

## Research Article

ISSN 2320-4818  
JSIR 2014; 3(3): 303-307  
© 2014, All rights reserved  
Received: 12-03-2014  
Accepted: 01-06-2014

### Sibi P. Ittiyavirah

University College of Pharmacy,  
Cheruvandoor, M G University,  
Kottayam, Kerala

### Muraly A

University College of Pharmacy,  
Cheruvandoor, M G University,  
Kottayam, Kerala

### Rajiv P

University College of Pharmacy,  
Cheruvandoor, M G University,  
Kottayam, Kerala

### Raveendran R

University College of Pharmacy,  
Cheruvandoor, M G University,  
Kottayam, Kerala

### Correspondence:

#### Dr. Sibi P. Ittiyavirah

University College of Pharmacy,  
Cheruvandoor, M G University,  
Kottayam, Kerala

E-mail: [sibitho@gmail.com](mailto:sibitho@gmail.com)

## Inhibition of growth and induction of apoptosis in PC3 and A549 cell lines by hydro alcoholic fruit extract of *Morinda tinctoria* roxb: in vitro analysis

Sibi P. Ittiyavirah\*, Muraly A, Rajiv P, Raveendran R

### Abstract

The present study aimed to investigate the effect of hydroalcoholic extract of *Morinda tinctoria* Roxb fruit on inhibition of growth and induction of apoptosis in human prostate carcinoma cells (PC-3) and lung carcinoma (A549) cells. *Morinda tinctoria* Roxb hydro alcoholic fruit extract (MTHFE) was taken to in vitro studies, like phytochemical screening, MTT assay, cell induced apoptosis. Dose dependant cytotoxic activities were exhibited by PC-3 cell lines and A549 cells. The result showed that, as the dose of the extract increased, the no of viable cells were found to be decreased, which confirms the anti-cancer and cytotoxic activities of the fruit extract.

**Keywords:** *Morinda tinctoria* Roxb, MTT assay, Apoptosis, PC3 and A549 cell lines.

### Introduction

Cancer may also spread to more distant parts of the body through the lymphatic system or blood stream. Not all tumors are cancerous; benign tumors do not invade neighboring tissues and do not spread throughout the body. Lung cancer is a disease characterized by uncontrolled cell growth in tissues of the lung. If left untreated, this growth can spread beyond the lung in a process called metastasis into nearby tissues or other parts of the body. Most cancers that start in the lung, known as primary lung cancers, are carcinomas that derive from epithelial cells. The main types of lung cancer are small-cell lung carcinoma (SCLC), also called oat cell cancer, and non-small-cell lung carcinoma (NSCLC). The most common symptoms are coughing (including coughing up blood), weight loss and shortness of breath.<sup>1-3</sup> Adenocarcinoma of the prostate is the most common form of cancer in men and the second leading cause of cancer death. It is typically a disease of men over age 50. Little is known about the cause of prostate cancer. Several risk factors such as age, race, family history, hormone levels and environmental influences are suspected of playing roles. *M. tinctoria* commonly known as Aal or Indian Mulberry, is a species of flowering plant in the family Rubiaceae native to southern Asia. It is an evergreen shrub or small tree growing to 10-15m tall. The leaves are 15-25 cm long, oblong to lanceolate. The flowers are tubular, white, scented, about 2cm long. The fruit is a green syncarp, 2-2.5cm in diameter.<sup>4</sup> All parts of *M. tinctoria* has medicinal properties. Leaves are useful as a tonic, febrifuge, deostruent and emmenagogue. It is also used for curing dyspepsia, diarrhoea, ulceration, stomachitis, digestion, wound and fever. The leaf juice is useful as a local application. Root is used to cure inflammation and boils. Therefore, in the present study, we investigated the effect of hydro alcoholic extracts of

*M. tinctoria* Roxb fruit on cytotoxicity studies.

## Materials and Methods

### Plant materials

The fruit of *Morinda Tinctoria* Roxb collected from Kundara, Kollam and authenticated by Dr.K.V George, Department of Botany, CMS College, Kottayam.

### Preparation of hydro alcoholic extract

The fruit of *Morinda Tinctoria* Roxb was air dried, chopped into small pieces and pulverize. The dried powder (25g) was extracted with 70% of ethanol at room temperature for 48 hrs by cold extraction method. The separated hydro alcoholic extract was subjected to phytochemical screening and in vitro pharmacological studies, like MTT assay, cell induced apoptosis.

### Phytochemical Screening

Phytochemical screening of MTHFE was performed using the reagents and chemicals as follows.<sup>5</sup>

Alkaloids were screened using Mayer's, Hager's and Dragendroff's reagent, while Flavonoids with the use of sodium acetate, ferric chloride and amyl alcohol. Lead acetate and gelatin were used to find out the presence of Phenolic compounds in the extract. The presence of carbohydrates in the extract was confirmed by using Molisch's, Fehling's and Benedict's reagent.

Proteins and amino acids in the extract were found out using Millions, Biuret, Xanthoprotein test. Saponins were tested using hemolysis method while sterols with 5% potassium hydroxide. Steroids with Libermann-Burchard's test and terpenes with thionyl chloride. Ferric chloride, acetic acid and concentrated sulphuric acid were used to find out the presence of glycosides while gum was tested using Molisch's reagent.

### MTT Assay

MTT Assay is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-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 (eg. isopropanol) and then released, solubilised formazan reagent is measured spectrophotometrically. The reduction of MTT can occur only in metabolically active cells and

hence the level of activity is a measure of the viability of the cells.<sup>3</sup>

Traditionally, the determination of cell growth is done by counting the viable cells after staining with a vital dye. Trypan blue is a simple way to evaluate cell membrane integrity (and thus assume cell proliferation or death) but the method is not sensitive and cannot be adapted for high throughput screening. Measuring the uptake of radioactive substances, usually tritium labelled thymidine, is accurate, but it is also time consuming and involves handling of radioactive substances. Yellow MTT (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. The absorbance of this coloured solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer.

The absorption max is dependent on the solvent employed. This reduction takes place only when the mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated cells, the effectiveness of the agent in causing the death of cells can be deduced, through a dose response curve. Solutions of MTT solubilised in tissue culture media or balanced salt solutions, without phenol red, are yellowish in colour. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance.

The use of MTT method does have limitations influenced by 1) the physiological state of cells and 2) variance in mitochondrial dehydrogenase activity in different cell types. Nevertheless, the MTT method of cell determinations is useful in the measurement of cell growth in response to mitogens, antigenic stimuli, growth factors and other cell growth promoting reagents, cytotoxicity studies, and in the derivation of cell growth curves. The MTT method of cell determination is most useful when cultures are prepared in multiwell plates.

Determination of in vitro anti proliferative effect of *Morinda tinctoria* fruit extract was conducted on cultured

A549 cell line. The cell culture suspension was washed with 1x PBS and then added 30 µl of the MTT solution to the culture (MTT -5mg/ml dissolved in PBS). It was then incubated at 37°C for 3 hours. MTT was removed by washing with 1x PBS and 200µl of DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cell got lysed and colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank.

**Determination of apoptosis by Acridine Orange (AO) and Ethidium Bromide (EB) double staining**

Apoptosis is the process whereby individual cells of multicellular organisms undergo systematic self destruction in response to a wide variety of stimuli. Apoptosis is a genetically controlled preprogrammed event which eliminates cells during development when they have become redundant, or, alternatively, a process which functions as an emergency response following radiation damage, viral infection, or aberrant growth of cells as induced by the activation of oncogenes.

Acridine orange is taken up by both viable and dead cells. It would fluoresce green when bound to double stranded DNA in living cells and fluoresce red when bound to single stranded DNA, which dominates in dead cells. Ethidium bromide was excluded from living cells. However, late apoptotic or necrotic cells have a ruptured membrane that allows the entrance of ethidium bromide to intercalate into DNA and fluoresce red.

DNA-binding dyes AO and EB (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells.<sup>7</sup> AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA). EB is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. A549 (Lung carcinoma) cells were cultured in Dulbeccos modified Eagles media and grown to 60-70% Confluency and treated with extracts at a final concentration of 100mcg/ml for 24h. The cells were washed with cold PBS and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at room temperature for 10min. The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope in blue filter of fluorescent microscope (Olympus CKX41 with Optika Pro5 camera). The cells were divided into four categories as follows: living cells (normal green nucleus),

early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei).<sup>6,7</sup>

**Results**

The result of the phytochemical screening revealed that the fruit extract contains a broad spectrum of secondary metabolites. Alkaloids, phytosterols, flavanoids, phenols and carbohydrates are the major secondary metabolites (table: 1).

**Table 1:** Phytochemical screening of MTR fruit extract as follows

Phytoconstituents	Positive	Negative
Alkaloids	+	
Carbohydrates	+	
Proteins and amino acids	+	
Sterols	+	
Phenols	+	
Flavonoids	+	
Glycosides	+	
Saponins		-
Tannins		-

Treatment of A549 (lung cancer) cells with *M. tinctoria* fruit extracts show inhibition of growth of cancer cells. As dose of the extract increased, the viability of cancer cells decreased. (Table-2).

**Table 2:** Dose dependant cytotoxic effect of MTR fruit extract on A459cells by MTT assay

Sampleconcentration (µg/ml)	Average OD at 540nm	% viability
Control	0.7232	100
10µg/ml	0.5549	76.72
50µg/ml	0.4486	62.02
100 µg/ml	0.3472	48.08

% viability = (OD of Test/ OD of Control) X 100

Treatment of PC3 (Prostate carcinoma) cells with *Morinda tinctoria* Roxb fruit extracts shows induction of apoptosis. The presence of darker red stained nuclei in samples treated with *Morinda tinctoria* fruit extracts confirms nuclear membrane damage and apoptosis bodies. (Fig: 3)

## Discussion

Metastatic prostate carcinoma is associated with a high morbidity and mortality rate with a medium survival of approximately 12-15 months. The available treatment alternatives include radiotherapy, after radical retropubic prostatectomy, external beam radiation, prostate brachytherapy and androgen ablation of the prostate. Lung cancer, characterized by uncontrolled cell growth in the tissue of the lung can be metastatic, if the left can be treated. Most cancers that start in the lung, known as primary lung cancers, are carcinomas that derive from epithelial cells. Treatment and long term outcome depend on the type of cancer, the stage (degree of spread) and person's overall health. Common treatments include surgery, chemotherapy and radiotherapy. Overall, 15% of people diagnosed with lung cancer survive 5 years after diagnosis. In recent years, considerable efforts have been made to identify naturally occurring compounds and related synthetic agents that can prevent the development and recurrence of cancer. A wide variety of natural food and food products can induce apoptosis in various tumor cells. There is strong evidence supporting the positive role of some natural materials and medicinal plants in oncology and their ability to affect all phases of tumorigenic process. Therefore, it is very important to screen the natural products either as crude extracts or as isolated components for apoptotic properties to identify potential anti-cancer compounds. Also, with a view on the risk of side effects caused by most therapeutic agents used, it has been more important to opt for more

natural products. Over 60% anticancer agents used are derived from natural sources, including plants, marine organisms and micro-organisms. Plants belonging to the genus *Morinda* have shown to be promising alternatives in various ailments. In the present study the plant *M. tinctoria* was selected and screened for the cytotoxic potential. The phytochemical screening of the plant revealed the presence of alkaloids, flavanoids and sterols (Table.1.). These phytoconstituents may be responsible for the various activities. Flavanoids are a diverse family of compounds, commonly found in fruits, vegetables and are generally safe. Alkaloids, as recent studies reveal, can also be used as promising anti-cancer agents.<sup>8</sup> PC3 (PC-3) human prostate cancer cells are one of the cell lines used in prostate cancer research. These cells are useful in investigating the biochemical changes in advanced prostatic cancer cells and in assessing their response to chemotherapeutic agents. Moreover, they can be used to create subcutaneous tumors in mice in order to investigate a model of the tumor environment in the context of the organism. MTT assay confirms the dose dependent cytotoxic effect (fig 2) of crude hydroalcoholic extracts of *M. tinctoria* in lung cancer cell line (fig:1a, fig:1b, fig:1c). In the case of Acridine orange (AO) and Ethidium bromide (EB) double staining confirms the anti-proliferative effect of crude hydroalcoholic extracts of *M. tinctoria* in prostate cancer cell lines (fig:3). As the dose of the extract increases, the number of viable cells decreases and confirms the cytotoxic activity.

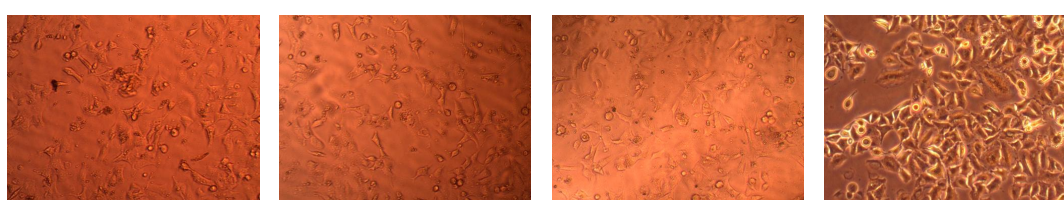
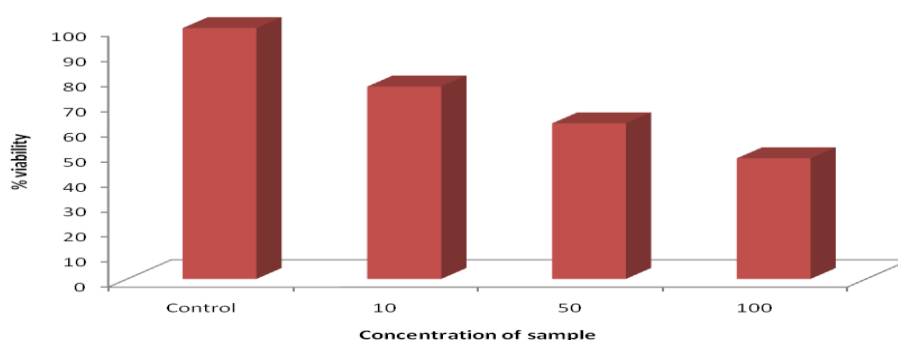


Fig:1a-10 µg/ml      Fig:1b-50 µg/ml      Fig:1c-100 µg/ml      Fig:1d-control

**Figure 1:** Cytotoxic effect of crude hydroalcoholic extracts of *M. tinctoria* in lung cancer cell line



**Figure 2:** Effect of hydroalcoholic extract of *M. tinctoria* on inhibition of growth of A549 cells by MTT assay

