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Research Article

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INDUCTION OF APOPTOSIS AND CYTOTOXIC ACTIVITIES OF METHANOLIC EXTRACT OF *Careya arborea* Roxb BARK

V. Subhadradevi*, J. Christy, K. Asok Kumar, M Umamaheswari and A. T. Sivashanmugham

Department of Pharmacology, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, India.

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ABSTRACT

In the present study anticancer activity of the methanolic extract of bark of *Careya arborea* Roxb was evaluated. Cytotoxicity was assayed by Trypan blue dye exclusion method and MTT assay and apoptosis was determined by DNA fragmentation assay and morphological studies were carried out. CABE were found to be cytotoxic to DLA and EAC cell lines in a dose dependent manner in Trypan blue dye exclusion method. Concentration needed for 50% inhibition was found to be 200µg/ml in DLA cell lines and 120µg/ml in EAC cell lines respectively. In MTT assay concentration needed for 50% inhibition was found to be 17µg/ml in L929 cell lines. We found that cytotoxic effect of CABE was associated with apoptosis on DLA cell lines by determination of morphological changes and DNA fragments. Thus it indicates that this substance can show different activities and has potential for cancer prevention which was dose dependent.

Keywords: *Careya arborea, C*ytotoxicity, Apoptosis, DNA fragmentation.

INTRODUCTION

Cancer is a serious clinical problem that possesses significant social and economic challenges to the healthcare system. Despite improved imaging and molecular diagnostic techniques, cancer continues to affect millions of people globally.[1] In many countries, cancer is the second leading cause of death after heart diseases.[2] Lung, colorectal and stomach cancer are among the five most common cancers in the world for both men and women.[3]

Plants have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer. *Careya arborea* Roxb. Family Lecythidaceae, is named 'the slow match tree'. It is a large tree found throughout India in deciduous forests and grassland. Stem bark of *Careya arborea* is traditionally used in the treatment of bronchitis, epileptic fits, astringents, antidote to snake-venom and skin disease. It is also used as remedy for diarrhea, dysentery with bloody stools and ear pain.[4] The leaves are useful in ulcers. The flowers are useful in healing vaginal ruptures caused by child birth. The fruits are acrid, astringent, and aromatic and are useful in dyspepsia.[5]

MATERIALS AND METHODS

Plant material

The bark of *Careya arborea Roxb* was collected from Kottayam district in Kerala, India during the month of May 2009. The plant was identified and authenticated by Mr. G.V.S. Murthy, Joint Director, Scientist, C-I/C, Botanical survey of India, Tamil Nadu Agricultural University Campus, Coimbatore bearing the reference number BSI/SC/5/23/09-10/Tech-285.

***Corresponding Author:**
Varadharajan Subhadra Devi
Lecturer, Department of Pharmacology, College of Pharmacy,
Sri Ramakrishna Institute of Paramedical Sciences,
Coimbatore – 641044, India
Contact no: +91-9994284820,
Email: subhaakshay@gmail.com

Preparation of the extract

The fresh bark of *Careya arborea* Roxb was collected. The collected bark were dried in shade under room temperature, powdered mechanically and sieved through No.20 mesh sieve. The extraction was carried out by continuous hot percolation method using Soxhlet apparatus. The solvent used was a mixture of methanol. About 50 g of powder was extracted with 400 ml of solvent. The extract was concentrated to dryness under controlled temperature between 40-50 ºC. The bark extract of *Careya arborea* was used for the experiment. The percentage yield of *Careya arborea* Roxb bark was 7.45%w/w.

Chemicals

DMEM medium, phosphate buffered saline, streptomycin, penicillin from Himedia Laboratories, Mumbai. RNA-ase, proteinase K, 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT), agarose, ethidium bromide were purchased from Sigma Aldrich, St. Louis, USA. All chemicals were of analytical grade.

Cell lines

Ehrlich ascites carcinoma (EAC) cells and Dalton's lymphoma ascites (DLA) cells were used for short term *in vitro* cytotoxicity experiments. Mouse L929 cell lines (Lungs fibroblast) were used for long term *in vitro* cytotoxicity experiments.

Short term in vitro cytotoxicity assay by trypan blue dye exclusion technique

Cells were aspirated from the peritoneal cavity of tumor bearing mice. The cells were washed three times using PBS. The viability of the cells was checked using trypan

blue. The cell suspension was added to tubes containing various concentrations of the test compounds and the volume was made up to 1ml using phosphate buffered saline (PBS). These assay mixtures was incubated for 3h at 37^{0}C and then 1ml of trypan blue was added after incubation and the no. of dead cells was counted using a haemocytometer.[6]

Long term in vitro cytotoxicity by MTT assay

Cells were seeded in 96-well flat-bottom plates and allowed to adhere for 24h at 37^{0}C with 5% CO_2 atmosphere. Different drug concentration was added and incubated further for 48 hrs. Before 4h of the completion of incubation, 20µl of MTT (5mg/ml) was added. Percentage of dead cells was determined using an ELISA plate reader set to record absorbance at 570nm.[7]

Apoptosis Detection Assay

Prepare DMEM medium (912mg/100ml) containing benzyl pencillin 1mg/litre, Ambistyrin (1mg/litre) adjusts the pH to 7.0 with sodium bicarbonate. To the medium add 2 × 10^6 DLA cells, 2µl trypsin and different drug concentration and then make the final volume to 2ml. Incubate at 37^0 for various hours. After incubation centrifuge the cells at 10,000 rpm for 10min, discard the medium and wash the cells for 2 times in NTE buffer. Suspend the cells in 2ml NTE buffer and 2% trypsin (100µg/ml) and add 20% SDS (25µl/ml). Incubate the cells at 37^{0}C for overnight. Add 1ml NTE buffer saturated phenol and 1ml chloroform and shake the vial 12 times. Centrifuge at 10,000 rpm for 10 min and transfer the upper portion to another vial and add 1ml chloroform, repeat this for 4 times. Take the pellet (DNA) and dissolve in TAE buffer. Then the dissolved DNA is subjected to horizontal electrophoresis.[8]

Morphological studies

To detect the morphological changes during apoptosis, 5×10^6 DLA cell pellets were smeared on a clean glass slide. The slides were fixed in methanol and stained with hematoxylin-eosin method. The slides were observed at 100X for the changes.

RESULTS

Cytotoxic activity of *Careya arborea* bark on DLA cells and EAC cell lines was evaluated by Trypan blue dye exclusion method. Cytotoxicity of CABE to DLA and EAC cells culture is shown in Table **1**.

Table 1. Effect of CABE against DLA and EAC cell lines by Trypan blue dye exclusion method

Sample	Concentration (µg/ml)	Percentage Cytotoxicity (DLA cell lines)	Percentage Cytotoxiciy (EAC cell lines)	CTC_{50} (DLA cell lines)	*CTC_{50} (EAC cell lines)*
Control		1.12± 2.41			
CABE	50	23.13± 3.10	29.62± 1.41	200.23± 2.12	120.21±11.2
	100	30.31± 2.87	44.14± 8.52		
	200	48.58± 3.60	66.95± 4.21		
	500	91.95± 3.00	96.31± 4.80		
Curcumin	50	38.14± 1.01	40.12± 3.54	97.22± 6.51	85.56± 2.51
	100	52.39± 7.12	69.38± 1.43		
	200	70.62± 7.14	85.24± 2.12		
	500	100.24± 1.12	100± 3.62		

Values are mean± S.D, n=3; P<0.01 when compared with control

Viable cells which remained unstained by trypan blue were counted in a haemocytometer. The percentage cytotoxicty of the DLA cells at different concentrations ranging from 50µg/ml to 500µg/ml. Extracts showed a dose dependent inhibition of the growth of DLA cells. Curcumin was used as the reference drug and it produced 100% cytotoxicity at 500µg/ml. Compared to standard, CABE was found to have cytotoxic effect but slightly weaker when compared to standard drug. This result emphasized cytotoxic nature of CABE against DLA cells.

The percentage cytotoxicity of the EAC cells at different concentrations ranging from 50µg/ml to 500µg/ml. Extracts showed a dose dependent inhibition of the growth of EAC cells. Curcumin was used as the reference drug and it produced 100% cytotoxicity at 500µg/ml. Compared to standard, CABE was found to have cytotoxic effect but slightly weaker when compared to standard drug. This result emphasized cytotoxic nature of CABE against EAC cells.

The relationship between concentration of CABE and their cytotoxic effect on L929 cells was investigated by MTT assay (Table **2**).

Cells were treated at concentrations ranging from 10-100µg/ml for 48h and then the percentage of cell viability was analysed. CABE was significantly inhibited the proliferation of L929 cells in a dose-dependent manner. Extract showed a concentration dependent cytotoxicity to cultured L929 cells that was almost comparable with that of curcumin.

Table 2: Effect of CABE against L929 cell lines by MTT assay

Sample	Concentration (µg/ml)	Absorbance at 570 nm	Percentage Cytotoxicity	*CTC_{50}*
Control		0.306±0.10		
CABE	10	0.212±0.023	30.64±6.12	17.14±3.23
	25	0.118±0.054	61.50±5.23	
	50	0.012±0.043	95.12±2.10	
	100	0.000±0.000	100.00±4.12	
Curcumin	10	0.196±0.067	40.12± 2.88	15.45± 2.45
	25	0.085±0.098	75.00± 4.12	
	50	0.000±0.000	100.00± 2.97	
	100	0.000±0.000	100.00± 3.09	

Values are mean± S.D, n=3, P<0.01 when compared with control

Microscopic examination of CABE treated and control DLA cells revealed that the cell death is due to apoptotic characteristics (Figure **1**). On studying the morphology of cultured DLA cells, apoptotic cells were distinguished as

highly condensed/shrunken cell with nuclear elongation, margination, fragmentation, cell blebbing and presence of apoptotic bodies. Active normal DLA cells were characterized by less eosinophilic cytoplasm and nucleus with uniform distribution of chromatin material.

Figure 1. Cell Death

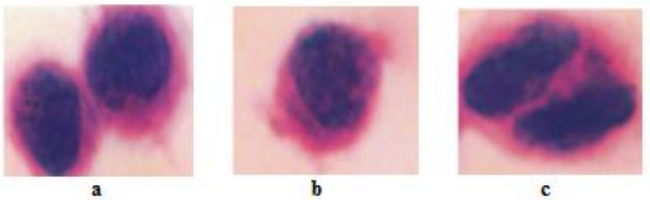
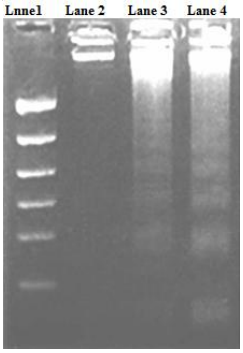


Figure a. showing normal DLA cells with uniform distribution of basophilic chromatin material.
Figure b and **c.** CABE showing vertical cleavage of nucleus, vaccuolation and chromatin condensation of the nucleus.

We observed a significant increase in DNA fragmentation with increasing concentration of CABE (Figure **2**). Agarose gel electrophoresis of DNA isolated from cells also showed the presence of typical DNA ladder. At exposure to 50µg/ml of CABE, fragmented DNA was clearly observed in DLA cells, whereas control cells did not provide ladders.

Figure 2. DNA Fragmentation of CABE



Lane 1: DNA molecular weight marker.
Lane 2: DNA isolated from fresh DLA cells.
Lane 3: DNA isolated from CABE treated (25µg/ml) DLA cells.
Lane 4: DNA isolated from CABE treated (50µg/ml) DLA cells.

DISCUSSION

Cancer is the leading cause of mortality worldwide, and the failure of conventional chemotherapy to effect a major reduction in mortality indicates that new approaches are critically needed.[9]

Plants have played an important role as a source of effective cancer agents, and it is significant that 60% of currently used anticancer agents are derived from natural sources, including plants, marine organisms and microorganisms. The control of cell proliferation is crucial in maintaining cellular homeostasis and loss of this mechanism is a principle hallmark of cancer cells. Thus the inhibition of tumor cell growth without side effects is recognized as an important target for cancer therapy.[10] In this study CABE inhibits growth in DLA and EAC in a dose dependent manner. The results of the trypan blue dye exclusion assay indicated that CABE extract could inhibit the growth of DLA and EAC cells.[11]

The current investigation was also designed to explore the CABE extracts for their oncolytic properties using standard MTT assay. These cancer effects were further studied using morphological assessment of cancer cells using agarose gel electrophoresis. The MTT reduction as a cell viability measurement is now widely chosen as the most advantageous end point.[12] The results of the MTT assay indicated that CABE significantly inhibited the proliferation of L929 cells in a dose-dependent manner.

Anti-tumor agents, that can modulate apoptosis, may be able to affect the steady state of cell populations that are helpful in the management and therapy of cancer.[13] Since it has been suggested that apoptosis plays a critical role in tissue homeostasis and cancer development, apoptosis modulation has become an interesting target for both therapeutic and preventive approaches to cancer treatment.[14]

CONCLUSION

Results of the present study suggested that CABE could induce tumor cell death by physiological and pathological means. It has been established that apoptotic cells display DNA fragmentation at internucleosomal sites followed by morphological changes and loss of membrane integrity. This study revealed that the potency of CABE to bring about apoptotic changes decreases with dose. Thus induction of apoptosis in tumor cells that make them more render for host phagocytic clearance without initiating inflammation, could be attributed for *Careya arborea's* tumoricidal activity.

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