

## Novel Boswellic acids Nanoparticles induces cell death in Prostate cancer cells

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(Received 08 January 2012; Revised 11-24 January 2012; Accepted 26 January 2012)

### ABSTRACT

Boswellic acid was successfully extracted from gum resin and its nanoparticles were prepared without using any surfactants or polymer at room temperature. Boswellic acid nanoparticles were prepared by controlled nanoprecipitation method and were characterized by dynamic light scattering (DLS), scanning electron microscope (SEM), atomic force microscopy (AFM) which shows that the size of the particle less than 200nm and spherical in shape, x-ray diffraction (XRD), fourier transformed infra-red spectrum (FTIR), thermo gravimetric analysis (TGA) and differential thermal analysis (DTA) were also done. Its in-vitro cytotoxicity was assessed against different human cancer cell lines and it showed a greater level of cytotoxicity towards prostate cancer. In order to assess whether these particles causes apoptosis DNA fragmentation assay was also carried out, results of which showed that the nanoparticles causes fragmentation of DNA which is a hall mark of apoptosis. So the developed boswellic acid nanoparticles formulation is a promising anticancer agent in the treatment of prostate cancer.

**Keywords:** Boswellic acid; Nanoparticles; Nanoprecipitation; DNA; Apoptosis.

### INTRODUCTION

Cancer is a disease characterized by a group of cells that grow and divide uncontrollably with respect to normal cells, invading and destroying adjacent tissues and even to distant anatomical sites, by a process called metastasis. Basically cancer is a genetic disease. The vast catalogue of cancer cell physiology that collectively dictate malignant growth, self sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replication potential, sustained angiogenesis, tissue invasion and metastasis (Douglas, et al., 2000). Evasion of apoptosis is a key defence strategy of cancer cells. Hence any anticancer agent that activates apoptosis would be a potential anticancer therapeutics. The anticancer agents

may kill the cells either by activation of extrinsic pathway (death receptor mediated) or intrinsic pathway (mitochondrion dependent) apoptotic pathway (Gousia, et al., 2011). Many approaches have been made over the past three decades to tackle cancer. Most recent of these approaches is the use of natural or their semi synthetic products.

*Boswellia serrata* is a kind of deciduous tree found in dry parts of India and China. The gum resin of it has been used for the treatment of inflammatory and arthritic diseases from the very beginning of human civilization (Hans, et al., 1994). The pentacyclic triterpenic acids named Boswellic Acid; present in the gum resin is responsible for its anti-inflammatory property (Safayhi, et al, 1992). Besides anti-inflammatory effects many recent research studies showed that Boswellic Acid also have anticancer effects. Studies shows that Boswellic Acids trigger apoptosis via a pathway dependent on caspase 8 activation (Jian, et al., 2002), inhibit several leukemic cell lines in vitro, inhibit melanoma growth and also dual inhibitor of human topoisomerase I and II (Gousia, et al., 2011; Zhao, et al., 2003), it modulate immune response (Ammon, et al., 2010), possess anti-tumour properties (Agrawal, et al., 2011) and leukotriene inhibitor (Singh, et al., 2008) and against gastric ulcer (Singh, et al., 2008).

The objective of this study was to prepare nanoformulation of Boswellic Acid extracted from natural *Boswellia serrata* gum resin without using any polymer at room temperature and also to check the anticancer activity of the nanoformulation. According to Noyes-Whitney equation, the administration of a substance in a reduced particle size is a promising way to improve bioavailability of poor soluble substances (Noyes and Whitney, 1987). The technique used here is nanoprecipitation which is a physical method to prepare ultrafine or nano-sized particles on the basis of change in the super saturation caused by mixing solution and anti-solvent. This technique has been earlier used in many studies to prepare nano-sized hydrophobic drugs (Zhang, et al., 2006; Wang, et al., 2007). In this method ethanol was used as solvents and distilled water was as anti-solvent. Pure Boswellic acid nanoparticles were successfully precipitated without using any surfactants at room temperature. The Boswellic nanoparticles were characterized with dynamic light scattering (DLS), scanning electron microscope (SEM), atomic force microscopy (AFM), x-ray diffraction (XRD), fourier transformed infra-red spectroscopy (FTIR), thermo gravimetric analysis (TGA) and differential thermal analysis (DTA). Its in-vitro cytotoxicity was assessed against different human cancer cell lines.

## MATERIALS AND METHODS

**Plant materials:** Gum resins of *Boswellia serrata* were purchased from the Holistic Medicine Pharmacy, Amrita Institute of Medical Science, Kochi.

**Chemicals:** Cell culture media like Minimum Essential Medium (MEM), Dulbecco's modified eagle medium (DMEM), 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium (MTT) purchased from Sigma Aldrich. Mouse fibroblast cell line (L929), human prostate cancer cell line (PC3), human amelanotic melanoma cell line (A375), human pancreatic carcinoma cell line (MIA-PaCa 2) for cell culture use were purchased from NCCS, Pune. All other chemicals used were of analytical grade.

**Extraction of Boswellic Acid and Preparation of Nanoformulation:** Nanoparticles of boswellic acids were prepared by nanoprecipitation technique. Briefly 100mg of *Boswellia serrata* gum resin was added to 300ml of methanol and kept for 24h stirring at room temperature. After 24h the solution was filtered and the supernatant was collected. The total extract was concentrated in vacuum and kept for lyophilisation for

48h. The lyophilized powder was dissolved in ethanol and water (anti solvent), was added drop wise under constant stirring condition till the solution turns turbid in colour. The turbidity shows the onset of nanoparticles formation. The solution is the gently heated to remove the excess ethanol content. The resulting nanoparticles were pelleted out by centrifugation at 20,000 rpm for 30 minutes. The pellets so obtained were washed and resuspended in distilled water and kept for lyophilisation. The lyophilized powder was used for further characterization and studies.

**Estimation of total organic acids (boswellic acids) in the extract:** The total organic acid content of the extract prepared was done by volumetric analysis. Briefly adequate amount of lyophilized extract powder was dissolved in ethanol. Few drops of the indicator phenolphalein are added to it. Under stirring condition the solution was titrated with 0.1M NaOH solution.

**Nanoparticle size analysis and stability studies:** The size distribution of the boswellic acid nanoparticles was analysed by dynamic light scattering (DLS using DLS ZP or particle sizer Nicomp<sup>TM</sup>3680 ZLS). The surface morphology and average size of the nanoparticles was further analysed by scanning electron microscopy (SEM using JEOLJSM-6490LA) and atomic force microscopy (AFM using JEOLJSPM5200) respectively. For SEM and AFM sample preparations the nanoparticle suspensions were diluted and dropped on to metallic studs and mica sheet pasted on AFM stud respectively. The stubs were then dried, sputter coated and scanned. The surface charge and thereby colloidal stability of the nanoparticle were also analysed by zeta potential measurement (DLS-ZP or Particle sizer Nicomp<sup>TM</sup>380 ZLS).

**Nanoparticles Characterizations:** Spectral analysis of the extract before and after nanoparticles preparation was analysed by fourier transformed infra-red spectroscopy (FTIR) using KBr method. The physical nature and the thermal behaviour of above said samples were done by X-ray diffractometer (XRD using PAN analytical X'pert PRO XRD), thermogravimetry and differential thermal analysis (TG/DTA using SIITG/DTA6200EXSTAR) respectively.

**Cell Culture:** L929, A375, MIA-PaCa cell lines were maintained in MEM while PC3 cell line in DMEM respectively supplemented with 10% foetal bovine serum (FBS). The cells were incubated at optimal temperature of 37°C in CO<sub>2</sub> incubator with 5% CO<sub>2</sub>. Upon confluency the cells were trypsinized and the cell suspension was centrifuged at 3000 rpm for 30 min and then resuspended in growth medium for further experiments.

**Cytotoxicity studies:** For cytotoxicity experiments, cells were seeded at a density of 10,000 cells/well into a 96 well plate. MTT assay was performed to evaluate cytotoxicity of the prepared nanoparticles. Briefly MTT is a colorimetric test based on the selective ability of viable cells to reduce the tetrazolium component of MTT in to purple colored formazan crystals. Four different concentrations of (2, 1, 0.5 and 0.25mg/ml) were prepared by diluting the sample with the media. After reaching 90% confluency, the cells were incubated with different concentrations of the nanoparticles for a period of 24h. Cells in media alone devoid of nanoparticles acted as positive control and wells treated with Triton X-100 as negative control. The cells were incubated with MTT solution for 4h followed by 1h incubation with solubilization buffer. The optical density of the solution was measured at a wavelength of 570nm using a Beckman Coulter Elisa plate reader (BioTek Power Wave XS). Triplicate samples were analyzed for each experiment. Cell viability was expressed as the percentage of the negative control.

## RESULTS AND DISCUSSION

**Volumetric analysis:** Volumetric analysis shows that the total organic acid (boswellic acid) in the extract is 60%.

**DLS and zeta potential:** The size distribution of the nanoboswellic acid particles in solution is determined by DLS and showed (Figure 1) that the average particles size of nanoboswellic acid particles in solution is 259nm (hydrodynamic diameter) and the prepared particles showed zeta potential value of -42.88 mV, which indicates that the nano-suspension is having a good stability. In order to confirm the size and morphology of the prepared particles SEM and AFM analysis were carried out.

**Scanning electron microscopy (SEM) and Atomic force microscopy (AFM):** SEM image of the nanoboswellic acid particles were shown in Figure 2. SEM image of the nanoparticles clearly shows the size and shape of the particle. The size of the particle less than 200nm and it has a spherical morphology. The two dimensional and three dimensional picture of nanoboswellic acid particle were analysis by AFM and shown in Figure 3, which also shows the size of the particle is about 190nm and spherical in shape. In order to be used for drug delivery application the nanoparticles size should be tuned in such a way that the NPs should be large enough to prevent the rapid leakage into blood capillaries at the same time they should escapes the capture by macrophages lodged in the reticuloendothelial system (Zahr, et al., 2006). Hence the boswellic acid nanoparticles lying in the optimal size range (below 200nm) is suitable for drug delivery applications.

**X-Ray diffraction analysis (XRD):** XRD graph of the crude boswellic acid extract and nanoboswellic acid were shown in Figure 4. Both crude boswellic acid extract and nanoboswellic acid showed a crystalline nature. However a reduction in the intensity of the peak was observed in nanoboswellic acid particle which is the due to the reduction in the size of the particle and there by leading to peak broadening.

**Thermo gravimetric analysis (TGA):** In order to assess the thermal stability and degradation of both crude boswellic acid and nanoboswellic acid TGA were done and shown in Figure 5. It was shown that up to 200°C both the samples were thermally stable and above 200°C both started degradation but the nanoformulation had a faster degradation rate compared to crude.

**FTIR spectra:** FT-IR spectra of crude boswellic acid extract and boswellic acid nanoparticles were shown in Figure 6. On conversion from crude to nanoformulation most of the peaks were retained which shows that the constituents in nanoformulation are same as that of crude extract. In the crude boswellic acid, the peaks were found at 3448  $\text{cm}^{-1}$  corresponding to hydroxyl group, 2954  $\text{cm}^{-1}$  of C=C and 1703  $\text{cm}^{-1}$  of the carboxyl group. Similar peaks were found in boswellic acid nanoparticles too.

**MTT:** Different concentrations of boswellic acid nanoparticles were treated with different cancer cell lines such as Pancreatic, Breast, Melanoma and Prostate in order to study the anti-cancerous activity. For that concentrations ranging from 0.25mg/ml to 2mg/ml were used. MTT results were shown in Table 1, in all the cell lines selected for the study anti-cancerous activity of the boswellic nanoparticles was found to be concentration dependent, which is as concentration decreases the percentage of viability increases. In all cell lines the highest percentage of viability was observed for 0.25mg/ml concentration. Overall from the table we can conclude that 0.25mg/ml concentration is relatively non cytotoxic concentration in most of the cancer cell lines while the higher concentration of 1mg/ml and 2mg/ml are highly cytotoxic. In PC3 cell lines except for 0.25mg/ml rest all concentrations showed much similar percentage of viability in comparison with the negative control. Hence the novel

boswellic nanoparticles showed a greater cytotoxicity activity towards prostate cancer cell lines (PC3), melanoma cell lines and pancreatic cell lines in comparison to that of breast cancer cell line.

**DNA fragmentation assay:** To test whether boswellic acid nanoparticles causes apoptosis in PC3 cells, DNA fragmentation assay was carried out and it is shown in Figure 7. From which, it is clear that the nanoboswellic acid particles in Lane B and Lane C causes DNA fragmentation which is known as hallmark of apoptosis. Lane A having the positive control, camptothecin did not show any DNA fragmentation as the PC3 cells may have develop resistance to it (Hasegawa, et al., 2006).

## CONCLUSION

Boswellic acid, the active component in the gum resin of *Boswellic serrata* was successfully extracted and made in to nanoformulation using a controlled precipitation technique. Size analysis by SEM and AFM revealed that the prepared nanoparticles have spherical shape with a size below 200nm. *In vitro* cytotoxicity studies of the nanoparticles towards various cancer cell lines confirmed its anticancer activity against prostate cancer especially. In order to understand the mechanism of action, 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.

**Acknowledgements:** We acknowledge the financial support from Department of Science and Technology, India under the Nano-initiative Program monitored by Prof. C. N. R. Rao. One of the authors, Saji U would like to acknowledge the Department of Science and technology, M-Tech grant for the financial support. The authors are also thankful to Mr. Sajin P Ravi, Mr. Girish, Mr. Sarath, Mr. Sudheesh Kumar and Miss Sreeja V Nair for their help in SEM, AFM, TG/DTA and XRD studies.

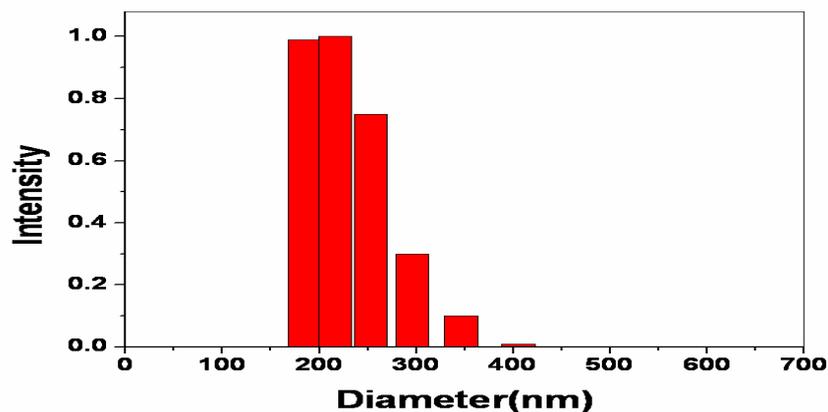
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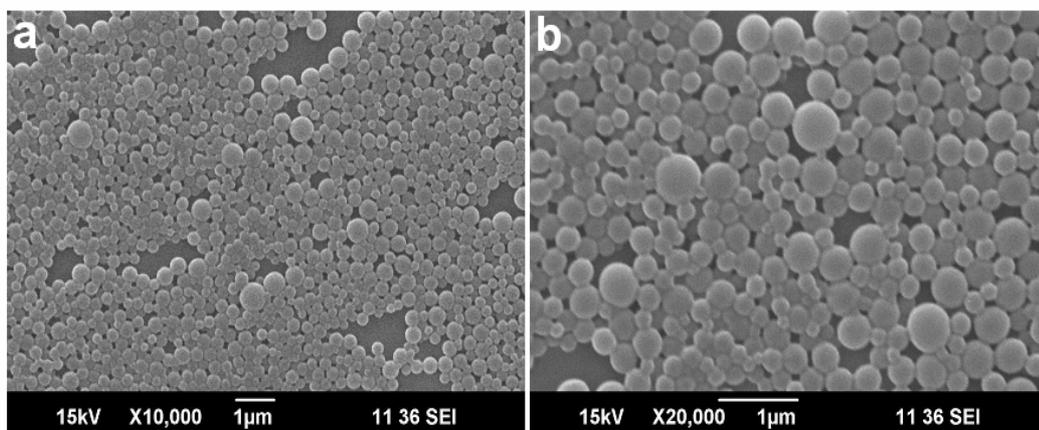
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**Table-1: MTT assay of nanoboswellic acid particles in different cancer cell lines at different concentrations.**

Different cancer cell lines	% of cell viability at different concentration					
	Ctrl+	Ctrl-	2mg/ml	1mg/ml	0.5mg/ml	0.25mg/ml
MIAPaCa	100	12	14	15	26	96
MCF7	100	24	25	26	48	84
A375	100	12	11	12	55	82
PC3	100	10	13	12	14	96



**Figure-1: Size distribution of nanoboswellic acid particle in DLS.**



**Figure-2: SEM picture of nanoboswellic acid particle.**

- (a) Lower magnification (b) Higher magnification.

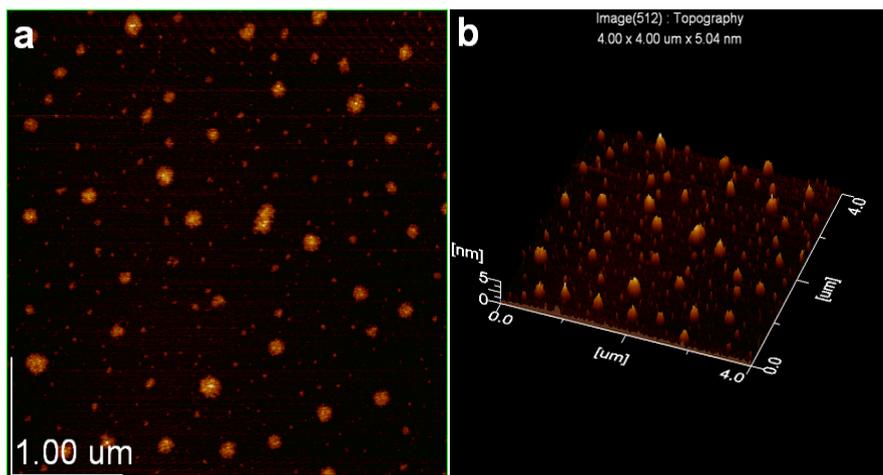


Figure-3: AFM picture showing the surface morphology of the nanoboswellic acid particles.

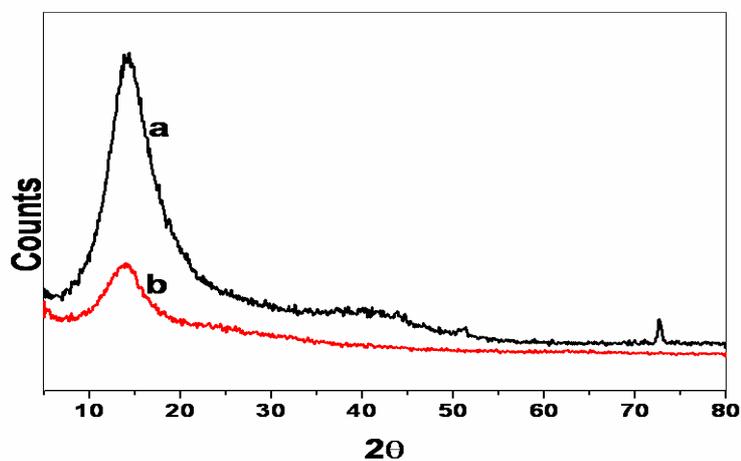


Figure-4: XRD graph of crude boswellic acid and nanoboswellic acid.

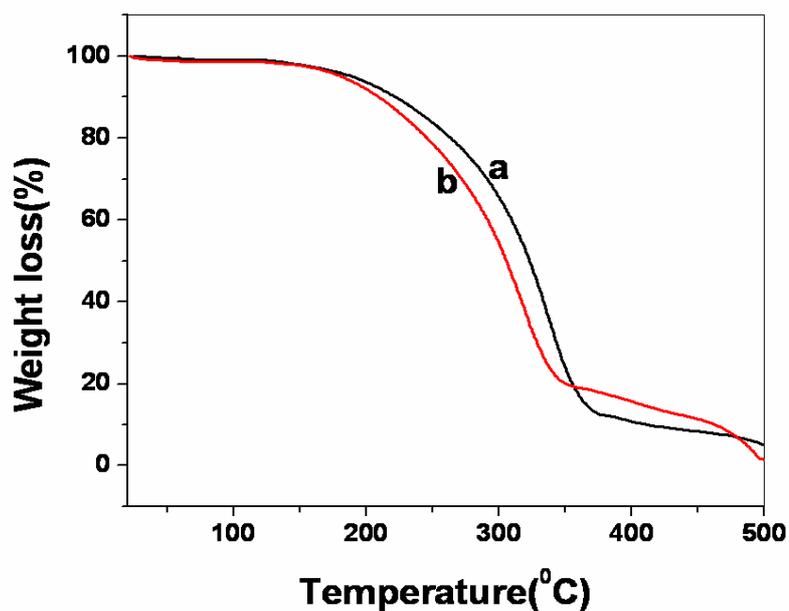


Figure-5: Thermo gravimetric analysis of crude boswellic acid and boswellic acid nanoparticles.

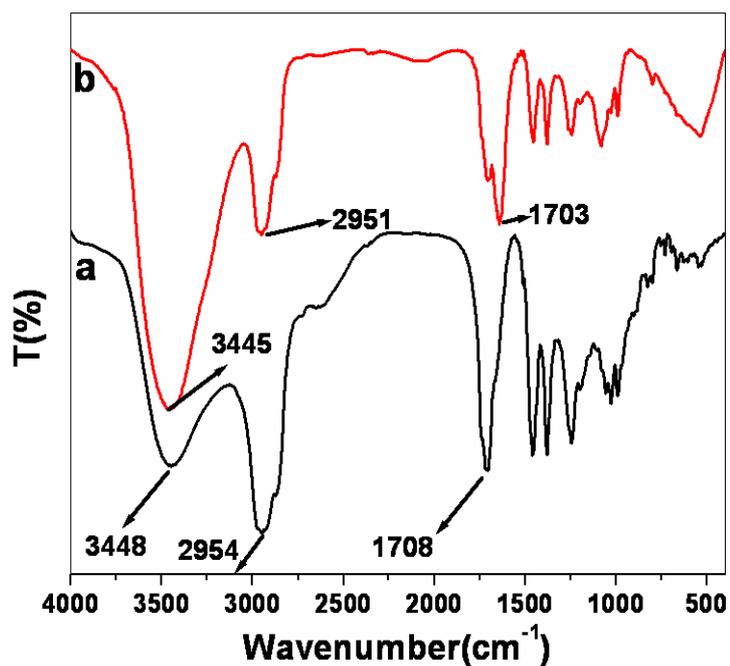


Figure-6: FTIR spectra of crude boswellic acid (a) and boswellic acid nanoparticles (b).

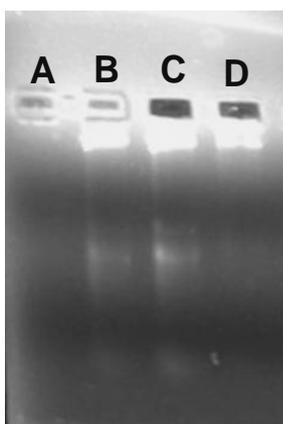


Figure-7: Shows the DNA fragmentation assay of boswellic acid nanoparticles in PC3.

- Lane A; 4 $\mu$ M camptothecin drug, Lane B; 2mg/ml boswellic acid nanoparticles, Lane C; 1mg/ml nanoboswellic acid and Lane D; without any treatment.