

Synthesis and characterization of *Careya arborea* nanoparticles for assessing its in vitro efficacy in pancreatic cancer cells

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ABSTRACT

Pancreatic cancer has an infaust prognosis and is the fifth common cause of cancer related death in India. As it can only be diagnosed at an advanced stage, most of the conventional treatment modalities such as chemotherapy, radiation therapy and surgery would become inefficient. *Careya arborea* is an ayurvedic plant that has shown increased anticancer effect in various cancers. Induction of tumor cell death by *Careya arborea* bark extract through physiological and pathological means is reported in breast cancer cells. In the present study, we explored the anticancer effect of nanoparticles prepared from methanolic extract of *Careya arborea* bark in pancreatic cancer cells. The nanoparticles were prepared and size analysis was performed by DLS and SEM which depicted the average size of nanoparticles as 195 ± 50 nm. *In-vitro* cytotoxicity assay showed upto 60% inhibition in pancreatic cancer cell (MiaPaCa-2) proliferation. The *C. arborea* nanoparticles exhibited induction of apoptosis through DNA fragmentation in pancreatic cancer cells.

Keywords: Pancreatic cancer; Methanolic extract; *Careya arborea*; Nanomedicines.

INTRODUCTION

Pancreatic cancer is the fifth leading cause of cancer related death in United States and the rate of incidence is increasing in India (Seigal, et al., 2013). Among all the cancers, human pancreatic cancer is well known to have the worst prognosis (Jemal, et al., 2008). By the time of diagnosis, the vast majority of the cancer extends beyond the pancreas and direct invasion to nearby organs such as stomach, duodenum, colon, spleen and kidney is observable (Yohe, et al., 2010), (Warshaw, et al., 1992). Although progress has been made in reducing incidence and mortality rates and improving survival, pancreatic cancer still accounts for only 5-year overall survival in patients undergone pancreatectomy (Riediger, et al., 2009). A large number of natural products have never been replicated by synthetic medicinal chemistry, which illustrates the importance of drug discovery to identify active compounds and define novel pharmacophores (Leach, et al., 2010). *Careya arborea* commonly known as Wild Guava is a deciduous tree found in India that has tremendous effect on various

diseases. The stem bark of *C. arborea* has been widely used in the treatment of tumors, cough, toothache, wounds, dyspepsia, colic, haemorrhoids, intestinal worms, dysentery, leucoderma, epilepsy, abscesses, ulcers and eruptive fever particularly smallpox and antipruritic (Sharma, et al., 2001; Kritikar, et al., 1991; Yoganarasimhan, et al., 2000). Interestingly, the anticancer activity *in vitro* (Subhadradevi, et al., 2010) and *in vitro* (Ramesh, et al., 2013) of the methanolic extract of *C. arborea* has been reported against Dalton's lymphoma ascites (DLA) induced tumor in rats.

However, the major challenge that restricts the development of phytochemicals based therapeutics is their low aqueous solubility. Many phytochemicals are poorly absorbed by human body, hence reducing its bio-availability. With the emergence of nanotechnology, the concept of nanomedicines has provided unique formulation routes to improve the solubility of water insoluble drugs (Liversidge, et al., 2003). An oldest text in ayurveda, the Charak Samhita, mentions the particle size reduction for enhanced therapeutic efficacy of ayurvedic preparations, even though the concept of particle size reduction using nanotechnology has been implemented by modern scientific techniques very recently. Nanoparticle size of Ayurvedic 'bhasmas' (residue of metals/minerals obtained after calcination, incineration or powdering, treated along with herbal products which is used for treatment of critical ailments in ayurveda) has been confirmed in various studies where it is proposed that nanoparticles are responsible for its fast and targeted action and subsequent action upon DNA/RNA molecule and protein synthesis within the cells (Bellini, et al., 2006).

In this work, we had evaluated the effect of nanoformulations of stem bark of *C. arborea* in pancreatic cancer cell lines. *C. arborea* nanoparticles were synthesized by precipitation method and further nanoparticles were characterized using Atomic force microscopy (AFM), Dynamic light scattering (DLS) and Scanning electron microscopy (SEM). These nanoparticles were treated with pancreatic cancer cells to understand the cytotoxic effect of *C. arborea* nanoparticles and its apoptosis.

MATERIALS AND METHODS

Materials: The bark of *C. arborea* was obtained from the Department of Holistic Medicine, Amrita Institute of Medical Sciences and Research centre, Kerala, India. The solvent DMSO (99% pure) was purchased from Merck, India. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich, UK. FBS and Penicillin/streptomycin used for cell culture were purchased from Gibco- Invitrogen, USA. Camptothecin drug for DNA fragmentation analysis was purchased from Sigma-Aldrich, UK.

Cell culture: Human pancreatic cancer, MiaPaCa-2 cells were purchased from NCCS, Pune. The cells were cultured on standard polystyrene tissue culture flasks with a seeding density of 5×10^5 cells/well and incubated at 37°C, 5% CO₂ and 85% relative humidity in an incubator using DMEM medium supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and L- glutamine.

Nanoparticle synthesis of *Careya arborea*: The barks of *C. arborea* were collected, dried and later cut into small pieces and crushed into a fine coarse powder. The powder was subjected to methanolic extraction using soxhlet apparatus for 3 days at 60°C. The methanolic content of the extract was evaporated using IKA(R) ETS D5 at 55°C. The resulting extract was in the form of paste which was stored in a packed container at 4°C for further use. 10mg/ml of solution was prepared in methanol. Adjust the pH to neutral. The nanoparticles of *C. arborea* were prepared by the

process of precipitation. By the controlled addition of distilled water into methanolic extract with continuous stirring, the components got precipitated. This approach leads to generate stable nanoparticles of plant extract.

Nanoparticle characterization: The hydrodynamic particle size of the nano formulations of *C. arborea* were analyzed by using the technique of dynamic light scattering (DLS). The samples suspended in MilliQ water were analyzed using Nicomp™ 380 ZLS-ZP/Particle Sizer (Santa Barbara, USA) with a laser source emitting at 632.8nm, and the scattered intensity measured at an angle of 90°. The surface morphology of the nanoparticles was analyzed by scanning electron microscopy (SEM using JEOLJSM-6490LA). The topography and size of the prepared nanoparticles were further studied using AFM (JEOL SPM 5200). For this, 10µl of diluted nanoparticles solution were drop casted on to a flat mica surface and the dried samples were taken for AFM imaging. 5mg each of the lyophilized sample powder were subjected to thermal degradation studies in the temperature range of 35-45°C at a progressive rate of 10°C/min under inert nitrogen environment.

Cell viability analysis: The viability of cells with prepared nanoformulation was evaluated in pancreatic cancer cell lines and normal cell lines by direct contact method. Analysis of cell proliferation and cytotoxicity was carried out using the colorimetric MTT assay. MTT is an indicator of cell viability and its yellow coloured tetrazolium salt gets reduced to purple coloured formazan crystals by mitochondrial reductase enzyme that can be seen only in metabolically active cells. MiaPaCa-2 cells were seeded in 96 well plates at a seeding density of 8×10^3 cells/well. The cells were treated with varying concentrations of plant extract nanoparticles and incubated for 48 h. The untreated cells served as the positive control (PC) and 1% Triton X-100 treated cells serve as the negative control (NC). After incubation, samples were removed and cells were incubated with 100µl of MTT (0.5mg/ml) at 37°C in a humidified incubator for 4h. 100µl of solubilisation buffer were added to dissolve the sharp formazan crystals. Absorbance of the colour was then measured on a microplate reader (Power Wave XS, BioTek, Vermont, U.S.A.) at 570nm.

Hemolysis: The compatibility of red blood cells (RBC) towards *C. arborea* nanoparticles were analyzed using hemolysis assay by examining the leakage of hemoglobin from the RBC treated with samples. 10ml of fresh blood was collected in a vial containing 1.5ml of acid citrate dextrose (ACD). Two different concentrations of *C. arborea* nanoparticles i.e., 3.6mg/ml and 1.8mg/ml served to be the samples used in the test. 100µl of the sample were mixed with 900µl of blood. The blood samples were treated with 1% (v/v) Triton X-100 as a positive control whereas 0.9 % saline served as negative control. The samples were then incubated for 3 h in an incubator with gentle shaking at 37°C. Further, the samples were centrifuged at 4500 rpm for 10 min to obtain the plasma, which would be in red color indicative of hemolysis. To the upper layer obtained, 900µl of 0.01% sodium bicarbonate was added and the hemoglobin content in the plasma (100µl) was quantified by measuring the optical density at 450 nm, 380 nm and 415 nm respectively (Ashokan, et al., 2012). Hemolysis percentage was calculated as:

$$\text{Hemolysis (\%)} = \frac{\text{Plasma Hb content in sample}}{\text{Total Hb content}} \times 100$$

DNA Fragmentation Assay: The possibility of DNA degradation upon treatment was determined by DNA fragmentation analysis in MiaPaCa-2 cells. The cells were cultured in a 96-well plate with a seeding density of 2×10^5 cells/ml followed by treatment with two different concentrations of the plant extract (3.6mg/ml and 1.8 mg/ml) for 48h. Untreated cells served as positive control and 20 μ M camptothecin served as negative control. After 48 hours of incubation, cells were trypsinized and centrifuged to obtain the cell pellet. The obtained cells were re-dispersed in 1 ml of 1X TE buffer (10mM Tris and 1mM EDTA). 10% SDS and 10mg/ml proteinase K was added to a final concentration of 0.5% and 200mg/ml respectively and incubated at 55°C for 2h. After incubation, 400 μ l of saturated sodium chloride (NaCl) was added and incubated at 4°C for 10min. Centrifugation was done at 13,000 rpm for 10min and supernatant obtained was incubated with 2-3 ml of ice-cold ethanol for 30 minutes to precipitate the DNA. Further, centrifugation was done at 10,000 rpm for 10 minutes and the pellet was resuspended in 10 μ l of 1X TE buffer. Then the DNA obtained was qualitatively analyzed by agarose gel electrophoresis.

Further, agarose gel electrophoresis was performed in order to analyze the presence of fragmented DNA in the treated MiaPaCa-2 cells. 1.6% agarose was prepared in 1X TAE buffer and dissolved completely by heating. 4 μ l of ethidium bromide was added to the agarose solution and poured to a sealed plate in order to make gel with the combs placed on it. Once the gel is solidified, the combs were removed and the gel was placed inside the electrophoresis chamber filled with 1X TAE buffer. 4 μ l of DNA samples mixed with 4 μ l of gel loading dye were loaded into wells on agarose gel. The gel was run at 50V for 1 hour and the DNA fragments were visualized in a UV transilluminator (BioRad Chemidoc).

RESULTS

Nanoparticle characterization: The particle size and topography of the *C. arborea* nanoparticles were analyzed using DLS, SEM and AFM. Figure- 1 showed DLS particle size distribution (a) SEM analysis (b) and AFM topographic image (c) which depicts average particle size to be in the range of 135-150nm.

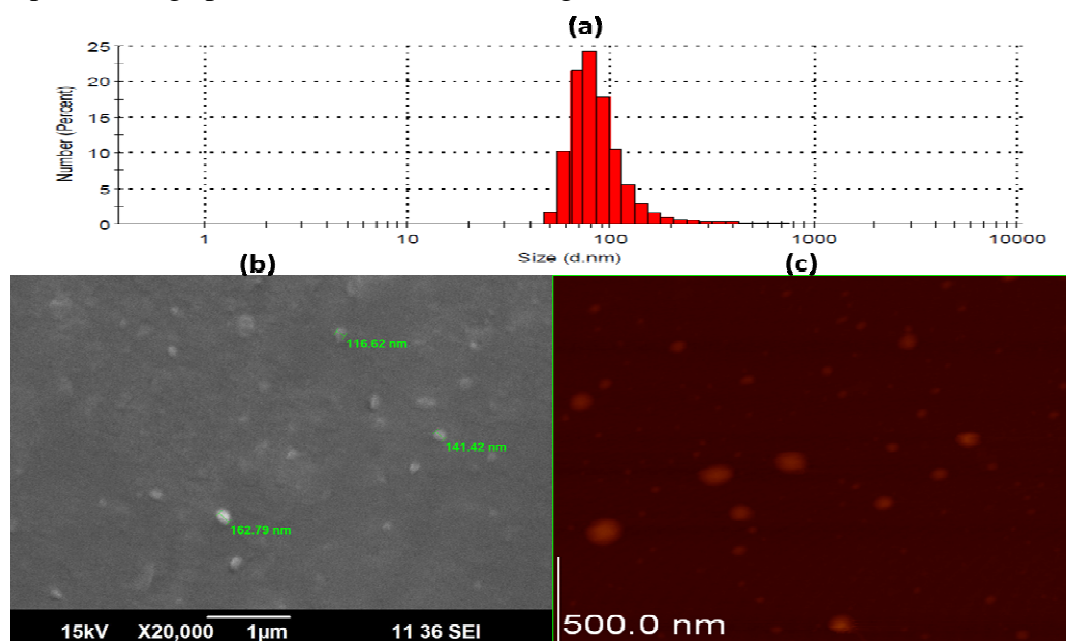


Figure-1: Characterisation of *C.arborea* nanoparticles with a size range of less than 150 nm.

(a) shows the result of Dynamic light scattering (b) shows the SEM (Scanning Electron Microscopy) analysis with an average size of 140 nm and (c) shows the topography of the nanoparticle with AFM (Atomic force microscopy)

Cell Viability Assay: The cytotoxic effect of the plant extract was examined in pancreatic cancer cells (MiaPaCa-2). Cells were exposed to varying concentration of *C. arborea* nanoparticles (0.5mg/ml, 0.9mg/ml, 1.8mg/ml, 2.7mg/ml and 3.6mg/ml). It was observed that concentration dependent reduction was clearly depicted as the concentration increased from 0.5mg/ml to 3.6 mg/ml. A maximum toxicity of 90% was attained with 3.6mg/ml of the *C. arborea* nanoparticles shown in Table-1.

Table-1: The cell viability of MiaPaCa-2 cells treated with the *C. arborea* nanoparticles showing dose dependent cytotoxicity.

Samples	Cell viability in MiaPaCa-2 cells
3.6mg/ml <i>C. arborea</i> nanoparticles	2.84%
2.7mg/ml <i>C. arborea</i> nanoparticles	14.75%
1.8mg/ml <i>C. arborea</i> nanoparticles	25.55%
0.9mg/ml <i>C. arborea</i> nanoparticles	55.47%
0.5mg/ml <i>C. arborea</i> nanoparticles	94.43%
Positive control	100%
Negative control	0.34%

Hemolysis: The percentage of hemolysis after 3 h incubation with two different concentrations of *C. arborea* nanoparticles are shown in Table 2.

Table-2: Showing percentage of hemolysis versus sample *C. arborea* in different concentrations.

Samples	Hemolysis
Positive control (Triton)	90.32%
Negative control (Saline)	1.61%
3.6mg/ml <i>C. arborea</i> nanoparticles	3.22%
1.8mg/ml <i>C. arborea</i> nanoparticles	2.68%

The blood plasma separated from the whole blood after the treatment with positive control, negative control, 1.8mg/ml and 3.6 mg/ml is depicted in figure-2. While, table-2 indicates that the hemolysis induced by the samples were below 5%. Thus, the results proved the compatibility of the plant extract nanoparticles towards red blood cells (RBC).

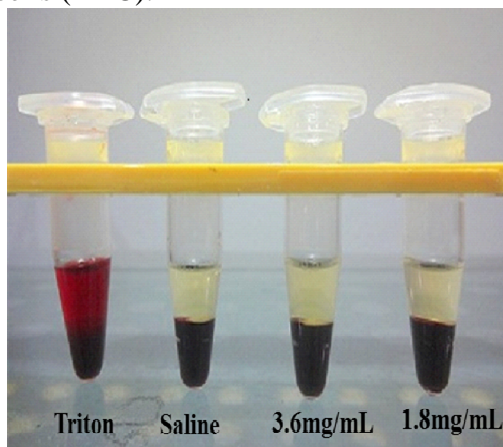


Figure-2: The hemocompatibility of the *C. arborea* nanoparticles with concentrations 3.6 mg/ml and 1.8 mg/ml respectively in saline.

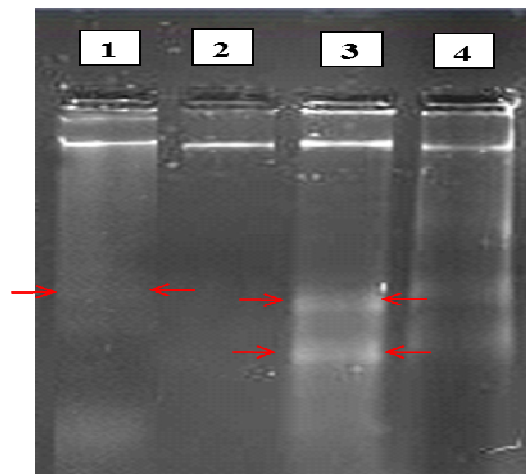


Figure-3: The image indicating DNA fragmentation for the MiaPaCa-2 cells treated with 20µM Camptothecin (Lane 1), positive control (Lane 2), and two different concentrations of *C. arborea* nanoparticles (3.6mg/ml (Lane 3), and 1.98 mg/ml (Lane 4).

DNA fragmentation analysis: DNA fragmentation assay was carried out to check whether the DNA in the pancreatic cancer cells, MiaPaCa-2 cells has degraded upon treatment with the prepared nanoparticle. The DNA of untreated cells served as control. The cells treated with 20 μ M camptothecin served as negative control and two different concentrations of *C. arborea* nanoparticles were incubated for 48h and the procedure for isolation of DNA was efficiently performed. The degradation of isolated DNA was analyzed using agarose gel electrophoresis. The concentration dependent action of *C. arborea* nanoparticles was depicted by the fragments in the DNA of the treated cells (Figure-3).

DISCUSSION

A typical characteristic of pancreatic cancer is aggressive metastasis (Lakshmanan, et al., 2011), including local invasion to adjacent structures and metastasis to lymph nodes and liver in the very early stages. Therefore, efforts must be focused not only on targeting the primary tumor but also controlling metastasis of pancreatic cancer cells. Nanotechnology has already advanced cancer detection and treatment. Abraxane is a major success story in the treatment of metastatic breast cancer, and has extended the scope of nanomedicine to other cancers and chemotherapeutic drugs (Khanna, et al., 2011). The main focus of the present study was to develop nanoformulation of *C. arborea* to enhance its efficacy for pancreatic cancer treatment. Several studies have been accompanied on herbs under a multitude of ethno-botanical levels and a large number of plants possessing anticancer properties have been documented (Aoki, et al., 2005; Gupta, et al., 2004; Kim, et al., 2005; Saito, et al., 2004; Shimizu, et al., 2004 Spiridon, et al., 2006) and this is the first report of nanoformulation effect of *C. arborea* plant on pancreatic cancer cell lines. In this study, we have characterized the nanoformulation from the plant extract by DLS, SEM and AFM, which revealed that the prepared nanoparticles were of size range 135-150nm. *In vitro* cytotoxicity studies of the nanoparticles towards various pancreatic cancer cell lines confirmed its anticancer activity. The cell viability for the pancreatic cancer cells treated with varying concentration of nanoparticles were obtained to be less than 20% in the broad range of analysis. This may be due to the methanolic action of the plant extract and the increased cytotoxic nature of the same. We further confirmed the compatibility of nanoparticles towards haemoglobin by the *in-vitro* hemolysis assay which depicted to be highly compatible. To understand the cell death induced by *C. arborea* DNA fragmentation assay was done and the results showed that the prepared nanoparticles had the potential to fragment the DNA which is the hall mark of apoptosis.

CONCLUSION

Thus nano formulation of 150 nm from methanolic extract of *C. arborea* stem bark was established and its efficient action towards pancreatic cancer cells was tested. We conclude that the phyto nanomedicine of *C. arborea* showed concentration dependent cytotoxicity in the pancreatic cancer cells (MiaPaCa-2) suggesting this nanoformulation of *C. arborea* can be further explored in the *in vivo* mouse models.

Conflict of interest: The author(s) confirm that this article content has no conflict of interest.

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