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Original article

Effect of *Baliospermum montanum* nanomedicine apoptosis induction and anti-migration of prostate cancer cells

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ABSTRACT

Prostate cancer has been diagnosed as the second most frequent and the sixth among the cancer causing deaths among men worldwide. There is a limited scope for the prevalent therapies as prostate cancer advances and they present adverse aftermaths that have put way for us to delve into naturally available anticancer agents. The main objective of the present work is to compile the advantages of ayurvedic herbal formulations with modern technology. *Baliospermum montanum* is a plant that is used in ayurveda for the treatment of cancer and the plant is studied to possess various constituents in it that are responsible for its anticancer activity. Stable nanoparticles of *B. montanum* were prepared from both the aqueous and ethanolic extracts of the plant and its cytotoxic effects were studied on prostate cancer and normal cell lines. Size analysis by DLS and SEM revealed the average size of nanoparticles prepared was 100 ± 50 nm and 150 ± 50 nm for the nanoparticles prepared from aqueous and ethanolic extract respectively. *In vitro* cytotoxicity showed a concentration and time dependent toxicity on prostate cancer cells with cell viability of 22% and 6% with maximum concentration of aqueous and ethanolic nanoparticles respectively, in 48 h. *In vitro* hemolysis assay confirmed that the prepared nanoparticles were compatible with blood with no occurrence of hemolysis. The nanoparticles showed a significant reduction in the colony forming ability and wound healing capacity of the prostate cancer cells. These studies hold the anti cancer potential of the *B. montanum* nanoparticles making it an important candidate for prostate cancer therapy.

1. Introduction

Prostate cancer is ranked the second leading cause of cancer related death in men, globally. It has been diagnosed the second most frequent and the sixth among cancer-causing deaths in men worldwide [1,2]. Some prostate cancers grow and spread rapidly while some others progress very slowly. But as prostate cancer advances, the disease becomes aggressive [3]. Increasing mortality rate in cancer patients has led many researchers to source for potential natural-product based therapeutic compounds. Alternative medicine has evolved as promising nanomaterials and a large number of natural products are being researched as therapeutic targets toward prostate cancer [4]. The solubility issues and the unfavorable biodistribution of these plant-derived compounds in the body are addressed with the recent technological interventions such as nanotechnology [5]. The activity of herbal medicines depends on overall function of a variety of active components, as all the constituents provide synergistic action and thus enhances the therapeutic value [6]. In phyto-formulation research, developing nano dosage forms has large number of advantages for herbal drugs, which includes solubility and bioavailability enhancement, diminution of toxicity, improvement in the pharmacological activity and stability among others [7]. The benefits of using nanotechniques in anticancer therapy are the targeted delivery of anticancer drugs to the site of action with reduced side effects. The combined effects of nanotechnology with ayurveda provide an effectual strategy in intriguing an herbal medicine with enhanced bioavailability profile and minimal toxicity [8].

Baliospermum montanum (Willd.) Muell Arg (*BM*) is a predominant woody medicinal plant categorized in the family Euphorbiaceae and is noted to possess extensively diverse medicinal properties [9,10]. The various parts of the plant such as the roots, leaves, and seeds are used traditionally for the treatment of different ailments. The roots of *B. montanum* are ascribed to possess anticancer [11], antimicrobial [12], immunomodulatory

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[13] and antihelmintic [14] properties. Its root is proclaimed to contain axillarenic acid, baliospermin, and montanin which is responsible for all the wide range of activities [15]. Five phorbol esters (montanin, baliospermin, 12-deoxyphorbol-13-palmitate, 12-deoxy-5β-hydroxyphorbol-13-myristate, and 12-deoxy-16-hydroxy phorbol-13-palmitate) were isolated from *B. montanum* roots and was reported for their antitumor activity [11]. Preliminary phytochemical analysis of *B. montanum* revealed the presence of compounds such as glycosides, flavonoids, tannins and triterpenoids in aqueous and alcohol extracts of the plant [12]. *B. montanum* was a rich source of flavonoids that also accounted for the anti-oxidant and anticancer property of this plant [16].

Herbal decoctions composed of multiple herbs, each possessing a tremendous potential for cancer cure are frequently used in Ayurveda. These herbs own scientifically proven anti-cancerous properties and are used for the treatment of various types of cancers. A paste containing *B. montanum*, *Plumbago zeylanica*, *Euphorbia neriifolia*, *Calotropis procera*, *Semecarpus anacardium* and jaggery (traditional non-centrifugal cane sugar) were applied over the tumors' to treat them [17]. *B. montanum* has been in use since ancient times for the treatment of abdominal tumors. Aqueous and alcoholic extracts of the plant present an interesting cytotoxic activity on HT-29 human colon cancer cell lines. *In vitro* cytotoxic activity against HT-29 cell line at different concentrations was evaluated and the IC50 value calculated was below 50 μg/mL, indicating the potentiality of *Baliospermum montanum* Muell Arg extracts [18]. An aqueous alcoholic extract of the root of *B. montanum* exhibited an activity against the P-388 lymphocytic leukemia *in vivo* [11].

In this study we prepared nanoparticles from the aqueous and ethanol extracts of the plant *B. montanum*, and the cytotoxicity, anti-migrating effect, effect on the colony forming ability and compatibility with blood were evaluated to determine the potency of our nanomedicine.

2. Materials and methods

2.1. Materials

Roots of *B. montanum* were a kind gift from the Holistic Medicine Pharmacy, Amrita Institute of Medical Sciences, Kochi. Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS) and Penicillin/streptomycin used for cell culture were purchased from Gibco-Invitrogen, USA. MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium] was procured from Sigma–Aldrich.

2.2. Cell culture

Human prostate cancer PC3 cells and normal mouse embryonic fibroblast NIH3T3 cells were acquired from the National Centre for Cell Sciences (NCCS), Pune. The cells were cultured on standard polystyrene tissue culture flasks with a seeding density of 5×10^5 cells/mL in DMEM medium supplemented with 10% fetal bovine serum (FBS), and penicillin/streptomycin and maintained at 37 °C, 5% CO_2 and 85% relative humidity in an incubator. Media was changed every 2 days. After the desired confluency (about 90%) was attained, both the prostate cancer (PC3) and normal (NIH3T3) cells were trypsinised from the flask and centrifuged at 1200 rpm for 3 min. The cells were then resuspended in its growth medium for further studies.

2.3. Extraction of Baliospermum montanum

The anticancer activity of *B. montanum* has been attributed to a variety of bioactive compounds in the extract. Presence of compounds such as glycosides, flavonoids, tannins, triterpenoids and phorbol esters in aqueous and alcohol extract of the plant were responsible for its anticancer effect. Since the roots of the plant had most of the anti cancer compounds, it was used for the extraction procedure. Both the aqueous and ethanolic extractions were carried out to do a comparison study of the anticancer activity of both the extracts.

Fresh roots gathered from the plant were shade dried for a week and powdered using a grinder after it was cut down into small pieces. The fine coarse powder was then subjected to successive solvent extraction using soxhlet apparatus for 3 days at 60 °C with water and ethanol as the solvent. A dark brown filtrate was obtained. The aqueous content of the filtrate obtained was vaporized at 100 °C and the ethanol content was at 65 °C using IKA® ETS D5. The resultant paste of the aqueous and ethanolic crude extract was lyophilized and stored in eppendorfs at 4 °C for further use.

2.4. Nanoparticles synthesis of Baliospermum montanum

Nanoparticles of *B. montanum* were prepared by nano-precipitation method. 4 mg of the aqueous extract was weighed and dissolved in 1 mL of distilled water and stirred well. The pH of the solution was made acidic by adding a drop of 1N HCl. With continuous stirring, 10 μL of isopropyl alcohol was added dropwise to the 4 mg/mL of the BM solution at room temperature and the solution was left to stir for 2 h. The solution was then centrifuged at 13,000 rpm for 5 min, to obtain the pellet. The pellet so obtained was washed thrice using distilled water and PBS was added to neutralize the pH. The dry weight of the pellet was noted down to determine the yield of the nanoparticles prepared. The yield was calculated as the percentage dry weight of the nanoparticle pellet formed to the weight of crude extract taken for the preparation. The pellet was then resuspended in distilled water and kept for lyophilization. The lyophilized powder was used for further characterization and cell studies. For the preparation of nanoparticles from the ethanol extract, 4 mg of the crude was weighed and 5 μL of DMSO was added to it. After vortexing it properly, ethanol was added to make up to 1 mL. This 4 mg/mL of the solution was then added dropwise with continuous stirring to water. After centrifugation the pellet obtained was redispersed in water and washed twice before lyophilization.

The nanoparticles prepared from the aqueous extract had a high yield (approx 65%), though the yield of the nanoparticles prepared from the ethanolic extracts were slightly lower. The isopropyl alcohol and DMSO used for the preparations were in diminutive amounts (lower than 0.01%). The nanoparticles were prepared from both the extracts using the nano precipitation technique. Turbidity was observed and that represented the formation of nanoparticles. The particles formed were not visible to the naked eye. The nanoparticles prepared were redispersed in water and a stable suspension was obtained. This solution was then lyophilized to remove the water content, and the powder obtained was stored for further studies. The nanoparticles formed were easily soluble in water whereas it was difficult to dissolve the crude extract in water, especially the ethanol extract.

2.5. Nanoparticles characterization

2.5.1. Dynamic light scattering and zeta potential

The average particle size and polydispersity index of the nanoparticles were analyzed using the technique of Dynamic light scattering (DLS). DLS is widely used for NP size determination in suspension. DLS is based on scattering intensity fluctuations due to Brownian motion of NPs in suspension and the measured hydrodynamic diameter reflects the dimension of NP's together