ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

Vol 7, Suppl 1, 2014

ISSN - 0974-2441

Research Article

APOPTOSIS AND FLOWCYTOMETRIC STUDIES OF *BAUHINIA VARIEGATA* BARK EXTRACT

SHYAM KUMAR B*[1], K. ISHWAR BHAT[2]

[1]Department of Quality Assurance, Shree Devi College of Pharmacy, Airport Road, Mangalore -574142, [2]Department of Pharmaceutical Chemistry, N.G.S.M Institute of pharmaceutical sciences, Nitte University, Deralakatte, Mangalore. Email: shyambknair@gmail.com

Received: 14November 2013, Revised and Accepted: 13December 2013

ABSTRACT

Objectives: The present investigation is focused on *in vitro* anticancer activity of flavonoid rich fraction of *Bauhinia variegata* bark ethanolic extract (BBEE) on HeLa cell lines.

Method: The Ic_{50} value of extract was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) and SRB (sulforhodamine B) assay. The extract treated HeLa cell lines were subjected to apoptosis determination by Ethidium bromide/Acridium orange fluorescent staining method. Flow cytometric analysis was also carried out to analyse the effect of plant extract on cell cycle progression.

Results: The Ic_{50} value of the extract was found to be 191.5 µg/ml against HeLa cell lines by MTT and SRB assay. BBEE extract induced apoptic cell death in HeLa cell lines. The flow cytometric analysis on BBEE treated HeLa cell lines shows the arrest of cell cycle in G0/G1 phase.

Conclusion: The studies indicate cytotoxic activity of *Bauhinia variegata* bark extract on HeLa cell lines.

Keywords: *Bauhinia variegata* bark, HeLa cells, apoptosis, Flow cytometry, MTT, SRB

INTRODUCTION

Herbal medicines play a vital role in traditional method of healing around the world. Almost 70 % of modern medicines in India are derived from plant species. Among the vast reserve of plant wealth few of them were studied for their biological activity and screened for phytoconstituents which are responsible for therapeutic effect. These aspects give enormous potential for research in herbal medicines and establish their credibility in modern settings.

Bauhinia variegata Linn (Family- Fabaceae) is an herbaceous medicinal plant found in the sub Himalayan tracts and forests of India. The plant is called Kanchanara in Sanskrit and bark of this plant is found to possess many medicinal properties. Kanchanara guggula is an ayurvedic preparation made from the bark of *Bauhinia variegata* against scrofulous tumours [1]. An emulsion prepared from the bark is used along with ginger against scrofulous enlargement on glands of the neck [2]. In view of these facts the antitumor studies of *Bauhinia variegata* bark was carried out to ascertain the claims made about this plant in traditional literature.

MATERIALS AND METHODS

Collection of plant material and preparation of the extract

The bark of the plant *Bauhinia variegata* was obtained from Kulai, Mangalore. It was authenticated by Dr. Neoline J. Pinto, H.O.D, Dept. of Botany, St. Agnes College, Mangalore, India. The dried bark was coarse powdered in a cutter and grinding mill. The extract was defatted with petroleum ether (60-80°C) and then subjected to continuous extraction in a soxhlet apparatus with ethanol (90 %) as solvent [3]. It was finally concentrated with the help of a vacuum evaporator and kept in a dessicator.

Phytochemical studies

The *Bauhinia variegata* ethanolic bark extract (BBEE) prepared was then subjected to qualitative analysis for the identification of various plant constituents [4]. The presence of flavonoids indicated in the chemical tests upon the extract was confirmed by thin layer chromatographic studies (TLC). To detect the flavanoids, 0.2 mg of extract was applied on a silica plate and chromatogram was developed in a solvent system of chloroform and acetone (6:4). The plates were sprayed with 1% $AlCl_3$, observed under UV light [5].

Cell culture

The HeLa cell lines were purchased from National Centre for Cell Science, Pune, India. The cells were cultured as monolayer in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10 % FBS, 1 % glutamine and 100 U/ml penicillin-streptomycin at 37°C in 5 % CO_2 atmosphere, stocks were maintained in 25 cm^2 tissue culture flasks. A stock solution of 1mg/ml of BBEE was prepared in 0.5 % dimethyl sulfoxide (DMSO) and diluted for further use.

Cytotoxic screening

MTT assay

Different concentrations of drug extract BBEE were prepared (500, 250, 125 and 62.5) by serial dilution and cytotoxicity was observed by MTT assay [6]. MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5 dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent DMSO (Himedia) and the released, solubilised formazan product was measured at 540 nm. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. MTT assay were performed to determine the *in vitro* cytotoxicity of the extracts. The monolayer cell culture was trypsinized and the cell count was adjusted to $1x10^5$ cells/ml using a medium containing 10 % newborn calf serum. 0.1 ml of diluted cell suspension (approximately 10,000 cells) was added to each well of 96 well microtitre plates. When a partial monolayer was formed, after 24 hours, the supernatant was flicked off, and washing of the monolayer with the medium and 100 µl of different drug concentrations were added to the cells in micro titre plates. Incubation of the plates was carried out at 37 °C in 5 % carbon dioxide atmosphere. The microscopic examination and recording of observations were done every 24 hours. The drug solutions in the well were discarded and 50 µl of MTT in DMEM without phenol red was added to each well after 72 hours. The plates were gently shaken and incubated for three hours at 37°C (5 % carbon dioxide atmosphere). The supernatant was removed and 50 µl of propranolol was added. The plates were gently shaken to solubilise the formed formazan. The absorbance was measured

using a microplate reader at a wavelength of 540 nm. The percentage of growth of inhibition was calculated using the formula:

Percentage of growth inhibition = 100- (mean OD of individual test group /mean OD of control group) x 100

SRB assay

The monolayer cell culture (HeLa cells) was trypsinized and the cell count was adjusted to1.0 x 10^5 cells/ml using a medium containing 10 % new born calf serum. 0.1 ml of the diluted cell suspension (approximately 10000 cells) was added to each well of the 96 well micro titre plate (Torreson). After 72 hours, 50 µl of 50 % trichloro acetic acid was added to the wells gently in such a way that it forms a thin layer over the drug dilutions to form an overall concentration of 10 %. The plates were then incubated for one hour at 4°C. The plates were flicked and washed five times with tap water. This is to remove the traces of medium, drug and serum and was then air dried. The dried plates were stained with SRB for 30 minutes. The unbound dye was removed by washing. Tris base (100 µl of 10 mM) was added to the wells to solubilise the dye. The plates were shaken vigorously for five minutes. The absorbance was measured using micro plate reader at wavelength of 540 nm and calculated by the formula:

Percentage of growth inhibition = 100- (mean OD of individual test group /mean OD of control group) x 100

Determination of apoptosis by fluorescent staining method

DNA-binding dyes Acridine orange (AO) and Ethidium bromide (EB) (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells. BBEE (191.5 µg/ml) was added to 80 % confluent HeLa cells and incubated for 24 hours followed by washing with PBS (phosphate buffered saline). A mixture of AO (100 µg/ml) and EB (100 µg/ml) was added and incubated at room temperature for 10 min. After washing with PBS the cells were observed in fluorescent microscope (Olympus CKX41Optika Pro5 camera). 5- Fluorouracil was used as standard [7].

Analysis of DNA content and cell cycle distribution by flow cytometry

Muse cell cycle analysis kit (Millipore, USA) utilizes a premixed reagent which includes the nuclear DNA intercalating stains propidium iodide (PI) which discriminates cells at different stages of the cell cycle based on the differential DNA (G0/G1, S and G2/M). For fluorescence activated cell sorting (FACS) analysis, HeLa cells were treated with BBEE for 24 hours. After overnight incubation, cells were trypsinzed and spun at 300Xg for 5 minutes and washed once with 1X PBS. The cells were fixed with 1ml of ice cold ethanol and incubated at -20°C for 5 hours [8]. The ethanol fixed cells were washed once with PBS followed by 200 µl of Muse cell cycle reagent and incubated for 30 minutes and analyzed on Muse flow cytometer (Millipore, USA).

RESULTS

Preliminary phytochemical screening

The preliminary phytochemical screening of the extract showed the presence of flavonoids. The TLC plates after being sprayed with $AlCl_3$ and visualised under UV light of 365 nm showed two yellow coloured spots of Rf values 0.85 and 0.57 respectively which is characteristic to flavonoids.

Cytotoxic screening

BBEE was evaluated for its anti tumour potential against HeLa cell lines. IC_{50} value was recorded as 191.5 µg/ml. The extract showed moderate cytotoxicity against the tested cell line. The average cytotoxicity was determined by MTT and SRB values (Table 1).

Table 1: IC_{50} value of *Bauhinia variegata* bark extract on HeLa cell line (µg/ml)

Test samples	Cell line	MTT	SRB	Mean ± SEM
BBEE	HeLa	184 ± 6.16	199 ± 3.34	191.5 ± 4.75

Values are expressed as mean ± SEM (n = 6); BBEE-*Bauhinia variegata* bark ethanolic extract; CTC_{50}- Concentration of the sample required to kill 50 % of the cells; MTT- 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide.

Apoptosis study

The HeLa cell lines were checked for apoptosis determination by treating with BBEE (191.5 µg/ml) and investigating for morphological changes. The induction of early apoptosis was detected by the presence of chromatin condensation and bright green stained nuclei when compared with control (untreated) and standard (5-flouro uracil). The results are shown in (Figure 1, Figure 2 and Figure 3).

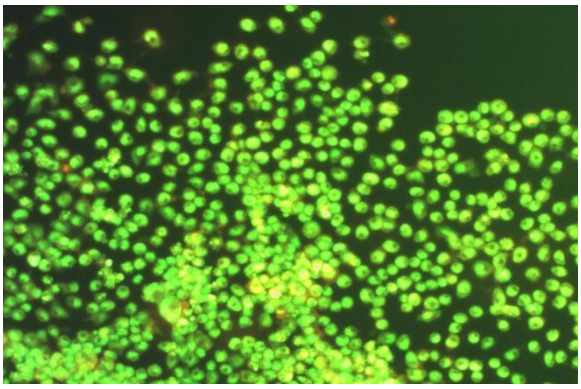

Fig. 1: Untreated HeLa cell lines (Control) shows no apoptosis as cells stained green

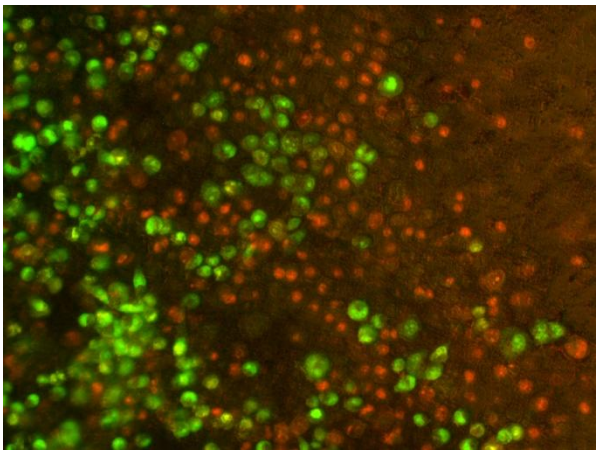

Fig. 2: BBEE treated HeLa cells showing apoptosis with orange stain

Analysis of DNA content and cell cycle distribution using Muse cell cycle kit by flow cytometry

Flow cytometric analysis of HeLa (191.5 µg/ml) cells treated with ethanolic extract showed significant inhibition of cells at G0/G1 phase. There was 72.9 % increase in cells arrested at G0/G1 phase when compared with untreated control where as the S phase cells decreased proportionally. This finding indicates that cell cycle distribution was blocked significantly in the G0/G1 phase in cells treated with ethanolic extracts of *Bauhinia variegate* (Figure 4 and Figure 5).

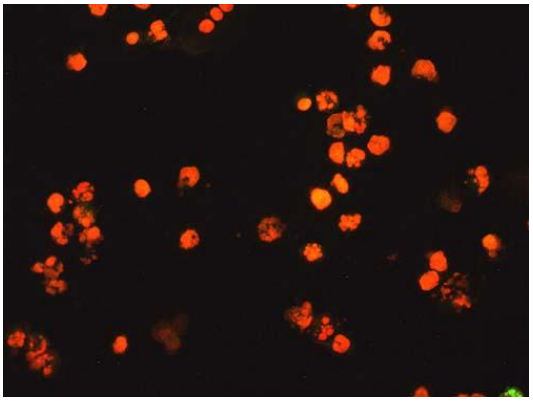

Fig. 3: 5-Flouro uracil treated HeLa cells showing apoptosis with orange stain

	G0/G1	S	G2/M
% Gated :	10.6	62.6	24.5
Mean :	2449.7	4802.1	6088.3
% CV :	17.5	10.2	7.3

Fig.4: Flow cytometric analysis of HeLa cells untreated

	G0/G1	S	G2/M
% Gated :	84.5	8.5	6.7
Mean :	1223.0	3822.8	5548.8
% CV :	51.5	14.8	7.7

Fig. 5: Flow cytometric analysis of HeLa cells treated with sample (BBEE)

DISCUSSION

Cancer can be described as the major non communicable disease that India is going to face in the coming decades with projected cases of over 10 lakhs by 2015[9]. The increased socio economic burden arising out of the treatment regimens and adverse side effects is supposed to affect a larger population which raises concerns. Exploring ayurveda and validating the herbal medicines that was used since historic time can be promising. In this aspect the indigenous plant *Bauhinia variegata* was studied for its anticancer potential, owing to the fact that apart from screening studies no scientific evidence is available till date regarding the anticancer potential of *Bauhinia variegata* and the mechanism of cytotoxicity.

A preliminary screening in different cell lines was performed in view that cytotoxicity of compound can vary significantly with the sensitivity of the cell lines. MTT assay was performed in HeLa cell lines which confirmed cytotoxicity. Apart from purifying and isolating crude extracts for pure compounds we were mainly concerned with validating the antiproliferative potential of crude extracts. The failure in replicating the beneficial antagonistic activities of two or more phytochemicals is the major factor that limits the development of anticancer drugs from plants described in ayurvedic medicine. Hence the current study gave significance in establishing anticancer potential of flavonoid rich fractions of *Bauhinia variegata* [10].

Cell death through apoptosis is most preferred over necrosis which can induce inflammatory responses. Acridine orange/ethidium bromide double staining results clearly depict increased number of apoptotic cells rather than necrotic nuclei [11]. This confirms induction of apoptosis by flavonoid fractions of *Bauhinia variegata*. The variability of cancer cells towards chemotherapy can have grave implications in development of multi drug resistance and cancer relapse. It is apparent that survival of a single cancer cell can lead to failure of chemotherapy induction of cell death by apoptosis can be promising.

Flow cytometric analysis offers a precise technique to check the effect of plant extracts on cell cycle progression and check points. The process of replicating DNA and dividing a cell can be described as a series of coordinated events that compose a cell division cycle [12]. Cell cycle regulation will become aberrant or deregulated in most of the neoplastic cells and thus the activity of compound over the regulation of cell cycle attains prior significance. We first reports G0/G1 phase arrest in HeLa cells when treated with flavonoid rich fractions of *Bauhinia variegata*. The mechanism of G0/G1 phase arrest can be attributed to inhibition of pry phosphorylation at multiple sites or the suppression of c-Abl function as a consequence of the perturbation of cyclin D1 expression [13]. These aspects need to be studied in detail.

CONCLUSION

This study confirms that ethanolic extract of *Bauhinia variegata* bark possesses anti cancer properties. The extract induced apoptosis in malignant HeLa cell lines at a dose level of 191.5 µg/ml and also arrested the replication of cells at G0/G1 phase.

AKNOWLEDGEMENT

The authors are thankful to M/s Biogenic Research Centre, Thiruvananthapuram for the help extended for this study

CONFLICT OF INTEREST - None

REFERENCES

1. Rajkapoor B, Jayakar B, Murugesh N. Antitumour activity of *Bauhinia variegata* on Dalton's ascitic lymphoma. J Ethanopharmacol 2003; 89: 107-109.
2. Nadkarni KM, Nadkarni AK. Indian Materia Medica. 3rd ed. New Delhi: Vallabh Prakashan; 1954.
3. Kokate CK. Practical Pharmacognosy. 1st ed. New Delhi: Vallabh Prakashan; 1986.
4. Trease GE and Evans WC. Pharmacognosy. 10th ed. London: Bailer Tindall; 1972.
5. Harbone JB. Methods of extraction and isolation, In: Phytochemical Methods, London: Chapman and Hall; 1998.
6. Srividya AR, Shalom A, Chandrashekar R, Vijayan P, Vishnuvarthan VJ. Cytotoxic, antioxidant and antimicrobial activity of *Polygonum Chinensis* Linn. Int J Pharm Sci Nanotech 2012; 4(4): 1569-1574.
7. Biba Veerabhadran S, Remani P. Cytotoxic and apoptic effect of *Annona squamosa* seed extract on human and murine tumour cells. Int J Pharm Bio Sci. 2013; 4(4): 682-687.
8. Martineti V, Tugnarini J, Carbonell SS, Clematis F, Dola M, Lansotti V et al. Inhibition of *in vitro* growth and arrest in the G_0/ G_1 phase of HCT8 line human colon cancer cells by Kaempferidetriglycoside from *Dianthus caryophyllus*. Phytother Res 2010; 24(9): 1302-1308.
9. Ali I, Wani WA, Saleem K. Cancer scenario in India with future prospectives. Cancer Therapy 2011; 8: 56-70.
10. Cechinel FV. Chemical composition and biological potential of plants from the genus Bauhinia. Phytother Res 2009; 23: 1347-1354.
11. Sharma RA, Gescher AJ, Steward WP. Curcumin: the story so far.Eur J Cancer 2005, 41:1955-1968.
12. Tripathi KD. Essentials of Medical Pharmacology. 5th ed. New Delhi: Jaypee Publishers; 2003.
13. Lukas J, Pagano M, Staskova Z, Draetta G, Bartek J. Cyclin D1 protein oscillates and is essential for cell cycle progression in human tumour cell lines. Oncogene 1994; 9(3): 707–718.
14. Prabhavathy D, Valli Nachiyar C. Cytotoxic potential and phytochemical analysis of *Justicia beddomei* and its endophytic *Aspergillus sp*. Asian J Pharm Clin Res 2013; 6(5): 159-161.
15. Devarajan S, Venugopal K, Indira VA. Pharmacognostic and physicochemical studies of the leaves and stem of *Actinodaphne madraspatana bedd*. Asian J Pharm Clin Res 2013; 6(5): 114-118.