



## Anticancer Activity of *Hibiscus sabdariffa.L*(Flowers) against Human Liver Cancer (HepG2) Cell Line

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**Abstract : Objective:** To investigate the anticancer activity of the flower of *Hibiscus sabdariffa Linn*(Roselle) against human hepatoma cell line (HepG2).

**Methods:**In vitro anticancer activity was carried out to screen cytotoxicity potency of the solid obtained from ethyl acetate fraction from *Hibiscus Sabdariffa.L* flower extract at different concentrations against HepG2 cell line. The MTT (methylthiazolyl diphenyl- tetrazolium bromide) assay for cell viability and markers is predictable to confirm the cytotoxicity.

**Results:**The solid obtained from ethyl acetate fraction from the flower extract of *Hibiscus Sabdariffa.L* was tested for its anticancer activity against HepG2 cell lines (liver cancer) at various concentrations by MTT assay. It was confirmed that the IC<sub>50</sub> value of this sample was 171 ± 8.3 µg/ml against Liver Cancer HepG2 cell line.

**Conclusions :** *Hibiscus Sabdariffa.L* is a potential plant with anticancer activity. The isolation of the pure compounds and determination of the bioactivity of individual compounds will be further performed.

**Keywords:** *Hibiscus sabdariffa.L*; anti-cancer activity; MTT assay; HepG2 cell line; cytotoxicity

### 1. Introduction

Cancer is one of the most severe disease and a major public health problem in many parts of the country. Cancer continues to stand for the major cause of deaths in the world and claims over 6 million lives every year [1].The International Agency for Research on Cancer (IARC) estimates the incidence of mortality and prevalence from major types of cancer cell at national level. For 184 countries of the world revealed that there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people were living with cancer in 2012 worldwide. By 2030, it is projected that there will be 26 million new cancer cases and 17 million cancer deaths per year [2-5]. Primary liver cancer (PLC) has two major form of Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma accounting for approximately 90% and 5% respectively [6-7]. HCC causes high annual mortality rates particularly in Thailand, Cambodia and Laos where viral hepatitis is endemic [8]. The induction of HCC is preceded by the occurrence of hepatocellular damage via reactive oxygen species (ROS) and the generation of chronic inflammation related to hepatocarcinogenesis [9]. However, the main problem of chemotherapy to treat HCC is the cancer resistance mechanism, due to up regulation of the multi-drug resistance protein (MDR) and a decrease of apoptotic proteins [10]. Thus, more effective Chemotherapy is needed to control cancer and apoptosis induction, which is the desired effect for successful cancer treatment [11]. *Hibiscus* is one of the most common flower plants grown worldwide. There are more than 300 species of *hibiscus*

around the world. One of them *Hibiscus sabdariffa* Linn(roselle). This roselle belongs to the family Malvaceae. Its native distribution is uncertain; some believe that it is from India or Saudi Arabia. Nowadays, it is widely cultivated in both tropical and subtropical regions including India, Saudi Arabia, China, Malaysia, Indonesia, The Philippines, Vietnam, Sudan, Egypt, Nigeria and Mexico. *Hibiscus sabdariffa* is commonly known as roselle, hibiscus, Jamaica sorrel, Indian sorrel or red sorrel (English), karkadeh (Arabic) and in Indian language Gongura, Lal-ambari or Patwa (Hindi), Lal-mista or Chukar (Bengali), Lal-ambadi (Marathi), Yerra gogu (Telugu), Pulichchaikerai (Tamil), Pulachakiri or Pundibija (Kannada), Polechi or Pulichchai (Malayalam) and Chukiar (Assam). Roselle (*H. sabdariffa*) is an edible plant used in various applications including foods. The fleshy red calyces are used for making wine, juice, jam, syrup, pudding cakes, ice cream or herbal tea. Roselle flowers and calyces are also known for their antiseptic, diuretic, antioxidant and antimutagenic properties. The traditional medicine use the aqueous extract of this plant as diuretic, for treating gastrointestinal disorders, liver diseases, fever, hypercholesterolemia, and hypertension. So there is a need to assess the potential effects of this plant. Keeping this in view, the present study has been undertaken to investigate the anticancer potential of the solid obtained from the ethyl acetate fraction from the flowers of *H.sabdariffa.L*.

## 2. Materials And Methods

### 2.1 Collection of flowers

The fresh flowers of *Hibiscus sabdariffa.L* were collected from Z. Suthamalli, Ariyalur (Dt), Tamil Nadu, India, during the month of January and identified by Dr.S.John Britto, Director, The rapinat Herbarium and Centre for Molecular Systematics (Authentication No. DP003 dated: 22/01/2016). St.Joseph's College (Campus), Trichirappalli, Tamil Nadu, India.

### 2.2 Extraction and fractionation

Fresh flowers (3 kg) of *Hibiscus sabdariffa.L* were extracted with 90% ethanol (5x500ml). The combined alcoholic extract was concentrated in vacuum and the aqueous extract was successively fractionated with petroleum ether (60-80°C) (6x250ml), Peroxide free diethyl ether (4x250ml) and ethyl acetate (8x250ml). Petroleum ether fraction and diethyl ether fraction did not yield any isolable material. Ethyl acetate fraction on concentration yielded a dry powder which was dissolved in DMSO to get various concentrations and were used for further study.

### 2.3 In vitro Anti-cancer Activity

#### 2.3.1 Cell line and culture

HepG2 (liver) cell lines were obtained from National Center for Cell Sciences Pune (NCCS). The cells were maintained in Minimal Essential Media (MEM) supplemented with 10% FBS (Foetal Bovine Serum), penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml of CO<sub>2</sub> at 37°C.

#### 2.3.2 Reagents

MEM was purchased from Hi Media Laboratories, FBS was purchased from Cistron laboratories, Trypsin, methylthiazolyl diphenyl- tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) were purchased from Sisco research laboratory chemicals, Mumbai. All the other chemicals and reagents were obtained from Sigma Aldrich, Mumbai.

#### 2.3.3 Principle of MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS). The MTT tetrazolium assay technology has been widely adopted and remains popular in academic labs as evidenced by thousands of published articles. The MTT substrate is prepared in a physiologically balanced solution, added to cells in culture, usually at a final concentration of 0.2 - 0.5mg/ml, and incubated for 1 to 4 hours. The quantity of formazan (Directly proportional to the number of viable cells) is measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer.

Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm. When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of the viable cells. The exact cellular mechanism of MTT reduction into formazan is not well understood, but likely involves reaction with NADH (The redox reactions of nicotinamide adenine dinucleotide) or similar reducing molecules that transfer electrons to MTT. Speculation in the early literature involving specific mitochondrial enzymes has led to the assumption mentioned in numerous publications that MTT is measuring mitochondrial activity.

The formazan product of the MTT tetrazolium accumulates as an insoluble precipitate inside cells as well as being deposited near the cell surface and in the culture medium. The formazan must be solubilized prior to recording absorbance readings. A variety of methods have been used to solubilize the formazan product, stabilize the color, avoid evaporation, and reduce interference by phenol red and other culture medium components. Various solubilization methods include using: acidified isopropanol, DMSO, dimethylformamide, SDS (Sodium dodecyl sulphate) and combinations of detergent and organic solvent. Acidification of the solubilizing solution has the benefit of changing the color of phenol red to yellow color that may have less interference with absorbance readings. The pH of the solubilization solution can be adjusted to provide maximum absorbance if sensitivity is an issue; however, other assay technologies offer much greater sensitivity than MTT.

### 2.3.4 In vitro assay for cytotoxicity activity (MTT assay)

The cytotoxicity of sample (*Hibiscussabdariffa*) on HepG2 (liver) cell line was determined by the MTT assay. Cells ( $1 \times 10^5$ /well) were plated in 1ml of medium/well in 24-well plates (Costar Corning, Rochester, NY). After 24 hours incubation, the cell reaches the confluence. Then, cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 24 hours at 37°C. After removal of the sample solution and washing with phosphate- buffered saline (pH 7.4), 200  $\mu$ l/well (5 mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl- tetrazolium bromide (MTT) phosphate -buffered saline solution was added to cells. After 4 hours incubation, 0.04 M HCl/ isopropanol were added. Viable cells were determined by the absorbance at 570 nm. Measurements were performed and the concentration required for a 50% inhibition of viability ( $IC_{50}$ ) was determined by dose-response graphically using Graph PadPrism7 (USA). The absorbance at 570 nm was measured with a UV- Spectrophotometer using wells without sample containing cells as blanks. The effect of the samples on the proliferation of HepG2 was expressed as the % cell viability, using the following formula:

$$\% \text{ Cell viability} = (A_{570} \text{ of treated cells} / A_{570} \text{ of control cells}) * 100$$

### 2.3.5 Statistical analysis

All the data were reported as the mean  $\pm$  standard deviation (S.D.). All statistical analysis was performed by means of one-way analysis of variance (ANOVA) and Student's *t*-test using Graph Pad Prism statistical software package version 7.02. Only a value of  $p < 0.05$  and 0.01 was considered statistically significant.

## 3. Results

### 3.1 In vitro assays (Cytotoxic studies):

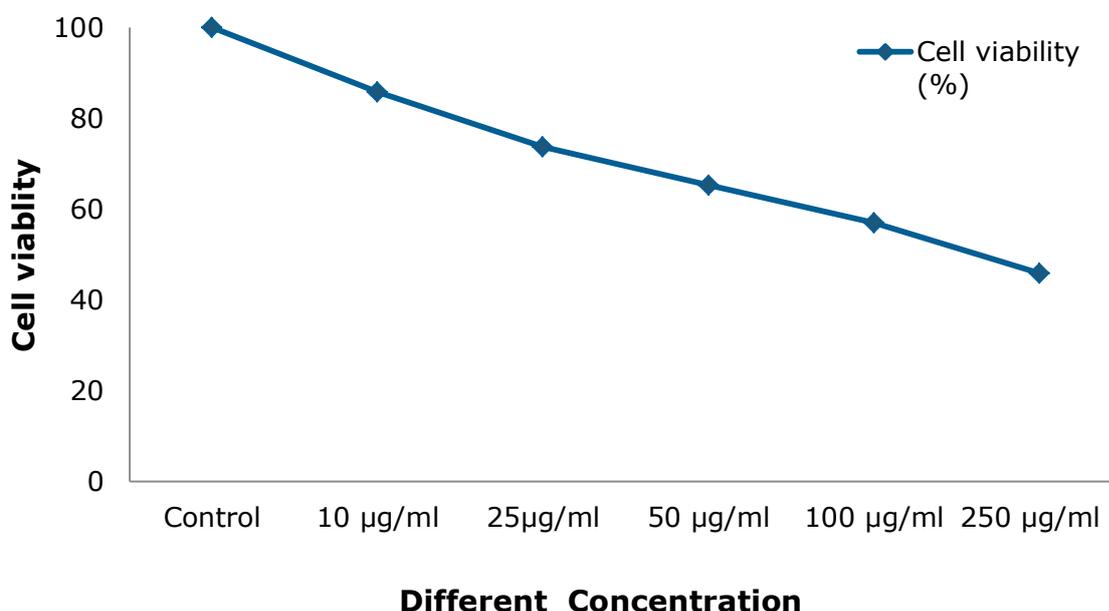
The anticancer activity of the *Hibiscussabdariffa* flower was confirmed by MTT assay (Table 1, Fig 1&2). The control HepG2 cells showed high proliferation that has been taken as 100%. Test samples showed effective  $IC_{50}$  value against HepG2 (liver) cell line was found to be  $171 \pm 8.3 \mu\text{g/ml}$ . It induced cytotoxicity in a significant manner which implicit the damage to the membrane integrity of the cell when compared with control. The cytotoxicity was increased with increase in concentration of the sample and near normal level was attained at various concentrations (10  $\mu\text{g/ml}$ , 25  $\mu\text{g/ml}$ , 50  $\mu\text{g/ml}$ , 100  $\mu\text{g/ml}$  and 250  $\mu\text{g/ml}$ ) and maximum effect was found when treated at 250  $\mu\text{g/ml}$ , which showed cell viability of  $45.86 \pm 0.8\%$ . From the above results, it was confirmed that the solid obtained from methyl acetate fraction of *Hibiscussabdariffa* (flowers) at 250  $\mu\text{g/ml}$  seems to offer significant protection and maintains the structural integrity of the hepatocellular membrane.

**Table: 1** Cell viability (%) of HepG2 cell line

Concentration ( $\mu\text{g/ml}$ )	Cell Viability (%)	IC <sub>50</sub> ( $\mu\text{g/ml}$ )
Control	100	171 $\pm$ 8.3
10	85.79 $\pm$ 0.6**	
25	73.77 $\pm$ 0.5**	
50	65.24 $\pm$ 0.6**	
100	57.00 $\pm$ 0.7**	
250	45.86 $\pm$ 0.8**	

Values shown as for means  $\pm$  S.D. of triplicate.

\*\*  $p < 0.01$  compared with control (one-way ANOVA and *t*-test)

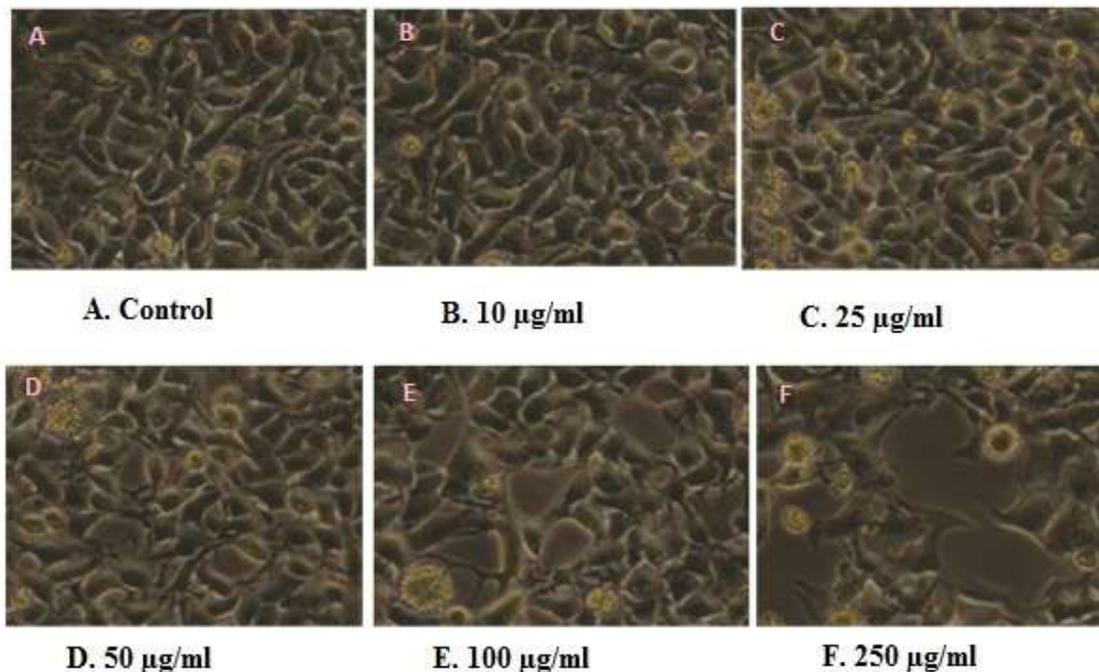


**Fig. 1.** Graphical representation of the cell viability (%) values of the solid obtained from the ethyl acetate fraction of *Hibiscus sabdariffa*(flowers)against HepG2 cell line.

#### 4. Discussion

Negative free radicals are produced in the body amid typical digestion and furthermore upon presentation to ecological toxins, for example, irresistible specialist, UV light, radiation et cetera. Hurtful free radicals are not killed by the body's essential and auxiliary resistance component on overabundance of ominous free radicals [20]. Existing clinical reviews have additionally demonstrated that supplemental levels of hostile to oxidant vitamins (E, C and B complex) lessen, the individual hazard for certain malignancy [20, 23, 24, 25]. Many of the medicinal plant have been found to be effective in experimental and clinical cases of cancer. Medical plants dominate immunomodulatory and anti-oxidant properties, leading to anti-cancer activity [20, 26, 27, 28]. This plant has been reported to be responsible for free radical scavenging effects and anti-oxidant property [21,22]. MTT is considered to be reliable assay to determine the extent of cell viability. In the present study, The cell treated with the test sample at various concentration (10, 25, 50,100 and 250 $\mu\text{g/ml}$ ) showed the protective nature of the sample act against the deleterious effects and the maximum effect was observed at 250 $\mu\text{g/ml}$ . The extract had an IC<sub>50</sub> value of 171  $\pm$  8.3 $\mu\text{g/ml}$  which assumed cell viability. From the results, it clear that the solid obtained from the ethyl acetate fraction of *Hibiscus sabdariffa* flower has cytotoxic effect against HepG2 cell line.

### MTT Assay on HepG2 Cell line (flowers extract)



**Fig.2. (B-F): Effect of the solid obtained from the ethyl acetate fraction of *Hibiscus sabdariffa* flowers against human Liver cancer HePG2 Cell line in different concentrations.**

### 5. Conclusion

The results obtained from the *in vitro* studies performed using the HepG2 cell lines reveals that the solid obtained from the ethyl acetate fraction of the EtOH extract of *Hibiscus sabdariffa* flowers has a moderate anticancer activity even though cell growth inhibition were increased when concentration of sample was increased. These concentrations were able to induce apoptosis on human cancer cell lines and its anticancer activity was found to be precise. Further work is required in order to establish the identity of the chemical constituent responsible for anticancer activity. Studies are in progress in our laboratory to elucidate the molecular structure of the compound. This contributes towards the development of potent anticancer drug.

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