Leucas aspera Nanomedicine Shows Superior Toxicity and Cell Migration Retarded in Prostate Cancer Cells

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Abstract Prostate cancer is one of the most common malignancies among men worldwide. The main aim of the present work was to clarify the advantages of a nanoformulation of ayurvedic herbal plants. Specifically, we assessed the improved anticancer activity of Leucas aspera nanoparticles compared with methanolic crude extract in PC3 prostate cancer cells and normal cells. L. aspera is a plant that is used in ayurveda due to the antirheumatic, antipyretic, anti-inflammatory, antibacterial, anticancer, and cytotoxic activities. Nanoparticles of L. aspera were prepared from plant methanolic extracts. Cytotoxic effect was studied in the normal and prostate cancer cells. Size and morphology of the formulated nanoparticles was assessed using dynamic light scattering and scanning electron microscopy. In vitro cytotoxicity of L. aspera nanoparticles for PC3 cells was concentration- and time-dependent. In vitro hemolysis assay, cellular uptake studies, cell aggregation studies, and cell migration assay established the anticancerous activity of L. aspera in prostate cancer.

Keywords Leucas aspera · PC3 · Cytotoxicity · Prostate cancer · Colony assay · Therapy
Introduction

Prostate cancer (PCa) is one of the most common age-related cancers affecting men. Prostate specific antigen (PSA) testing is the method that helps to diagnose 70 % of PCa cases at an early curable stage [1]. In 2015, the American Cancer Society estimated that 27,540 American men will die annually from PCa due to the progression of localized diseases into metastatic, castration-resistant PCa (CRPC) [2]. PCa is typically classified in three stages—low-risk, intermediate-risk, and high-risk—due to PSA level, tumor grade, and the extent of primary tumor in the prostate gland [3]. Low-risk or early-stage disease can be cured with radiotherapy or surgery; cure in high-risk disease was reported as about 25 % in one study [4]. Many therapeutic studies show PCa affects patients who are resistant to hormonal therapies that restrain the signaling of androgen receptors; the inhibited signaling drives tumor development and progression. Targeted therapy that induces cellular plasticity can prevent PCa progression [5]. The main advantage for the slowed or halted progression of disease is for early stage diagnosis that often occurs in men in their sixth or seventh decade of life [6, 7]. Clinical strategies like Gleason grade, serum PSA, TNM stage, surgical margin status, performance status, hemoglobin, and weight loss have improved the identification of high- and low-risk stage PCa, and are useful in predicting which therapy is most suitable for patients [8].

Ayurvedic medicinal plants have long been used to treat many diseases including cancer. Of the approximately 20,000 known medicinal plants, 90 % are found wild in different climatic regions and 15 % grow in India [9]. Plant secondary metabolites like tannins, terpenoids, alkaloids, flavanoids, steroids, glycosides phenols, and volatile oils have antibacterial, antimicrobial, cytotoxic, and other effects [10]. This therapeutic potential of the bioavailable material has driven the biosynthesis of nanoparticles of plant extracts [11–13]. Particle size reduction of ayurvedic preparations to the nano-scale enhances the therapeutic efficacy of plants [14–18] and different nanomedicines efficacy has been explored against prostate cancer cells [19–22].

*Leucas aspera* is an ayurvedic medicinal plant from the *Lamiaceae* family. It is commonly known as Thumbai and is found throughout India from the Himalayas to Ceylon. The plant is used traditionally as an antipyretic and insecticide; flowers have aperient, diaphoretic, and insecticide activities, and the leaves are useful in relief of chronic rheumatism, psoriasis, snake bites, and other chronic skin eruptions [23]. A preliminary phytochemical study reported that the methanolic extract of *L. aspera* is enriched in glycosides, alkaloids, flavonoids, phenols, steroids, and tannins and has antifungal, antioxidant, antimicrobial, anti-inflammatory, and cytotoxic activities [24]. Phytochemicals increase the cytotoxicity of cancer chemotherapeutic drugs [25, 26]. Flavonoids and phenolic compounds of *L. aspera* are efficient scavengers of free radical responsible for oxidative stress [27].

Different parts of the plant are active against different diseases. The ethanolic extract of *L. aspera* flowers is active against ulcers in rats [28]. *L. aspera* aerial extract shows higher antitumor activity, free radical scavenging, neoangiogenesis inhibition, and macrophage stimulation compared with the standard drug 5-flourouracil in a mouse model of Dalton’s lymphoma [29]. *L. aspera* root extract has antinociceptive, antioxidant, and cytotoxic activities [30].

Enhancing the anticancer activity of *L. aspera* extract is a worthwhile aim. The present study was undertaken to assess a novel method utilizing nanoparticles and compared the activity with crude extract in prostate cancer cells.
Materials and Methods

Materials and Chemicals

*L. aspera* plants were collected from the Perumbavoor area in Ernakulam District, Kerala. The methanol extraction solvent was purchased from MERCK (India). Commercially acquired PC3 human prostate cancer cells (NCCS Pune), Dulbecco’s minimum essential medium (DMEM), fetal bovine serum (FBS) were supplied by Invitrogen, and penicillin were used in cell culture. Phosphate buffered saline (PBS) was used to wash cells and remove dead cells and nutrient-exhausted medium and Trypsin for cell detachment. Tryphan was used for cell visualization using a hemocytometer. PC3 cells were maintained in culture flasks and well plates at 37 °C, 5 % CO₂, and 85 % relative humidity for the following studies. The cell maintained in an incubator that contains.

Preparation of *L. aspera* Methanolic Extract and Nanoparticles

Methanolic extract of whole *L. aspera* was prepared. Freshly harvested plants were washed and cut into smaller pieces and dried in shade at room temperature. The samples were powdered using a mortar and pestle. Each powdered sample was extracted using 300 mL using soxhlet apparatus at 65 °C in 6 days. The methanol content of the extract was evaporated using an IKA evaporation cylinder at 55 °C after proper cooling of the extract. *L. aspera* whole plant extract nanoparticles were prepared by the precipitation method. Ten milligrams of the methanolic extract of *L. aspera* was dissolved in 1 mL of methanol using a rotary mixer. The dissolved sample was added dropwise to 1 mL of Milli-Q water with constant stirring. HCL was used for pH stability. The solution was centrifuged and the recovered sample was lyophilized.

Size and Surface Morphology

Dynamic light scattering (DLS) is a characteristic method used to measure average particle size. In DLS, the polydispersity index of the nanoparticles is measuring as the intensity of the light scattered as particles undergo Brownian motion. The size of the particles and intensity changes are related. Nanoformulation was prepared as described above and material recovered after centrifugation was collected. The material was resuspended in water and serially diluted for size, surface charge, and stability analyses using a Malvern Zetasizer NanoZS. Scanning electron microscopy (SEM) was used for high-resolution surface structure examination of nanoparticles. The diluted sample was spread over double-sided carbon tape on conductive aluminum stubs and dried. SEM was then conducted.

Fourier Transform Infrared Spectroscopy (FTIR) and Thermal Gravimetric Analysis (TGA)

FTIR was used to determine functional groups present in nanoparticles at different temperatures. Dried crude extract and prepared nanoparticles (4 mg) obtained after lyophilization of *L. aspera* plants was loaded in the FTIR apparatus. Scan range was
400 to 4000 cm\(^{-1}\) and the peak values were recorded. Thermal stability of 9 mg of lyophilized nanoparticles was analyzed by TGA a temperatures ranging from 35–700\(^\circ\)C.

**In Vitro Analysis of Cell Proliferation**

Analysis of cell proliferation and cytotoxicity was carried out using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay. The yellow-colored tetrazolium salt is reduced to purple formazan crystals by mitochondrial reductase enzyme in metabolically active cells. PC3 cells were seeded in 96 well plates at a seeding density of 4 \(\times\) 10\(^5\) cells per well. The cells were treated with varying concentration of the free drugs or the nano-formulations and incubated for 48 h. Cells treated with 1 % Triton X-100 served as the negative control and untreated cells were the positive control. After incubation, 10 \(\mu\)L of MTT and 90 \(\mu\)L medium was added to each well, and the plate was incubated at 37 \(^\circ\)C in a humidified incubator for 4 h. of solubilization buffer (100 \(\mu\)L) was added to each well to dissolve the purple formazan crystals into a colored solution. The plates were incubated at 37 \(^\circ\)C for 1 h. Absorbance was then measured on a PowerWave XS microplate reader at 570 and 660 nm. Percentage cell viability was calculated as:

\[
\text{Cell viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of negative control}} \times 100
\]

**Cellular Uptake**

Cellular uptake of prepared nanoparticles into PC3 cells was determined by fluorescent microscopy. *L. aspera* nanoparticles were prepared with 5 \(\mu\)L Rhodamine dye, washed several times, and lyophilized. Coverslips were etched in concentrated HCL and dried. The cells were cultured on an etched cover slip in a 24-well plate with a seeding density of 15,000 cells per well. After 24 h, 0.25 mg/mL *L. aspera* and 500 \(\mu\)L of medium containing penestrep was incubated for 1 h prior to use for cell treatment. After 48 h, the wells were washed with PBS and the cells were fixed with 300 \(\mu\)L 4 % paraformaldehyde for 20 min. After two PBS washes, cells were exposed to 4′,6-diamidino-2-phenylindole (DAPI) for 5 min to stain cell nuclei. After two PBS washes, each cell preparation was dried overnight in the dark. The slides were then transferred and mounted on a glass slide using DPX (sigma). After complete drying, the prepared slides were viewed by fluorescent microscopy.

**Blood Cell Compatibility Assay**

Hemolysis assay was used to determine the compatibility of red blood cells to the nanoparticles. Nanoparticle-induce rupture of erythrocytes and release of hemoglobin into the surrounding fluids was assessed by a hemolysis experiment. Fresh blood (10 mL) was collected in a vial containing 1.5 mL of acid citrate dextrose. To 450 \(\mu\)L of the blood, 50 \(\mu\)L of each of the different concentrations (0.25, 0.125, and 0.0625 mg/mL) of the prepared nanoparticles and crude was added. Blood samples treated with 1 % (v/v) Triton X-100 and 0.9 % saline prepared similarly was used as positive and negative control, respectively. All the samples were incubated for 3 h in an incubator at 37 \(^\circ\)C. The samples were centrifuged at 4500 rpm for 10 min to obtain the plasma, which would be red in color if hemolysis had occurred. Aliquots
(50 μL) of plasma were collected and 450 μL of 0.01 % Na₂CO₃ was added and mixed slowly. Aliquots (200 μL) of the samples were dispensed into wells of a 96-well plate and the absorbance was read at 380, 415, and 450 nm. Plasma hemoglobin (Hb) concentration was quantified and percentage of hemolysis occurred was calculated as:

\[
\text{Hemolysis (\%)} = \left( \frac{\text{plasma Hb content in sample}}{\text{total Hb content}} \right) \times 100
\]

Isolation of Blood Cells

Ten milliliters of fresh blood was collected in a vial containing 1.5 mL of acid citrate dextrose. Leukocytes were isolated first from the blood. Five milliliters of blood was transferred to a 50 mL Falcon tube followed by addition of 5 mL RPMI 1640 medium. The suspension was gently mixed by inversion. The diluted blood was gently overlaid on Ficoll-Paque (GE Healthcare) in a 1:3 ratio. The material was centrifuged at 400 g for 30 min at 18–20 °C. The upper Ficoll layer was removed and the buffy coated layer containing leukocytes at the interface were carefully collected. Fresh blood (6.5 mL) was added to another Falcon tube and centrifuged at 2500 rpm for 10 min at 12–22 °C. The upper layer containing platelet-rich plasma (PRP) was collected. After separation of PRP, 2 mL normal saline was added to remaining RBC-containing pellet and the suspension was washed by centrifugation at 400 g for 5 min at 22–24 °C. The supernatant was removed the step was repeated two more times. RBCs were collected and diluted with normal saline in a 1:4 ratio.

Blood Cell Aggregation Treatments

Isolated cell fractions (RBCs, white blood cells [WBC], and PRP) were treated with 0.25 mg/mL concentration of L.aspera nanoparticles and crude extracts. Concentrated samples of 0.25 mg/mL were prepared using normal saline. Crude extract contained 0.4 % dimethylsulfoxide (DMSO). Ninety microliters isolated blood and 10 μl of a prepared sample were mixed. Blood cells treated with Triton X-100 or saline, respectively. Each sample was for

Fig. 1 Image of L. aspera plant extract after vaporization
30 min at 37 °C. A drop was dispensed on a glass slide, covered with a coverslip, and viewed by phase contrast microscopy.

**Scratch-Wound Assay**

Cell migration assay (scratch assay) was used to determine the effect of nanoparticles in inhibiting the cell migration process of PC3 cancer cells. PC3 Cells were seeded in wells of a 6-well plate at 2 × 10^5 cells/well and allowed to reach 80 % confluency. A scratch was drawn on the cell monolayer using a 200 μl pipette tip. The plate was washed twice with PBS to remove floating cells and debris. The adherent cells were treated with 0.0625 or 0.25 mg/mL of crude extract and the nanoformulation of the methanolic extract of *L. aspera*. Positive control was treated with medium alone. The wound was photographed at 0, 4, 8, 24, 30, and 48 h using a microscope fitted with digital camera. Area was calculated from a randomly selected field using ImageJ software and wound recovery by migrating cells was determined in percentage of wound closure by following equation:

\[
\text{wound closure (\%)} = \left( \frac{A_f}{A_0} \right) \times 100
\]

where \(A_f\) is the area at the end point and \(A_0\) is the area at the time zero.

**Fig. 2** a DLS histogram showing the size distribution of *L. aspera* nanoparticles. The average size ranged from 200–400 nm. b SEM image of surface morphology of *L. aspera* nanoparticles.

**Fig. 3** a FTIR data of *L. aspera* crude extract and nanoparticles. b TGA analysis of *L. aspera* crude extract and nanoparticles.
Results and Discussion

Plant Extraction and Nanoparticle Formulation

The main aim of this study was to develop a nanoformulation of *L. aspera* to enhance the efficacy of PCa treatment. Figure 1 displays part of the novel process.

Fig. 4  

(a) Images of PC3 cells treated for 48 h with 0.0625, 0.125, and 0.25 mg/mL of *L. aspera* crude extract or extract and nanoparticles. (b and c) Cytotoxicity assay analysis of *L. aspera* nanoparticles and crude extract at 24 and 48 h.
Nanoparticle Characterization

DLS analysis examined the size distribution of *L. aspera* nanoparticles. The peak value represents 220 nm range in 100 % intensity. The average size of nanoparticles range from 200 to 400 nm (Fig. 2a). Zetapotential distribution of the nanoparticles was −22 mv. SEM revealed the spherical shape of the nanoparticles (Fig. 2b) a spherical shape.

**FTIR and TGA**

Comparison of the FTIR spectra of nanoparticles appended methanolic extract and extract alone did not show appreciable differences (Fig. 3a). Significant losses of components were evident in both spectra, perhaps reflecting bondstretching in the functional groups. The functional groups represented phytochemical compounds including glycosides, alkaloids, flavonoids, phenols, steroids, and tannins. TGA analysis confirmed the thermal stability of both preparations. Crude extract degraded faster than nanoparticles.

![Image of cellular uptake of *L. aspera* nanoparticles in PC3 cells. Magnifications are 20× and 60×](image-url)

**Fig. 5** Images of cellular uptake of *L. aspera* nanoparticles in PC3 cells. Magnifications are 20× and 60×
Anticancer Activity of *L. aspera* Nanoparticles versus Crude Extract in PC3 Cells

To determine the anticancer efficacy of *L. aspera* nanoparticles compared with crude extract in PC3 PCa cells, the seeded PC3 cells were treated with different concentrations of nanoparticles PC3 for 24 and 48 h. The stock solution of nanoparticles crude extract was serially diluted to reach concentrations of 1, 0.5, 0.25, and 0.125 mg/mL. After 48 h, cells were treated with a combination of nanoparticles and DMSO. Nanoparticles were cytotoxic in a concentration-dependent manner (Figs. 4a, b).

**Cellular Uptake**

The cellular uptake of prepared nanoparticles was observed using fluorescent microscopy. *L. aspera* extract showed slight autofluorescence when hodamine dye was incubated with extract after nanoparticle preparation. Excess dye in the pellet was removed by PBS wash. PC3 cells were treated with 0.25 mg/mL nanoparticles and compared with the positive control. Microscopy images showed that prepared nanoparticles were easily taken up by the cells without any dose dependence (Fig. 5).

![Fig. 6 Images of hemocompatibility of *L. aspera*. a Photographic of hemolysis assay. b Graphical image of the same experiment](image-url)

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Blood Cell Compatibility

Compatibility of *L. aspera* nanoparticles in different concentrations was evaluated against the controls. Figure 6a plasma separation from whole blood after the treatment protocol. The percentage of hemolysis after 3 h incubation with 0.25, 0.125, and 0.0625 mg/mL *L. aspera* nanoparticles and crude extract is shown in Fig. 6b.

![Image](image_url)

**Fig. 7** Images of blood cell aggregation studies. **a** Isolation of leukocytes by buffy coated layer. **b** PRP, RBC’s, and WBC’s were treated with 0.25 mg/mL nanoparticles Nps and crude extract when combined with control.
Blood Cell Aggregation

RBCs, leukocytes, and PRP were isolated from blood and treated. Cell fractions treated with crude extract appeared crowded compared with fractions exposed to nanoparticles (Fig. 7).

Antimigratory Effect of *L. aspera* Treated PC3 Cells

The antimigratory effect of *L. aspera* nanoparticles and crude extract was higher. After 30 h incubation, cells were fully confluent. The cell morphology was changed with evidence of

![Scratch motility assay](image)

**Fig. 8** a Photographs of migration of *L. aspera* crude and nanoparticle treated PC3 cells compared with positive control. b Graph of percent wound closure
stress visible after 24 h incubation (Fig. 8a). After 30 h, cells treated with L. aspera nanoparticles had died and detached from the plate at the time of PBS wash. Therefore, the area of wound was increased and the graph expresses negative value at 30 h incubation. At prolonged incubation, wound closure was <30 %. L. aspera treated cells migrated 60 % less than control after 30 h incubation (Fig. 8b).

Conclusions

Stable nanoparticles of L. aspera were prepared by nanoprecipitation with methanol. Characterization of the nanoparticles was done. FTIR values represented the functional groups of the nanoparticles and crude extract and TGA analysis data showed the thermal stability of L. aspera nanoparticles were higher than crude extract. The anticancer activity or cytotoxicity of L. aspera nanoparticles was studied in PC3 cancer cells. Nanoparticles induced significant cytotoxicity compared with crude extract. Uptake of L. aspera nanoparticles by PC3 cells. Hemolysis was not evident in L. aspera treated blood, but was in Triton X-100 treated blood. Treated PRP, RBCs, and WBCs did not aggregate. Nanoparticles retarded cell migration. It can be concluded that the nanoparticles containing L. aspera extract are more cytotoxic to PC3 cells compared to crude extract.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References


