Available online at www.ijpsdr.com

International Journal of Pharmaceutical Sciences and Drug Research 2015; 7(4): 345-348

Research Article

ISSN: 0975-248X
CODEN (USA): IJPSPP

Study on Cytotoxic Effect of Root and Callus Extracts of *Myxopyrum smilacifolium* Blume

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ABSTRACT

Myxopyrum smilacifolium is a large woody climbing shrub belonging to the family Oleaceae which is known for many medicinal properties. In the present study, initially a short term cytotoxic study (3 hours) with hepatocarcinoma cell line HepG2 using Trypan Blue Assay was carried out against methanolic extracts of root and callus and found an appreciable result for both extracts. Then the study was extended to the same cell line employing MTT assay. In this procedure extracts were subjected to 24 hours treatment and cytotoxicity was measured using a colorimetric method based on the ability of metabolic active cells to cleave the yellow tetrazolium salt MTT to an insoluble purple formazan crystal. A concentration dependent activity was observed in both Trypan Blue as well as MTT assay. The results showed that both the extracts possess potent cytotoxic activity. IC_{50} value from MTT assay was found to be 63.75µg/l and 98.75µg/l for root and callus extracts respectively. To the best of our knowledge, this is the first study to demonstrate the cytotoxic potential of callus extracts of *Myxopyrum smilacifolium* Blume.

Keywords: Callus, HepG2 cells, Trypan Blue, MTT.

INTRODUCTION

The use of medicinal plants for the treatment of human diseases is an ancient practice; this has greatly increased in recent years. Nearly 80% of the world's population relies on traditional medicines for primary health care, most of which involve the use of plant extracts. [1] Medicinal plants are a boon to mankind as it contains many phytoconstituents of therapeutic importance as it has been already proved to be effective for the ailment of a vast majority of diseases from cold to cancer. The most important of these bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolics. [2]

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Received: 06 June, 2015; **Accepted:** 19 June, 2015

Cancer is the uncontrolled proliferation of cells and it is the second cause of death globally after cardiovascular diseases. [3] Plants are being used in the treatment of cancer since time immemorial and it is safe compared to synthetic drugs as it is free from side effects. The potential of using the natural products as anti cancer drugs was recognized in 1950's by U.S Natural Cancer Institute (NCI) Since 1950 major contributions have taken for the discovery of naturally occurring anti cancer drugs. [4] Plants have an almost unlimited capacity to produce compounds that attract researchers in the quest for new and novel chemotherapeutics. [5]

Myxopyrum smilacifolium is a large woody climbing shrub belonging to the family Oleaceae. Its root, stem, leaves are of much medicinally active and is employed in many traditional systems of medicines. The roots are used to treat various diseases like scabies, cough, rheumatism, fever, cuts and wounds. [6] The leaves are astringent, acrid, sweet, thermogenic, anodyne,

febrifuge and tonic. They are useful in vitiated conditions of kapha and vata, cough, asthma, rheumatism, cephalalgia, nostalgia, fever, otopathy, neuropathy and cuts and wounds. Pharmacognostical evaluation has been made for the plant and reported for the presence of terpenoids, flavones, anthraquinones, sugars, alkaloids, phenols, tannins, and saponins. Antimicrobial study has been carried out in leaves. [7] Previous studies have shown the presence of triterpenoid ursolic acid in leaves [8] and the iridoid glycoside myxopyroside. [9]

Various secondary metabolites present in the plant are responsible for its medicinal value. Callus culture could be employed for the production of these pharmacologically active compounds and thus it could help in preventing exploitation of plant materials *ex-vitro*. Callus is an undifferentiated mass of tissue which appears on explants within a few weeks of transfer onto growth medium with suitable hormones. [10] Callus formation occurs from reversed process of cell differentiation, known as dedifferentiation or redifferentiation. [11] Callus cultures are employed for shoot and root regeneration as well as micropropagation. Callus is also the starting material for suspension cultures for the mass production of secondary metabolites.

The present study was undertaken with the objective to determine the cytotoxic efficacy of methanolic root extracts as well as callus extract on hepatocarcinoma cell line HepG2 using Trypan Blue Assay followed by MTT Assay.

MATERIALS AND METHODS

Plant material and extraction

Fresh plant roots of *M. smilacifolium* were collected from Botanical garden, Dept. of Botany, University of Kerala, Kariavattom. The roots were washed thoroughly with running tap water followed by sterile distilled water. Then roots were dried under shade. Roots were then crushed to coarse powder and were stored at room temperature in air tight container bottles.

For callus induction surface sterilized internode explants were first inoculated in MS medium supplemented with 0.1% 2, 4 Dichlorophenoxy acetic acid (2,4-D) and then sub-cultured on to same medium supplemented with a combination of 0.1% 2,4-D and 1.0% Benzyl amino purine (BAP). Four week old callus was collected and dried in hot air oven at 50°C. Dried callus then powdered using mortar and pestle and stored in refrigerator.

Powdered roots of *M. smilacifolium* were extracted using methanol in soxhlet apparatus for 12 hours. The extracts were then filtered through Whatmann No.1filter paper and concentrated using vacuum evaporator. The extract value calculated and then stored in refrigerator for further use.

For callus extraction, callus was ground with mortar and pestle and then methanolic extract was taken using soxhlet apparatus and repeated the same procedure as for root extract.

In vitro cytotoxic studies

Trypan Blue Assay on HepG2 cells

Trypan Blue is an essential dye in estimating number of viable cells present in a population. [12] The test compounds were studied for short term *in vitro* cytotoxicity assay. Tumour cells aspirated from peritoneal cavity of tumour bearing mice were washed thrice with Phosphate buffered saline (PBS). A viable cell suspension (0.1 ml) was added to tubes containing varying concentration (10µg-200µg) of the test sample and the volume was made up to 1 ml using PBS. Control tube was taken containing only cell suspension. These assay mixture was incubated for 3 hours at 37°C. Cell suspension was then mixed with 0.1% of 1ml Trypan blue solution and left for about 3 minutes and then loaded on haemocytometer. Dead cells take up the blue colour of the dye while live cells do not take up the dye. Percentage living cell was calculated using the formula

$$\%\text{living cell} = \frac{\text{No. of viable cell}}{\text{Number of Dead cell} + \text{Number of viable cell}} X100$$

MTT assay on HEP-G2 cell lines

MTT is a colorimetric assay (12) that measures the reduction of yellow 3-(4, 5dimethythiazol-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 solubilized with an organic solvent Dimethyl sulfoxide (Himedia) and the released, solubilized 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.

Hep-G2 hepatic carcinoma cell line purchased from NCCS Pune were maintained in Dulbecco's modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37°C in 5 % CO_2 in a humidified atmosphere in a CO_2 incubator(NBS, EPPENDORF, GERMANY). The cells were trypsinized (500µl of 0.025% Trypsin in PBS/ 0.5mM EDTA solution (Himedia) for 2 minutes and passaged to T flasks in complete aseptic conditions. Extracts were added to grown cells at a final concentration of 6.25µg/ml, 12.5µg/ml, 25µg/ml, 50µg/ml and 100µg/ml from a stock of 1mg/ml and incubated for 24 hours. The % difference in viability was determined by standard MTT assay after 24 hours of incubation. Percentage viability was measured using the formula

$$\%\ \text{viability} = (\text{OD of Test} / \text{OD of Control}) \times 100$$

RESULTS AND DISCUSSION

Plants are important source of many biologically active compounds. Phytochemical examination has been making rapid progress and herbal products are becoming popular as sources of plausible anticancer

compounds. [13] Earlier studies on methanolic extracts of roots and callus of *M. smilacifolium* proved to have many phytochemical constituents such as flavonoids, terpenoids and tannins [14-15] and also antioxidant activities. [15] Taking the above fact into consideration the present study was conducted to have an insight into the anticancer potential of two extracts. Human hepatocarcinoma cell line, HepG2 was employed for the two *in vitro* cytotoxic assays viz: Trypan Blue Assay and MTT Assay. In both studies the extracts showed considerable cytotoxic potential.

Trypan Blue dye exclusion assay results are as shown in Table 1 and is graphically represented in Fig. 1. A dose dependent response is evident from the data with maximum cytotoxic activity at 200µg/ml concentration. This was short duration study i.e.; 3 hours incubation only with an IC_{50} value below 200µg/ml for both the extracts which is a good indication for its cytotoxic activity.

Then the study was extended to MTT Assay where the cancer cell line was exposed to increasing concentration (6.25µg/ml to 100µg/ml) of both extracts for 24 hours. Here also a dose dependent activity was observed with an IC_{50} value of 63.75µg/l and 98.75µg/l for root and callus extracts respectively. The results are as shown on Table 2 and plotted graphically in Fig. 2.

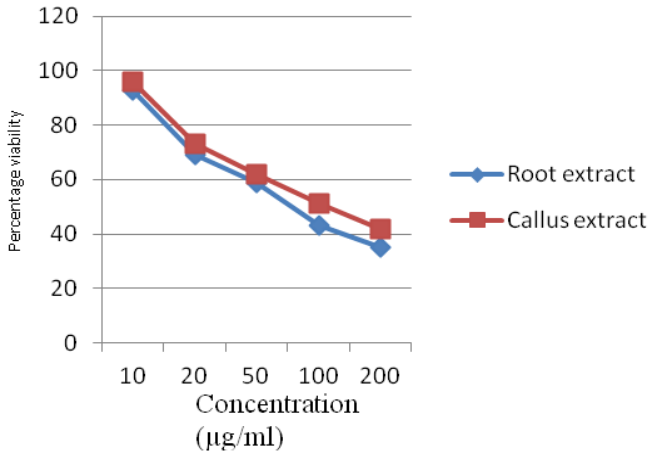

Fig. 1: Graphic representation of viable cells by Trypan blue assay

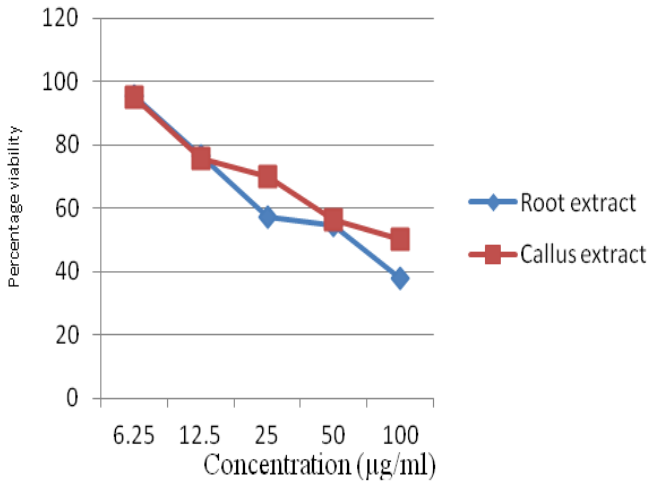

Fig. 2: Graphic representation of viable cells by MTT assay

Table 1: Percentage cell viability by Trypan Blue Assay

Sample Concentration (µg/ml)	Percentage Viable cells against Hep G2 cells	
	Root Extract (%)	Callus Extract (%)
10	93 ± 1.93	96 ± 1.52
20	79 ± 3.05	83 ± 2.51
50	63 ± 3.51	72 ± 2.87
100	53 ± 2.09	59 ± 1.86
200	44 ± 2.73	49 ± 3.02

Values are expressed as mean ± SD for triplicate

Table 2: Determination of cytotoxicity by MTT assay

Sample Concentration (µg/ml)	Percentage Viability	IC_{50}
Root extract		
6.25	95.48 ± 1.98	63.75
12.5	76.47 ± 1.34	
25	57.20 ± 0.85	
50	54.80 ± 1.76	
100	37.87 ± 0.34	
Callus extract		
6.25	94.88 ± 2.02	98.75
12.5	75.54 ± 0.98	
25	69.84 ± 1.57	
50	56.19 ± 1.44	
100	50.28 ± 0.92	

Values are expressed as mean ± SD for triplicate

Phase contrast microscopic views of treated cells by both the extracts are as shown on Fig. 3. From the figure one can observe the morphological changes in the cells on lower as well as higher concentration of the extracts. At lower concentration cells appeared to be normal shape but on higher concentration reduced cell density as well as loss of cell adhesion could be observed.

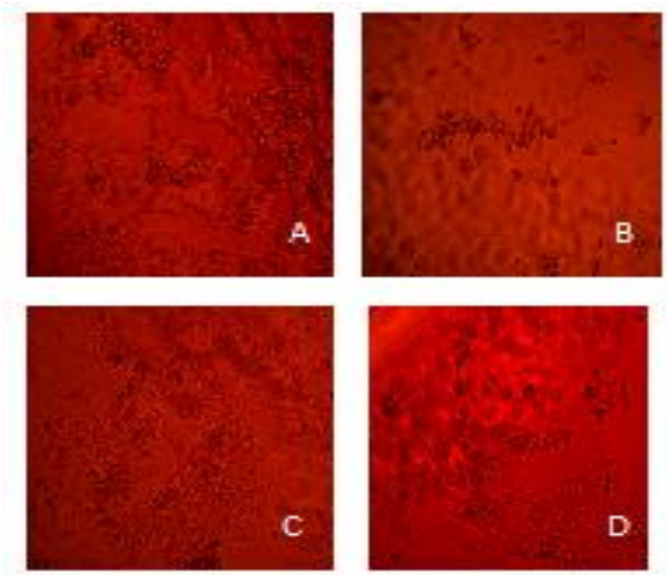

Fig. 3: Toxicity effects of *M. smiacifolium* methanol extract against HepG2 cell line after 24 hours of incubation. A. Root extract at 6.25µg/ml, B. Root extract at 100µg/ml, C. **Callus extract at 6.25µg/ml, D. Callus extract at 100µg/ml**

The study clearly provides a basement for further isolation and characterization of lead compounds and the exact mechanism by which it elicits its cytotoxic activity. The main highlight of present study is the anti-proliferative capacity of callus extracts which is comparable to the activity of root extracts thus

promising to be an alternative to avoid exploitation of the plant as a part of conservation strategy.

ACKNOWLEDGEMENT

The authors wish to thank Head of the Department for providing all the facilities to carry out the work as well as University of Kerala for the financial assistance.

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Source of Support: Nil, Conflict of Interest: None declared.