Original Article

Inhibition of Proliferation of Human Prostate Carcinoma Cell, PC3 by *Bauhinia racemosa* Lam. via Induction of Apoptosis

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ABSTRACT

Background: Prostate cancer is the second foremost cause of cancer death in Western males, the progression of which may be a consequence of defect in apoptotic machinery. **Objective:** Thus, aim of the study was designed to investigate inhibition of proliferation of human prostate carcinoma cell line, PC3 by Methanolic extract of *Bauhinia racemosa* Lam (MEBR). **Methods:** MTT assay was performed to evaluate antiproliferative effect of MEBR on prostate carcinoma cell line while DAPI and DCFH-DA staining studies were performed to investigate the underlying mechanism in antiproliferative effect of MEBR. **Results:** Total flavonoid content in MEBR was found to be 868 mg QE/g dried extract. MEBR exposure to PC3 cells significantly ($p<0.001$) increased the cytotoxicity in a concentration dependent manner. The percent cytotoxicity data indicates that exposure of PC3 cells to 25 and 50 μg/ml of MEBR, cytotoxicity was 15.42% and 36.67% respectively as compared to control, which further increased to 56.82% and 78.04% ($p<0.001$) at 75 μg/ml and 100 μg/ml respectively. IC_{50} value was found to be 66.85 μg/ml of the extract MEBR. MEBR induced significant ($p<0.001$) nuclear condensation in a concentration dependent manner depicting the induction of apoptosis in PC3 cells. MEBR elevated the significant ROS activity level and fluorescence intensity in a concentration dependent manner as compared to control in PC3 cells exposed to MEBR. **Conclusion:** MEBR possesses antiproliferative activity and leads to PC3 cell death via induction of apoptosis mediated through excessive ROS generation. Hence, the extract MEBR may be potentially precious for application in chemotherapeutic drug developments for prostate cancer..

Key words: Prostate cancer, PC3, *Bauhinia racemosa,* Apoptosis, Reactive oxygen species.

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INTRODUCTION

Nowadays, cancer has become the foremost cause of death in human beings where prostate cancer is the most frequently diagnosed one and second foremost cause of cancer death in males of Western countries.[1,2] Chemotherapy is mainly employed clinically for the management and treatment of such cancer.[3] Thus, scientists are struggling to find effective clinical treatment of such cancer and are investigating for novel anticancer agents from all sources. The investigation of anticancer agents from plant kingdom has been greatly emphasized.[4]

The plant *Bauhinia racemosa* Lam. belonging to the family Caesalpiniaceae is a small deciduous tree used in the indigenous system of medicine and is very common in foothills upto 1000 m in India and Srilanka.[5,6] It is commonly named as Mountain Ebony (English), Kachnal/ Kanchanara/ Sonpatta (Hindi), Gul-e-anehnal (Urdu) and Sona/ Sonpatta or Apta (Marathi).[7] The various parts of the plant viz., stem bark, leaves and roots are practiced in various indigenous systems of medicine and popular among the various ethnic groups in India for the

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cure of variety of ailments.[6,7] The bark of *B. racemosa* is reported to have antifilarial, abortifacient, anthelmintic, analgesic, antipyretic, antimalarial, anti-ulcerogenic, hepatoprotective and various other pharmacological activities.[7,8] Previously reported phytochemical constituents from its bark are octacosane, β-amyrin, β-sitosterol, triterpenoids and sterols.[7,9] Thus, in order to investigate the antiproliferative effect of Methanolic extract of *B. racemosa* (MEBR) on prostate cancer cell line and to elucidate the underlying mechanism of cancer prevention, MTT assay and assessment of apoptosis induction were carried out.

MATERIALS AND METHODS

Authentication of Plant Material

B. racemosa Lam bark collected from Chittoor of Andhra Pradesh was authenticated by the botanist, authentication office, Sri Venkateswara University, Tirupati, India (Authentication no.: SVUBH/LE/1876).

Preparation of Drug Extract

Collected *B. racemosa* Lam bark was shade dried, roughly powdered and extracted by methanol using Soxhlet extractor to get the extract, MEBR. Appearance of colorless methanol in the siphon tube of extractor was considered as the termination of Soxhlet extraction. The obtained extract was filtered while hot and then concentrated to dryness in a rotary evaporator (Buchi Rotavapor-R, Labco, India) under controlled temperature and pressure.[10]

Cell Line Culture

PC3, the human prostate carcinoma cell line obtained from National Centre for Cell Sciences (NCCS), Pune, India was cultured in Dulbecco's modified Eagle's medium (DMEM, Himedia) with added 10% (v/v) fetal calf serum (Himedia), 0.1mM non-essential amino acids (NEAA), 1mM sodium pyruvate, 1% antibiotic solutions, 2mM L-glutamine and 1.5g/l sodium bicarbonate. The cells were grown in CO_2 incubator (Excella ECO-170, New Brunswick) maintained at 37°C with 5% CO_2 in humidified air.

Determination of Total Flavonoid Content

One millilitre of 1mg/ml MEBR solution was added into a tube containing 2ml of double-distilled water. Then, 3ml of 0.5% $NaNO_2$, 0.30 ml of 10% $AlCl_3$ and 2ml of 1M NaOH were added to it at 0, 5 and 6 min sequentially. Volume was make upto 10ml with double-distilled water, thoroughly mixed and allowed to stand for 15 min. The absorbance was recorded at the maximum wavelength of 510 nm using UV-spectrophotometer (Shimadzu, Japan). Flavonoid content was estimated from quercetin standard curve. All the determinations were performed three times.[11]

MTT Assay for Cytotoxicity in PC3 Cells

The 1×10^4 PC3 cells per well were seeded in 100 µL complete medium in the wells of the 96-well culture plate for 24h in CO_2 incubator. Different concentrations of MEBR (25, 50, 75, 100 µg/ml) prepared directly in the medium were added to the wells in triplicate as per experimental design. After 21h of treatment, 10 µL of methyl-thiazolyl-tetrazolium dye (MTT, Himedia) solution was added in the wells and the plate was further incubated for another 3h in CO_2 incubator. Then, the supernatant was discarded and 100 µL of dimethyl sulphoxide (DMSO) was added to each well for 10 min at 37°C. The absorbance was read at maximum wavelength of 540 nm by a microplate Elisa reader (BIORAD-680) using the wells without MEBR as control. The percent cytotoxicity was calculated according to the equation [% cytotoxicity = $\{1 - (A_T/A_C)\} \times 100$], where A_C is the absorbance value of the control and A_T is the absorbance value of treated and concentration lethal to 50% of the cells (IC_{50}) was found out.[12]

Cell Morphology Analysis

The effect of MEBR was analyzed for morphological changes in the cultured cells. The 1×10^4 PC3 cells per well were seeded in 100 µL complete medium in the wells of the 96-well culture plate for 24h in CO_2 incubator. Then, the cells were exposed to different concentrations of MEBR (25, 50, 75, 100 µg/ml) prepared directly in the medium for 24h. The cellular morphology was observed by inverted phase contrast microscopy (Nikon Eclipse Ti-S, Japan).[12]

DAPI Staining for Analysis of Apoptosis Induction

The 1×10^4 PC3 cells per well were seeded in 100 µL complete medium in the wells of the culture plate for 24h in CO_2 incubator. The cells in triplicate were then exposed to two different concentrations of MEBR (25 and 75 µg/ml) for 12h. Then, extract plus media was removed, cells were washed with Phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 10 min. Subsequently, the cells were permealized with the buffer (0.5% Triton X-100, Merck, India and 3% paraformaldehyde) and stained with 50 µL of fluorescent nuclear dye 4',6-diamidino-2-phenylindole (DAPI, Himedia). After 1h, the cells were observed for the fluorescence intensity and apoptotic cells. Images were taken and number of cells was counted by using fluo-

rescent microscope (Nikon Eclipse Ti-S, Japan). The percent apoptotic cells was calculated according to the equation

$$\% \text{ apoptotic cell} = \frac{\{(\text{apoptotic cells} + \text{late apoptotic cells})}{(\text{total no of cells})\}} \times 100 \qquad 13$$

DCFH-DA Staining for Intracellular ROS Activity Level Analysis

Microscopic fluorescence imaging and quantitative fluorometric analysis were used to analyze generation of Reactive oxygen species (ROS) in PC3 cells exposed to MEBR. The 1×10^4 cells per well were seeded in culture plates, kept in CO_2 incubator for 24h and then exposed to two different concentrations of MEBR (25 and 75 µg/ml) for 12h. Then, mixture was again incubated with 10mM 2,7-dichlorodihydrofluorescein diacetate stain (DCFH-DA, Sigma-Aldrich, USA) for 30 min in CO_2 incubator. The mixture was aspirated and replaced by 200 µL of PBS in the wells. The plates were kept on a shaker for 10 min at room temperature in the dark. Intracellular fluorescence was analyzed by an inverted fluorescence microscope. For quantitative fluorometric analysis, 1×10^4 cells per well were re-seeded in black bottomed culture plate and kept in CO_2 incubator for 24h. Cells in triplicate were then exposed to two different concentrations of MEBR (25 and 75 µg/ml) for 12h. Then, cells were again incubated with 10 mM DCFH-DA for 30min in CO_2 incubator. Fluorescence intensity was measured with a multi-well microplate reader (Synergy H1 hybrid multi-mode microplate reader, BioTek) at wavelength of 528 nm relative to the control. Increased intensity of intracellular fluorescence was indicative of increased intracellular ROS activity level.[14]

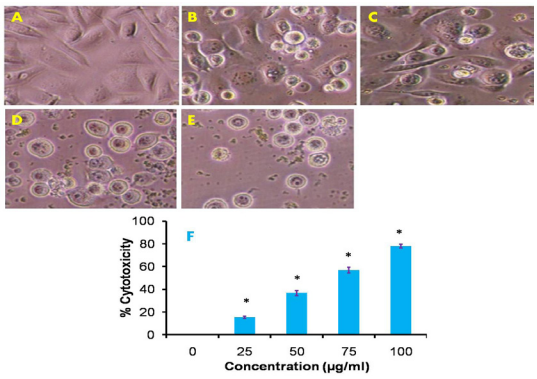

Figure 1: Morphological view (A-E) and percent cytotoxicity (F) of PC3 cells exposed to grading concentrations (0-100 µg/ml) of methanolic extract of *B. racemosa* bark, MEBR as measured by MTT assay at 24 h.

Statistical Analysis

All the data were expressed as Mean± Standard deviation (SD). Statistical significance was determined by one-way ANOVA using the GraphPad Prism program. Value of $P<0.05$ was considered statistically significant.

RESULTS

Determination of Total Flavonoid Content

Total flavonoid content in MEBR was found to be 868 mg QE/g dried extract where QE denotes to quercetin equivalent (Table 1).

MTT Assay for Cytotoxicity in PC3 Cells and Cell Morphological Analysis

MTT assay for cytotoxicity in PC3 cells demonstrated the antiproliferative effect of extract MEBR in prostate carcinoma, PC3 cell line. It was found that morphological shapes of the cells were drastically changed in concentration dependent manner. Images (Figure 1A, 1B, 1C, 1D and 1E) revealed that cells were detached themselves from the well surface and changed to round shape in the groups exposed to MEBR. Significant cytotoxicity characterized by deformation of cell bodies, cel-

Table 1: Determination of Total Flavonoid Content in MEBR.

	Conc (µg/ml)	Observed absorbance				Flavonoid content (mg QE/g extract)
		1st	2nd	3rd	Mean± SD	
Quercetin	50	0.18	0.18	0.19	0.18±0.01	
	100	0.27	0.28	0.29	0.28±0.01	
	200	0.45	0.47	0.50	0.47±0.02	
	300	0.70	0.72	0.73	0.72±0.02	
	400	0.87	0.88	0.89	0.88±0.01	
	500	0.99	0.99	0.99	0.99±0.01	
MEBR	500	0.54	0.53	0.54	0.54±0.01	868

All the values were expressed as mean± SD (n=3).

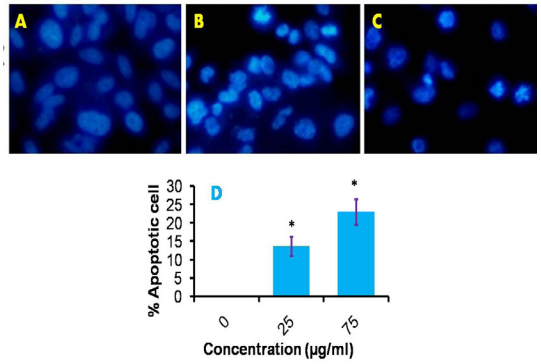

Figure 2: Apoptosis (A-C) and percent apoptotic cells (chromatin condensation) (D) of PC3 cells exposed to grading concentrations of methanolic extract of *B. racemosa* bark, MEBR (0-75 µg/ml) as measured by DAPI staining at 12 h.

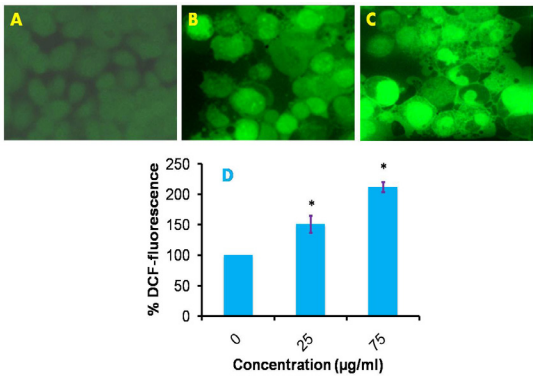

Figure 3: Intracellular ROS activity level (A-C) and percent DCF-fluorescence (D) in PC3 cells exposed to grading concentrations of methanolic extract of *B. racemosa* bark, MEBR (0-75 µg/ml) as measured by DCFH-DA staining at 12 h.

lular shrinkage and surface detachment was observed in the groups exposed to MEBR at higher concentrations. MEBR exposure to PC3 cells significantly (p<0.001) increased the cytotoxicity in a concentration dependent manner. The percent cytotoxicity data indicates that exposure of PC3 cells to 25 µg/ml and 50 µg/ml of MEBR lead to cytotoxicity to 15.42% and 36.67% respectively as compared to control, which further significantly (p<0.001) increased to 56.82% and 78.04% at 75 µg/ml and 100 µg/ml respectively. From the graph obtained (Figure 1.F), IC_{50} value was found to be 66.85 µg/ml of the extract MEBR.

DAPI Staining for Analysis of Apoptosis Induction

Images (Figure 2A, 2B and 2C) depict the details of MEBR induced apoptosis observed by using fluorescent DAPI staining. The cells with condensed and fragmented nuclei were regarded as apoptotic cells. As observed from the graph (Figure 2D), MEBR induced significant (p<0.001) nuclear condensation in a concentration dependent manner depicting the induction of apoptosis in PC3 cells.

DCFH-DA Staining for Intracellular ROS Activity Level Analysis

Fluorescent images (Figure 3A, 3B and 3C) of PC3 cells stained with DCFH-DA depicted the effect of MEBR-induced intracellular ROS activity level. The supplementary images and the graph of percent DCF-fluorescence (Figure 3D) suggested that MEBR elevated the significant ROS activity level and fluorescence intensity in a concentration dependent manner as compared to control in PC3 cells exposed to MEBR. It indicates that increased ROS activity level in PC3 cells is involved in the induction of apoptosis.

DISCUSSION

Prostate cancer is the most often diagnosed cancer and the second foremost cause of cancer death in Western males, the progression of which may be a consequence of defect in apoptotic machinery.[2] Hence, antiproliferative effect of MEBR was investigated in present study on prostate cancer cell line by MTT assay and underlying mechanism of cancer prevention was elucidated by assessment of apoptosis induction.

Yellowish MTT solution on reduction in metabolically active cells yields water insoluble purple colored needle-shaped MTT formazan crystals which get deposited as an extracellular deposit by exocytosis.[15] The amount of formazan increases with increase in number of viable cells while decreases with increase of cytotoxicity.[16] Results of the study clearly demonstrated that MEBR exposure to PC3 cells significantly (p<0.001) increased the cytotoxicity and decreased the amount of formazan in a concentration dependent manner. IC_{50} value was found to be 66.85 µg/ml of the extract MEBR.

Most of the cancer cells fail to undertake apoptosis due to mutilation of apoptosis.[17] Inducing apoptosis in cancer cells can hence be an efficient approach in anticancer therapy. The cells undergo changes during the process of apoptosis characterized by plasma membrane blebbing, chromatin compaction, DNA fragmentation, cell shrinkage and collapse into small intact fragments called apoptotic bodies.[18] Apoptosis increases the cell membrane permeability to DAPI, a DNA-specific dye that displays a blue fluorescence and thus leaves stronger blue fluorescence.[19] Results of the study clearly demonstrated that MEBR induced significant (p<0.001) nuclear condensation in a concentration dependent manner depicting the induction of apoptosis in PC3 cells.

Anticancer and chemopreventive agents prompt cells to produce ROS which induce apoptosis.[7,20] The DCFH-DA passes the cell membrane, gets cleaved by intracellular esterases to DCFH and thereby trapped within the cells. DCFH gets oxidized to the fluorescent DCF (2,7-dichlorofluorescein) emitting bright fluorescence.[21] Results of the study clearly demonstrated that MEBR elevated the significant ROS activity level and fluorescence intensity in a concentration dependent manner as compared to control in PC3 cells exposed to MEBR.

Phenolics and flavonoids are well known to have cytotoxic effect and induce apoptosis in various cancer cell lines due to their pro-oxidant property.[22] Results of the study have demonstrated the presence of flavonoid in MEBR as 868 mg QE/g dried extract. Hence, flavonoids present in MEBR may be responsible for the antiproliferative activity of the extract.

CONCLUSION

The present study shows a relationship between antiproliferative effect of MEBR and apoptotic induction and shows that the cytotoxicity was due to the induction of ROS mediated apoptosis. It confirmed the potential of MEBR in human prostate cancer and therefore, may be potentially precious for application in chemotherapeutic drug developments.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

MEBR: Methanolic Extract of *Bauhinia racemosa* Lam; **MTT:** Methyl-Thiazolyl-Tetrazolium; **$NaNO_2$:** Sodium Nitrite; **$AlCl_3$:** Aluminium Trichloride; **NaOH:** Sodium Hydroxide; **PBS:** Phosphate Buffered Saline; **DAPI:** 4',6-Diamidino-2-Phenylindole; **ROS:** Reactive Oxygen Species; **DCFH-DA:** 2,7-Dichlorodihydrofluorescein Diacetate; **ANOVA:** Analysis of Variance; **QE/g:** Quercetin Equivalent Per Gram; **IC:** Inhibitory Concentration.

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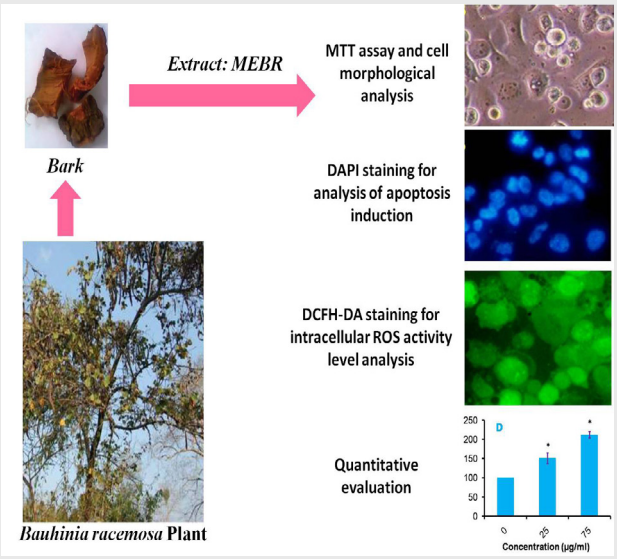
PICTORIAL ABSTRACT



SUMMARY

- Prostate cancer is the second foremost cause of cancer death in Western males, the progression of which may be a consequence of defect in apoptotic machinery. Thus, aim of the study was designed to investigate inhibition of proliferation of human prostate carcinoma cell line, PC3 by MEBR.
- Total flavonoid content in MEBR was found to be 868 mg QE/g dried extract. MEBR exposure to PC3 cells significantly increased the cytotoxicity in a concentration dependent manner. MEBR induced significant nuclear condensation in a concentration dependent manner depicting the induction of apoptosis in PC3 cells. MEBR elevated the significant ROS activity level and fluorescence intensity in a concentration dependent manner as compared to control in PC3 cells exposed to MEBR.
- MEBR possesses antiproliferative activity and leads to PC3 cell death via induction of apoptosis mediated through excessive ROS generation.

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