed in aqueous solution were thoroughly mixed with 0.8 ml of PBS and added to 0.1 mL of cell suspension containing 1×10^6 cells and incubated for 3 hours at 37 ºC. Soon after incubation, in order to count the non-viable (stained) and viable (unstained) cells using microscopic, a drop of cell mixture treated with 0.1 mL trypan blue dye were transferred to a haemocytometer. Counting of total viable and non-viable cells was carried out separately.

RESULTS AND DISCUSSION

Results of solid state C-13 NMR characterization of humic acid isolated from mangrove sediments is shown in Fig. 1 and Table 1. 13C NMR corroborates that humic acid isolated from mangrove sediment is amphiphillic in nature, and consist of polar groups such as carboxylic, hydroxyl, methoxy and quinoids[5,6]. Redox properties of humic acid have been generally validated to be linked mainly to hydroquinone and phenol groups[18]. Presence of polar and reducing groups in the humic acid molecule and unique amphiphilic composition aids in the synthesis of silver nanoplates and its stabilization[5]. Results of humic acid synthesized silver nanoplates are shown in Fig.2. The outcomes of this work demonstrate the properties of humic acid solution used as reducing and capping agent for the synthesis of silver nanostructures. These nanoplates possessed relatively high surface area and a thickness of around 41 nm. Approximately 45803874.649 silver atoms

Table 1: Assignments of 13C CPMAS NMR bands and C distribution (%) based on bands relative areas for the humic acids derived from mangrove sediments.

Chemical Shift Range δ values (ppm)	Inference: Carbon species[6]	Relative C %
0-45	Unsubstituted saturated Alkyl Carbon	30.13
45-65	Oxygen and Nitrogen substituted alkyl carbon	15.41
65-95	Alkyl C singly bonded to one oxygen atom and anomeric C	9.9
95-145	Proton- and alkyl-substituted aromatic C	31.53
145-160	Oxygen-substituted aromatic C	6.01
160-190	Carboxyl, ester, amide C and Quinone C	7.02

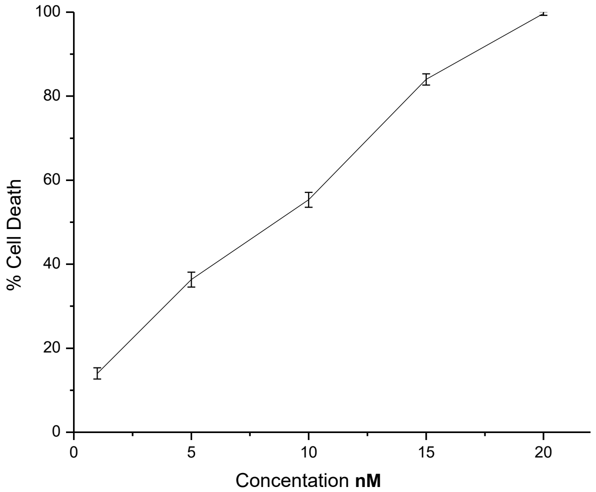

Figure 3: Dose-dependent effect of silver nanoplates over cell viability using Trypan blue exclusion assay. Data represent the mean ± standard error of the mean of triplicate experiments.

were present per HAgNpl[19]. The effect of HAgNp on viability of DLA was assessed using Trypan blue exclusion. HAgNpl was shown to decrease the viability of DLA cells in dose dependent manner as shown in Fig. 3. After incubation, the silver nanoplates were found to be cytotoxic against these malignant cells at dose of 1 nM and higher. Silver nanoplates at 10 nM reduced the viability of DLA cell to around 42% of the initial value and was chosen as IC_{50}. Clinical studies have shown that humic acid are capable of removing 95% plutonium and reducing the radiation dose to the living cells[20] hence decreasing the risk of lymphoma caused by ionizing radiation[21]. They effectively regulate the proliferation of lymphocytes of cortical thymus layer and their migration into thymus dependent zones of lymph node and spleen[22]. This assistance in rapid restoration of lymphocyte count in the blood, bone marrow and spleen could reduce malignancies caused by exposure to γ – radiation[23]. Consumption of mutagenic compounds particularly through chlorinated drinking water is another cause of lymphomas and it increases the risk by 1-1.3 irrespective of age, gender and social class[24]. This risk arising from mutagenic contaminated water can be effectively reduced by humic acids. Potential of humic acids to reduce the mutagenicity by adsorbing mutagens has been experimentally proven by Sato and co-workers. They effectively removed mutagens such as benzo[a]pyrene, 2 aminoanthracene, 2-nitrofluorene and 1-nitropyrene by humic acid[25]. Potassium salt of humic acid has been demonstrated to poses potential to modulate Nasal eosinophil counts[26]. This observation suggests humic acids could also find application in the treatment of lymphoid malignancies associated with eosinophilia such as lymphoblastic leukemia[27]. Another critical component influencing the progression of malignant lymphoma is inflammation, resulting from infections and chronic inflammation, capable of regulating neoplastic process, nurturing proliferation, its subsistence and relocation[28]. For example, occurrence of mucosa-associated lymphoid tissue lymphoma and follicular lymphoma of the spleen is seen to increase in person suffering from chronic *Helicobacter* infection[29-32] and parasitic infection[33] respectively. Toxicity studies have proved the potential of humic acid in reducing cutaneous inflammation and their anti-inflammatory potential is comparable to 1% dicofene sodium and 0.1% betamethasone[34] highlighting their potential in supressing inflammation induced lymhoma. In the presence of humic acids, expression of virus[35] and activity of pathogens in animals and humans[36] is suppressed. This is a direct consequence of non-covalent interactions of humic acids with protein and enzyme activity of these microbes. Further the activities of toxins released by them are neutralized by ionic bond formation by humic acid. Presence of polyphenolic groups in the humic acid molecule stimulates nonspecific resistance and immunity[37,38]. These properties of humic acids along with potential to supress DLA proliferation highlight the possibility of developing treatments to contain B-cell non-Hodgkin’s lymphoma caused by Epstein-Barr virus[39] and non-hodgkin lymphoma caused by HIV[40] by using HAgNpl. These observations cumulatively highlight the safe theraputical potential of humic acid and their products similar to that of sterols isolated from algae and mangrove ecosystems[41-43]. Bioactive prospective of humic acid coupled with strong potential of HAgNpl to suppress the DLA cells, observed in this investigation highlights the significance of their role in evolving treatment methods to successfully cure the patients suffering from lymphoma cancer without any side effects. However, further molecular level researches are required to recognise the mechanism by which humic acid synthesized silver nanoplates suppress these malignant cells. As this information are essential to predict collective outcome of test sample on gene expressions and their products prior to their in-cooperation in treatment methods.

CONCLUSION

So far there have been no data on the cytotoxic potential of humic acid against lymphoma cancer. To summarize, the present investigation documented the first ever synthesis, characterization and cytotoxicity of humic acid synthesized silver nanoplates against Dalton's lymphoma ascites. Composition of humic acid used in the present investigation was obtained using solid state C-13 NMR. Collectively, our result suggests that humic acid synthesized silver nanoplates possess superior cytotoxic potential and there is a wide-ranging possibility for comprehensive examination in the future for the application of humic acid synthesized silver nanoplates for treating cancer.

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