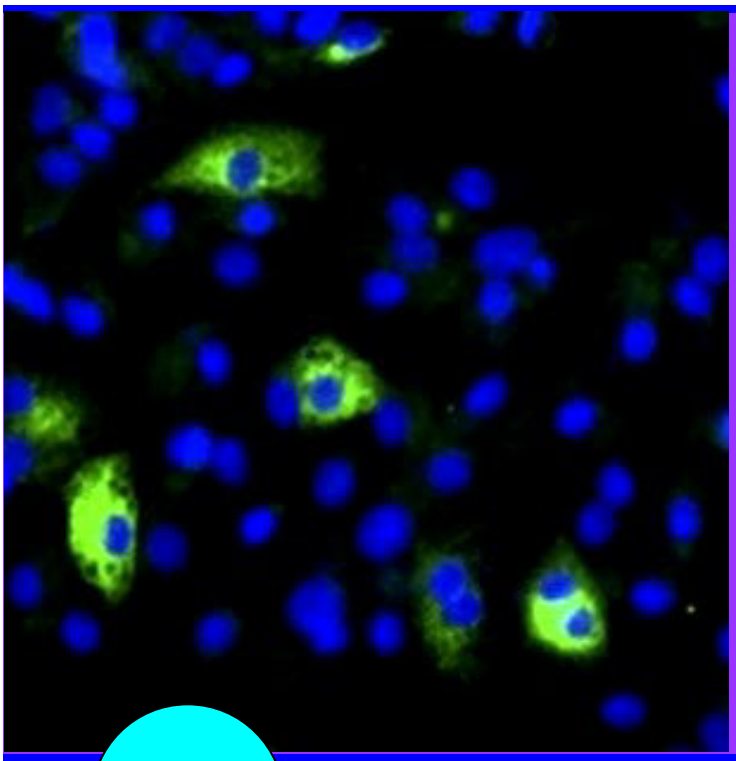

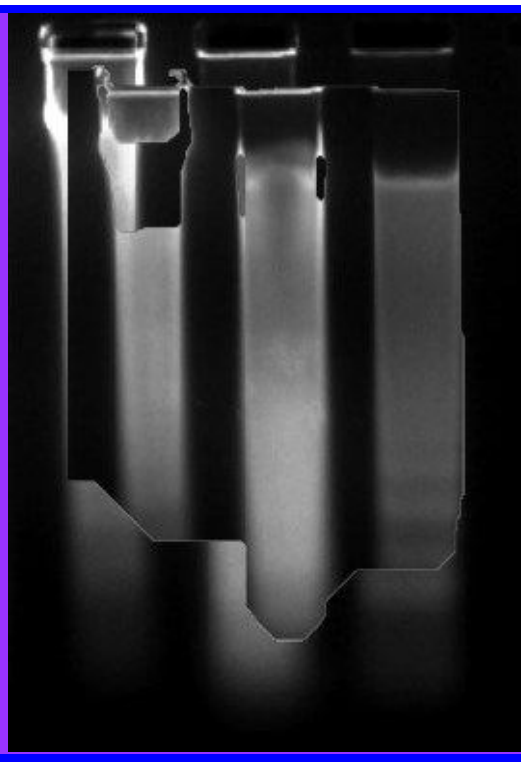

BJP

Bangladesh Journal of Pharmacology

Research Article

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A Journal of the Bangladesh Pharmacological Society (BDPS)
Journal homepage: www.banglajol.info
Abstracted/indexed in Academic Search Complete, Agroforestry Abstracts, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Global Health, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index
Bangladesh J Pharmacol 2014; 9: 604-09
ISSN: 1991-0088

Anti-cancer and apoptosis-inducing effects of *Moringa concanensis* using hepG2 cell lines

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Article Info

Received: 19 September 2014
Accepted: 7 October 2014
Available Online: 20 November 2014

DOI: 10.3329/bjp.v9i4.20481

Cite this article:
Balamurugan V, Balakrishnan V, Robinson JP, Ramakrishnan M. Anti-cancer and apoptosis-inducing effects of *Moringa concanensis* using hepG2 cell lines. Bangladesh J Pharmacol. 2014; 9: 604-09.

Abstract

The objective of the present investigation is focused on the anti-cancer activity of the ethanolic crude extract of *Moringa concanensis* leaf and bark against HepG2 cell line. The study was facilitated by collecting the plant sample and subjected to ethanol crude extraction. The anti-cancer activity of the crude extracted sample against HepG2 cell line was examined by MTT assay. The study confirms that the leaf crude extract of *M. concanensis* has pronounced anti-cancer potential against HepG2 cell lines while compared to that of the bark extract. The plant investigated possesses remarkable anti-cancer activity and hence isolation of the compound contributing to the activity may lead to develop at a novel and natural phytomedicine for the disease.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common tumor worldwide and the incidence of HCC has been rising over the past few decades in some areas such as Europe, USA and eastern Asian countries (Marrero, 2006). Malignant gliomas are one of the more lethal forms of cancer. An estimated 18,000 new cases of brain and central nervous system tumors are diagnosed each year and approximately 13,000 people died of their disease in the United States alone (American Cancer Society, 2006). Despite advances in diagnosis and standard therapies such as surgery, radiation, and chemotherapy, HCC remains a formidable challenge for clinical therapy systems (Okita, 2006; Bosch et al., 1999; Zhu, 2003; Obi et al., 2006).

The natural products including medicinal plants have become more and important in primary health care. Many pharmacognostical and pharmacological investigations are carried out to identify the new drugs or to find out new lead structures for the development of novel therapeutic agents for the treatment of human diseases such as cancer and infectious diseases (Newman et al., 1996).

Lung, breast and colon cancer are the three most common cancers worldwide, with an increasing annual incidence (Bingham and Riboli, 2004). Carcinogenesis is composed of a multi-stage process of initiation, promotion and progression. In the steady-state, cell division must be counterbalanced by cell death. This important active process of cell death is known as apoptosis or programmed cell death (Kerr, 1971). Apoptosis has been recognized as a tightly controlled mechanism involving death factors and death receptors in the control of cell proliferation. The recognition of tumor development involves an imbalance between cell proliferation and apoptotic cell death, which is the current dogma in tumor biology (Kanzler and Galle,

2000). HCC derived from hepatocytes is one of the most common malignancies. It is characterized by its high incidence in hepatitis B virus-associated cirrhotic liver disease and other risk factors such as hepatitis C virus, aflatoxin, sex, hormones and some metabolic diseases. The different epidemiology distributions of HCC have facilitated that the identification of these associated risk factors (Johnson, 1996; Graham and Alistar, 1996). Thus, a great deal of research has been turned towards novel chemotherapeutic drugs from the plant kingdom in search of cancer inhibitors and cures. The bioactive components obtained from various herbal plants have high potential in preventing and controlling carcinogenesis (Pezzuto, 1997).

One promising new source of therapeutic agents has been discovered in plant secondary metabolites, irregularly occurring compounds that characterize certain plants or plant species (Seigler, 1998). The secondary metabolite has been focused upon their medicinal properties (Harborne, 2000). Studies of flavonoids have produced the most compelling data for the antitumor activities of plant secondary metabolites in various types of cancers (Yang et al., 2001), and several flavornoids have been shows that to inhibit cancer development while exhibiting anti-oxidant activities in various animal models (Kuo, 1997; Lahiri-Chatterjee et al., 1999; Ingram et al., 1997; Messina et al., 1994; Barnes et al., 1995). Numerous cancer research studies have been conducted using traditional and folklore medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents. One of the more versatile plants used as a source of flavonoids is the root of the traditional Chinese medicinal herb Baikal skullcap (*Scutellaria baicalensis*), a member of the mint family (Chung et al., 1995). We previously reported the phytochemical evaluation and antimicrobial activity of *Moringa concanensis* leaf (Balamurugan and Balakrishnan, 2013b) and bark (Balamurugan and Balakrishnan, 2013a) extract. The objective of the present study was to evaluate the anticarcinogenic properties and mode of action of the *M. concanensis* leaf and bark crude extract in a human hepatoma cell line (HepG2).

Materials and Methods

Collection of plants

The *M. concanensis* plant was collected in the month of March 2010 from the Esanai Village of Perambalur District, Tamil Nadu, India, surrounded by South Arcot in the North (Villupuram and Cudda-lore), Trichirrappalli on south, and Salem on west and Thanjavur on the east and lies between Lat.11° 14' N; Long. 78° 56' E. *M. concanensis* is widely distributed on dry lands

Identification and confirmation of the plant

The plant material was identified by using the methods described by Jain (1983). The samples of plants were collected, identified and voucher specimens were deposited in the Department of Biotechnology, Sri Vinayaga College of Arts and Science, Ulundurpet, Tamil Nadu.

Preparation of ethanol extract

Required quantity (50 g) of powder was weighted and transferred to stopper flask and treated with the ethanol until the powder is fully immersed. The flask was shaken every hour for the first 6 hours and then it was kept, in shaken after 24 hours. This process was repeated for three days and then the extract was filtered. The extract was collected and evaporated to dryness by using a vacuum distillation unit. The final residue thus obtained was then subjected for further analysis

Cell growth inhibition study using the MTT assay

Cell growth inhibition of leaf and bark crude extracts were analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2, 5- diphenyltertrazolium bromide (MTT) assay. Briefly, HepG2 cells were seeded in 96 well plates at a density of 6 x 10^3 cells per well. After treatment with 2, 4, 6, 8, 10, 20, 30, 40, 50 and 100 µg/mL of leaf and bark crude extracts for 48 hours, 20 µL MTT (5 mg/mL) was added. Four hours later, 100 µL DMSO was added to each well to dissolve the resulting formazan crystals. Absorbance was read at 490 nm using an enzyme-linked immunosorbent assay reader (SpectraMax; Molecular Devices, USA).

Data were collected from the triplicate separate experiments and the percentage of leaf and bark crude extracts induced cell growth inhibition was determined by comparison to DMSO-treated control cells (Mosmann, 1983).

Assays for cell growth

Anti-cancer activity was determined using MTT assay (Alley et al., 1988). 40 to 50% cells were seeded on 96-well plates and cultured for 24 hours inside the CO_2 incubator at 37°C. After 24 hours of incubation media was removed and crude extract was added at various concentrations and incubated inside the CO_2 incubator for 24, 48 and 72 hours. Then MTT was added with media (500 µL) to each well. It was kept inside the CO_2 incubator for 2 hours in dark. Then 50 µL of DMSO was added to each well. Then it was shaken vigorously for 10 to 20 min for cells to lyses. The plate was read in ELISA Reader – measure OD in 570 nm. The cell growth in untreated control cultures was considered 100% and the growth of each treated group was compared relative to this value with positive control.

Analysis of DNA fragmentation

Cells were seeded at 2 × 10^5 cells/well in 6-well plate 24

hours before treatment. After 20, 40, or 60 min of incubation with cured extract the cells were harvested and then processed for analysis of DNA fragmentation. The cells were lysed with 0.5% sarcosyl in 50 mM Tris–Hcl and 10 mM EDTA (pH 8.0) in the presence of 0.5 mg/mL proteinase K. After a one hour of incubation at 50°C, RNaseA (0.15 mg/mL) was added to each sample. The lysate were analyzed by electrophoresis on 1.2% agarose gels containing 0.5 g/mL ethidium bromide (Shi, 1989).

Results

The results demonstrated that leaf and bark crude extracts had significant cytotoxicity on HepG2 cell line. Leaf and bark crude extracts decreased the viability of HepG2 cells in a dose-dependent manner. The maximum inhibition of cell growth (93.7% with IC_{50}) was observed at the concentration of 100 µg/mL of leaf crude extract and whereas inhibition of cell growth (93.44% with IC_{50}) was observed at the concentration of 100µg/mL of barks crude extract.

The decreased growth rates of HepG2 cells of about 26.9, 34.8, 48.3, 59, 61.5, 64.3, 69.8, 81.9, 84.4 and 93.7 percentage observed at the leaf crude extract concentration of 2, 4, 6, 8, 10, 20, 30, 40, 50 and 100 µg/mL, respectively and whereas growth rates of HepG2 cells of about 6.1% 34.9 , 48.3 , 57.1 , 59.2 , 69.8, 77.1 , 87.0 , 88.5 and 93.4 percentage observed at the bark crude extract concentration of 2, 4, 6, 8, 10, 20, 30, 40, 50 and 100 µg/mL, respectively. All the concentrations used in the experiment (leaf and bark crude extracts) decreased the cell viability significantly ($p<0.05$) in a concentration -dependent manner (Figure 1 and 2).

Treatments with leaf and bark crude extracts (100 µg/mL) effectively decreased the total number of HepG2 cells and were accompanied by cell shrinkage, condensed nuclei, blebbing and shape changes (Figure 3). When the cells were stained with trypan blue, which can discriminate between apoptosis and necrosis, condensed nuclei, blabbing, indicators of typical apoptosis manifestation were observed in plant crude extracts treated cells.

Leaf crude extract treated HepG2 cells exhibited more than 93.7% increase in apoptosis and whereas bark crude extract treated HepG2 cells exhibited more than 93.4% increase in apoptosis and investigation of the staining pattern indicated that the predominant cause of cell death in HepG2 was due to apoptosis.

To test whether leaf and bark crude extracts induced similar cell death in normal cells. 30 min of incubation with leaf and bark crude extracts (100 µg/mL) resulted in only 16.5% cell death. Concentrations used in the experiment (leaf and bark crude extracts) decreased the cell viability were not significantly ($p<0.05$) in a concentration 100 µg/mL.

To determine the mode of growth inhibition in HepG2 cells induced leaf and bark crude extracts, whether leaf and bark crude extracts were investigated if they induced inter nucleosomal degradation of DNA, a characteristic of apoptosis. In the present study reveals that the DNA ladders appeared in leaf and bark crude extracts treated HepG2 cells after exposure to concentrations of 100 µg/mL for 48 hours. The control cell of HepG2 shows any DNA fragmentation (Figure 4). The results of DNA fragmentation in leaf and bark crude extracts induced apoptotic cells which were confirmed by DNA ladder assay.

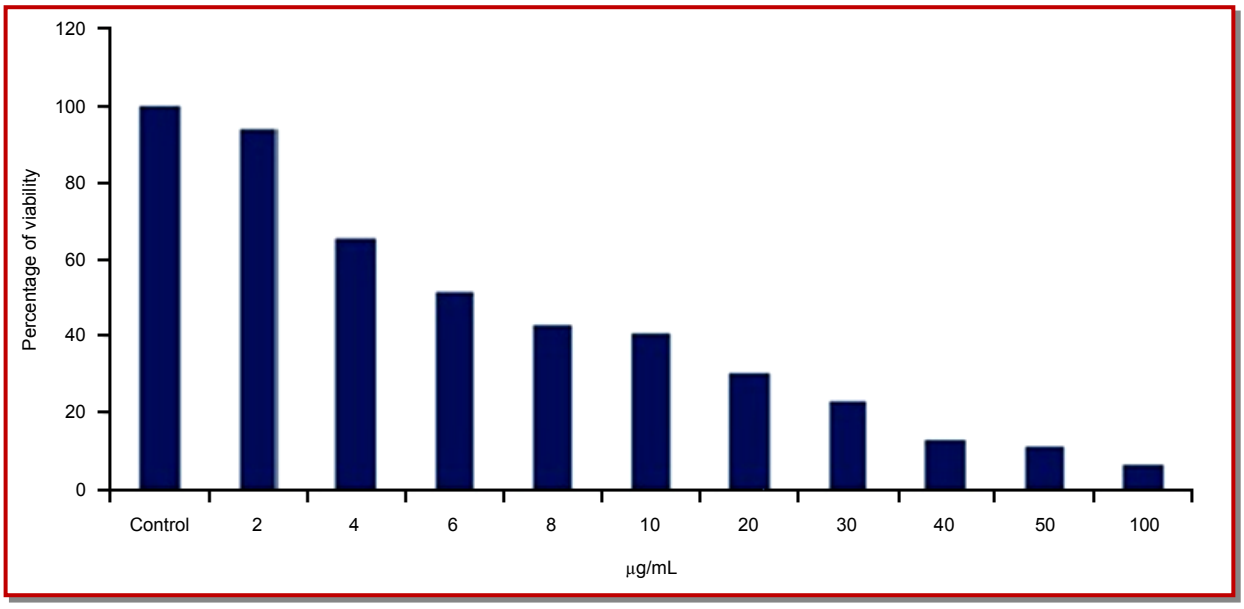

Figure 1: Cytotoxic effect of *M. concanensis* bark crude extracts against HepG2 cell line

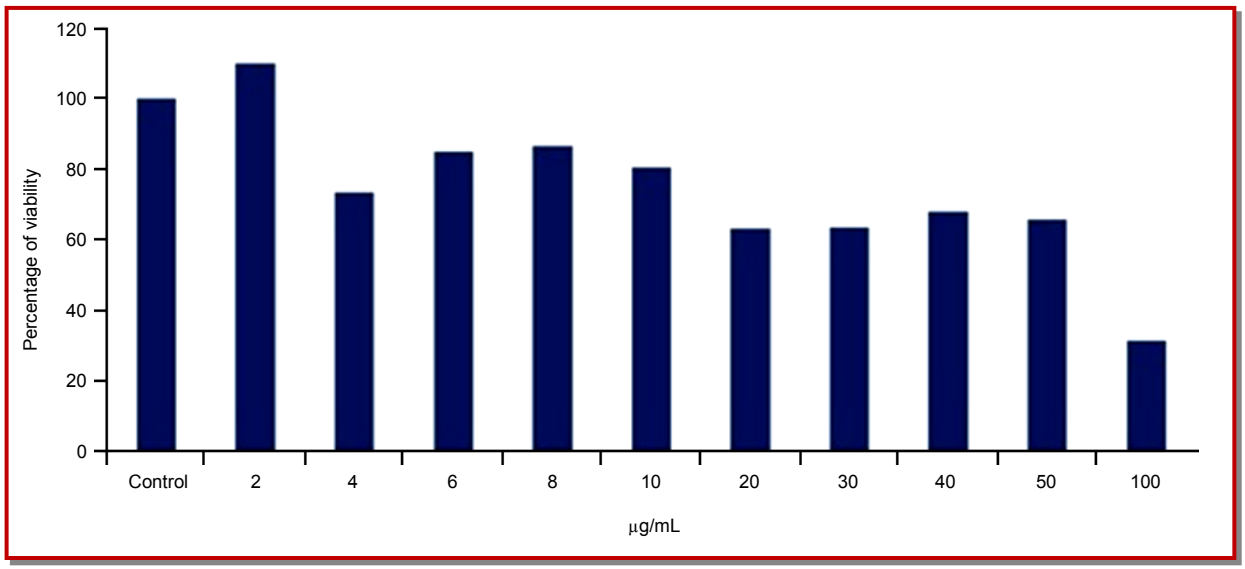

Figure 2: Cytotoxic effect of *M. concanensis* leaf crude extracts against HepG2 cell line

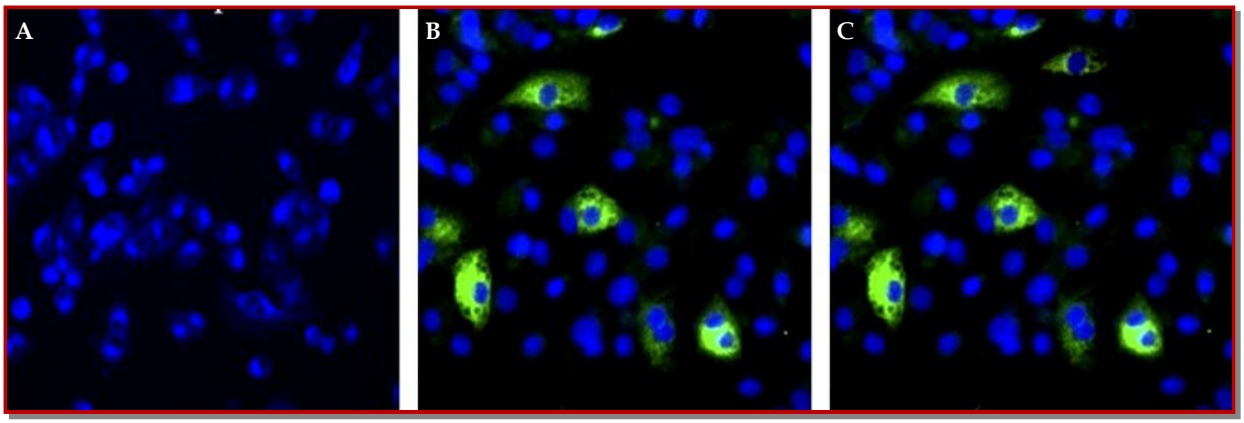

Figure 3: Cytotoxicity and apoptotic activity of *M. concanensis* bark and leaf plant crude extracts against HepG2 cell line

(A) Controls cells; (B) bark crude extracts treated HepG2 cells; (C) leaf crude extracts treated HepG2 cells showing cytotoxicity and apoptotic activity

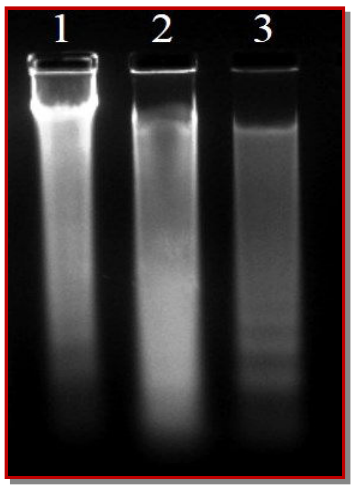

Figure 4: Apoptotic DNA fragmentation of HepG2 cells

(1) Control, (2) leaf treated showing apoptotic DNA fragmentation of HepG2 cell line (3) bark treated showing apoptotic DNA fragmentation of HepG2 cell line

Discussion

A major complication of chemotherapy is toxicity to normal cells, which is due to the inability of drugs to differentiate between normal and malignant cells. This often impacts the efficacy of the treatment and even it makes it impossible to cure the patients. One of the requisite of cancer chemo preventive agent is elimination of damaged or malignant cell through cell cycle inhibition or induction of apoptosis without or with less toxicity in normal cells (Srivastava and Gupta, 2006; Tian et al., 2006).

Apoptosis is a tightly regulated process, which is involves the changes in the expression of a distinct set of genes (Cummings et al., 1997; Tong et al., 2004). Two of the major genes responsible for regulating mitochondrial apoptosis pathway are antiapoptotic Bcl-2 and proapoptoticbax (Zhong et al., 1993; Bruce-Keller et al., 1997; Wolter et al., 1997).

Large scale screening of plant crude extracts on cell culture is an important initial step to determine their potential efficacy in clinical application. Several reports have shows that plant crude extracts replicate at tumor sites under hypoxic conditions and stimulate the host immune response and gene expression and leads to the inhibition of tumor growth.

Apoptotic cells often produce a unique ladder composed of nucleotide fragments which can be visualized by DNA-agarose gel electrophoresis. In general, cytotoxic drugs induce an enormous breakage of DNA into oligonucleosome fragments. The degradation of DNA down into oligonucleosomal fragments is a late event of apoptosis (Compton, 1992; Earnshaw, 1995; Lim et al., 2006). Thus, the leaf and bark crude extracts induces DNA damage in HepG2 cells, thereby causing apoptosis.

The result shows that the ethanolic extract of the *M. concanensis* was non toxic to normal cell and also anti-cancer activities individually. This study points to the probable anti-cancer potentials of ethanolic leaf and bark extracts of *M. concanensis*. The results of the study will also need to be confirmed using *in vivo* models.

Acknowledgement

The authors are thankful to Mr. M. Rajiv Gandhi, Research scholar, Anna University, Chennai, Tamil Nadu, India for successful completion of the work and assistance.

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