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Anticancerous Effect of *Hibiscus sabdariffa* Leaves on Hepatocellular Carcinoma Cell Line Hep 3B

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Abstract: *Hibiscus sabdariffa* L. (Malvaceae) is a natural plant containing a lot of pigments that was found to possess anti-oxidant activity. Therefore, the present study was aimed to evaluate the anticancer potential of *Hibiscus sabdariffa* (*H. sabdariffa*) leaves on Hep 3B. Different extracts of methanol, ethanol, ethyl acetate and chloroform were prepared and tested for their cytotoxic effect by MTT assay in a dose and time dependent manner. Among the different organic solvent extracts tested, methanolic extract showed a greater cytotoxic effect (i.e.,) IC 50 value of 50% reduction when compared to others. The time required to show 75% decrease in cell number was found to be 24 h.

Key words: *Hibiscus sabdariffa*, cytotoxic effect, MTT assay, cancer and Hep-3B

INTRODUCTION

Cancer is a disease that begins in the cells of the body. In normal situations, the cells grow and divide as the body needs them. No more, no less. This orderly process is disturbed when new cells form that the body doesn't need and old cells do not die when they should. These extra cells lump together to form a growth or tumor.

The liver is a common site of metastases from a variety of organs such as lung, breast, colon and rectum. When liver metastases occur at the time of initial diagnosis of the primary tumor, they are described as synchronous. If detected after the initial diagnosis, they are described as metachronous. The liver is frequently involved since it receives blood from the abdominal organs via the portal vein. Malignant cells detach from the primary cancer, enter the bloodstream or lymphatic channels, travel to the liver and grow independently. Potentially, the environment of the liver is suitable to the growth of certain tumor cells. Once a tumor begins to grow in the liver, it receives its blood supply from the hepatic artery.

Management of cancer will vary according to the individual. Some of the possible treatments are surgery, liver transplantation, chemotherapy and radiation. In recent times focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional system. Various medicinal plants have been studied using modern scientific approaches. The results from these plants have revealed the potential of medicinal plants in the area of pharmacology (Dahanukar *et al*., 2000). Recent research has identified food components (phytochemicals) that may have important anticarcinogenic activities (Mazur and Adlercreutz, 2000). These phytochemicals can suppress the initiation or reverse the promotion stage in multistep carcinogenesis.

Hibiscus sabdariffa commonly known as red sorrel and roselle. Roselle belongs to *Malvaceae* family. It is an erect, mostly branched, annual shrub. Stems are reddish in color and up to 3.5 m tall. Leaves are dark green to red, alternate, glabrous, long petiolate, palmately divided into 3-7 lobes, with serrate margins. Flowers are red to yellow with a dark center containing short peduncles (Yadong *et al*., 2005).

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Many parts of roselle including seeds, leaves, fruits and roots are used in various foods. The young leaves and tender stems of roselles are eaten raw in salads or cooked as greens alone or in combination with other vegetables and/or with meat. They are added to curries as seasoning. They have acid, rhubarb like flavor (Yadong *et al*., 2005). The leaves are antiscorbutic, emollient, refrigerant and sedative. The leaves are very mucilaginous and are used as an emollient and as a soothing cough remedy. They are used externally as a poultice on abscesses. The leaves and flowers are used internally as a tonic tea for digestive and kidney functions. Experimentally, an infusion decreases the viscosity of blood, reduces blood pressure and stimulates intestinal peristalsis. The plant is also reported to be antiseptic, aphrodisiac, demulcent purgative. It is used as folk remedy in treatment of abcesses, cancer, cough, debility, fever, heart ailments (Yadong *et al*., 2005).

In this present study, we have evaluated the *in vitro* anticancerous effect of *H. sabdariffa* leaves on hepatocellular carcinoma cell lines Hep 3B by MTT assay and it will be certainly helpful in chemoprevention and therapy.

MATERIALS AND METHODS

The present research was carried out in the Biotechnology Laboratory at Prince Shri Venkateshwara Arts and Science College, Chennai (India) during August 2006 and January 2007.

Plant Material

Plant samples of *H. sabdariffa* were collected in Chennai, Tamilnadu (India). The leaves were dried under shade and used for further experimentation.

Extraction Procedure

Five gram of *H. sabdariffa* was weighed, ground and dissolved in 50 mL of methanol. Similarly 5 g of the leaves were dissolved in 50 mL of chloroform, ethanol and ethylacetate. The solutions were stirred by placing them in a shaking incubator at 500 rpm at 20°C for 20 h. Later the solutions were filtered using filter papers and allowed to dry in the petridish separately for 3-4 days. After evaporation of the solvent, the powdered extract remaining in the plates was collected and weighed. About 0.1 g of the extract was dissolved in 5 mL of respective solvent. The extracts were stored at 4°C for further use.

Preparation of Extracts

Dry extracts of plant leaves were dissolved in 5 mL of respective solvents to give a desired stock solution of extracts (20 mg mL^{-1}). All the extracts were stored at 4°C till end of the experimentation. During the experiment, stock solutions were diluted to obtain an original concentration of 0.2 mg mL^{-1} (10 μL of stock solution made up to 1 mL with Minimum Essential Medium (MEM).

Cell Culture Conditions

Hepatocellular carcinoma Hep 3B cell culture was obtained from National Center for Cell Science, Pune, India and used in this study. The cell lines were grown at 37°C at humidified 5% CO_2 in Minimum Essential Medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g L^{-1} sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate, 90%, fetal calf serum, 10% (Jenkins, 1999).

Subculturing of Monolayer Cell Culture

Using a sterile pipette, the old medium was removed and discarded. Five milliliter of the trypsin versene glucose solution was added to the flask and left it for few min so as to detach the cells from

the flask. Then 5 mL of growth medium was added to the cell suspension. The suspended cells were collected in a 10 mL glass centrifuge tube and centrifuged at 1000 rpm for 10 min. The pellet obtained was collected, diluted appropriately and the correct amount of cells were added to each culture vessel. Then fresh medium was added to the culture vessel. The flasks were placed in the CO_2 incubator at 37°C with 5% CO_2 concentration (Jenkins, 1999).

Cell Counting and Cell Viability

Trypan Blue Exclusion Assay

0.5 mL cell suspension was mixed with 1mL of trypan blue by vortexing. Then, 20 µL of the sample was withdrawn and carefully loaded into a clean hemocytometer. The percentage viability was calculated (Chang *et al*., 2005)

MTT Based Cytotoxicity Testing

Concentration Dependent Dose

The subconfluent monolayer culture was trypsinized and collected in growth medium. The suspension was centrifuged to pellet the cells and resuspended in the growth medium and counted. The cells were diluted to 2.5-50×10^3 cell mL^{-1}. Two hundred microliter of the cell suspension was added into each well of the tube leaving a tube as blank. Two hundred microliter of growth medium was added to all the wells including the blank control medium. Serial dilutions of the cytotoxic drug (various extracts) were prepared for 15 concentrations. Then 200 µL of each drug concentration was added to the tubes excluding the blank. The tubes were then incubated at 37°C for 24 h. The contents in the tubes were transferred to the microtitre plates. At, the end of the drug exposure period, the medium was removed and fresh medium was added to the tubes. To it 50 µL of MTT was added to all the tubes. The tubes were incubated for 4 h at 37°C for 4 h. Medium and MTT from the wells were removed and the formazon crystals were dissolved by adding 200 µL 0.04 N HCl - isopropanol to all the tubes. The absorbance was taken immediately at 570 nm since the product was unstable. The results were tabulated (Table 1) and the IC_{50} concentration was determined, that was required to reduce the absorbance to half that of the control.

Table 1: The absorbance at 570 nm by the cells after treatment with various extracts of *H. sabdariffa* by concentration dependent manner

Types of extracts and concentration (%)	Absorbance (570 nm)
Methanol	
Control cells without extract	2.281
0.247×10^{-4}	1.015
0.494×10^{-4}	0.987
0.741×10^{-4}	0.920
0.988×10^{-4}	0.860
1.976×10^{-4}	0.847
2.964×10^{-4}	0.797
3.952×10^{-4}	0.660
4.94×10^{-4}	0.642
5.928×10^{-4}	0.618
6.916×10^{-4}	0.594
7.904×10^{-4}	0.575
8.892×10^{-4}	0.512
9.88×10^{-4}	0.503
10.868×10^{-4}	0.486
11.856×10^{-4}	0.463
Ethanol	
Control	2.192
0.247×10^{-4}	1.196
0.494×10^{-4}	0.962

Table 1: Continued

Types of extracts and concentration (%)	Absorbance (570 nm)
Ethanol	
0.741×10^{-4}	0.954
0.988×10^{-4}	0.945
1.976×10^{-4}	0.906
2.964×10^{-4}	0.881
3.952×10^{-4}	0.849
4.94×10^{-4}	0.825
5.928×10^{-4}	0.766
6.916×10^{-4}	0.606
7.904×10^{-4}	0.605
8.892×10^{-4}	0.591
9.88×10^{-4}	0.578
10.868×10^{-4}	0.543
11.856×10^{-4}	0.517
Ethyl acetate	
Control	2.206
0.247×10^{-4}	2.163
0.494×10^{-4}	1.015
0.741×10^{-4}	0.987
$0.988\ X10^{-4}$	0.929
1.976×10^{-4}	0.898
2.964×10^{-4}	0.874
3.952×10^{-4}	0.863
4.94×10^{-4}	0.792
5.928×10^{-4}	0.763
6.916×10^{-4}	0.747
7.904×10^{-4}	0.721
8.892×10^{-4}	0.698
9.88×10^{-4}	0.654
10.868×10^{-4}	0.632
11.856×10^{-4}	0.615
Chloroform	
Control	2.269
0.247×10^{-4}	2,284
0.494×10^{-4}	1.177
0.741×10^{-4}	1.105
$0.988\ X10^{-4}$	0.956
1.976×10^{-4}	0.920
2.964×10^{-4}	0.880
3.952×10^{-4}	0.886
4.94×10^{-4}	0.853
5.928×10^{-4}	0.818
6.916×10^{-4}	0.800
7.904×10^{-4}	0.763
8.892×10^{-4}	0.757
9.88×10^{-4}	0.683
10.868×10^{-4}	0.656
11.856×10^{-4}	0.632

Time Dependent Dose

The above mentioned procedure was followed but the drug exposure time was varied i.e., the time of drug exposure was maintained as 0, 6, 12, 24 and 48 h (Chang *et al*., 2005).

RESULTS AND DISCUSSION

In the search for new anticancer drugs, the most common screening methods employ cytotoxicity tests against panel of cancer cell lines. These are High Throughput Screening (HTS) assays, revealing compounds with the highest cytotoxic activity. Although anticancer drugs are designed to kill cells such activity should be selective towards tumor cells (Popiolkiewicz *et al*., 2005). The use of natural products as anticancer agents has a long history that began with folk medicine and through the years has been incorporated into traditional and allopathic medicine (Costa-Lotufo *et al*., 2005).

Cell Counting and Viability

The cells were maintained at a density of 3.4×10 cells mL^{-1} after subculturing and remained 86.66% viable.

MTT Based Cytotoxicity

Concentration Dependent Dose

Different concentrations of the extract were added to the cells and absorbance was taken at 570 nm and values in Table 1 were observed. It indicated that methanolic extract could inhibit the cells even at a lower concentration whereas other solvents did not show such effect at the particular concentration. The IC_{50} value for the methanolic extract was found to be 0.494×10^{-4} that showed cytotoxic effect on Hep3B cells to 50% reduction.

Hibiscus anthocyanins extracted from calyx of *H. sabdariffa* possess anti carcinogenic and antimutagenic activity (Tseng *et al*., 2000). Cancerous cell lines derived from different origins were used like Hep G2, NIH3T3, Caco-2, Hep3B, HL-60 and AGS. It was found that *H. sabdariffa* inhibited the growth of these cancerous cells through apoptosis. *H. sabdariffa* exhibited the strongest cytotoxicity potency towards Human Leukemia HL-60 and Hep 3B the next most sensitive. Present study was highly correlating with the aforesaid authors study but on the purified anthocyanins from the calyx on H.L 60 and Hep 3B cell lines. Christian *et al*. (2006) also evaluated the methanolic extract of *Hibiscus sabdariffa* flower had significantly higher activity than the other solvent extracts. This result agreed with our findings, the methanolic extract had higher cytotoxic activities than other solvent extracts.

Time dependent dose

The concentration of the drug was maintained as 11.856×10^{-4} and the time of exposure was varied and the absorbance was observed as in Fig. 1. The time required to show 75% decrease in cell number was found to be 24 h. Chang *et al*. (2005) found that *Hibiscus sabdariffa* anthocyanin treatment markedly induced apoptosis in HL-60 cells in a dose and time dependent manner. This result was comparable with that of present findings.

The present investigation suggests that the *H. sabdariffa* has potent anticancer activity against Hep 3B cell lines. Methanol was predicted to be the suitable solvent for the extraction of anticancer (Hepatocellular carcinoma) compounds from *H. sabdariffa* leaves. Further studies are needed for the identification of mechanism(s) of inhibition of cancerous cells. As leaves of *H. sabdariffa* are considered as dietary material by most of the Indians, this could reduce the chemotherapeutic side effects in the patients by suggesting herbal therapy.

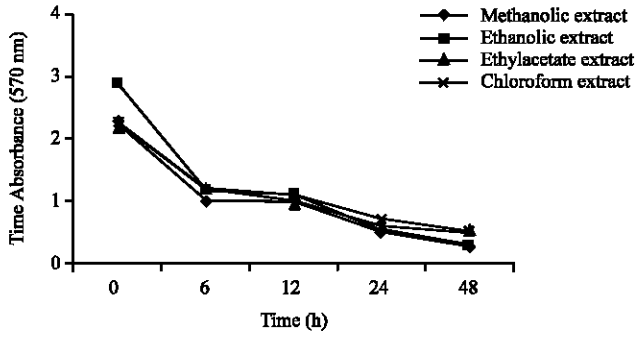

Fig. 1: The absorbance at 570 nm by the cells after treatment with various extracts of *H. sabdariffa* by time dependent manner

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