

# Growth Inhibition and Apoptosis of Ehrlich Ascites Carcinoma Cells by Methanol Extract from the Calyx of *Hibiscus Sabdariffa* Linn

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**Objectives:** This study focused on the antioxidant and anticancer activities of methanolic extract of *Hibiscus sabdariffa* calyx. **Methods:** *In vitro* methods were used to determine the different types of phytochemical content and antioxidant activity of methanolic extract of *Hibiscus sabdariffa* calyx as well as its cytotoxic effect against Ehrlich ascites carcinoma cells. *In vivo*, the antineoplastic effect of methanolic extract of *Hibiscus sabdariffa* calyx against Ehrlich ascites carcinoma was also evaluated by determining the viable tumor cell count, survival time, body weight gain, and hematological profiles of experimental mice along with observing morphological changes of Ehrlich ascites carcinoma cells by fluorescence microscopy and determining the expression of *p53* gene in Ehrlich ascites carcinoma cell assayed by reverse transcription-polymerase chain reaction. Chemical composition of methanolic extract of *Hibiscus sabdariffa* calyx was analyzed by gas chromatography–mass spectrometry. **Results:** Flavonoid rich methanolic extract of *Hibiscus sabdariffa* calyx had showed remarkable antioxidant activity and in *in vitro* assay, methanolic extract of *Hibiscus sabdariffa* calyx had potent inhibitory effect against Ehrlich ascites carcinoma cells with a half maximal inhibitory concentration of 28.16 µg/mL. Apoptotic morphological changes and increased expression of *p53* gene in Ehrlich ascites carcinoma cells derived from methanolic extract of *Hibiscus sabdariffa* calyx-treated (5 and 10 mg/kg/day) mice were also observed. Moreover, chemical constituents identified by gas chromatography–mass spectrometry analyses were likely related to this activity. **Conclusion:** Methanolic extract of *Hibiscus sabdariffa* calyx has promising antioxidant and antineoplastic potential and can be considered as a probable new source for antitumor agent.

**Keywords:** Hibiscus sabdariffa, antioxidant; antineoplastic, apoptosis, Ehrlich ascites carcinoma.

## Introduction

Research on the treatment of cancer is fundamental to improving outcomes for patients affected by this disease and the adverse effects of the existing drugs has increased the demand for new treatment options [1, 2]. Current research efforts focus on the development of more effective and less toxic treatments, such as targeted therapies, immunotherapies, and cancer vaccines, as well as the improvement of therapies that have existed for decades, such as chemotherapy, radiation therapy, and surgery. Recently, use of plant secondary metabolites has been identified as a promising approach for the prevention of human cancer due to their ability to scavenge reactive oxygen species, a factor contributing to cancer development, as well as induce apoptotic death of cancer cells [3]. Moreover, naturally occurring secondary metabolites of plants exhibit their antiproliferative action against cancer cells but not on host cells [4]. So, research on medicinal plants to identify new anticancer drugs with fewer or no side effects has significant value.

Roselle (*Hibiscus sabdariffa* L.), locally known as "Lal-mesta", belongs to the Malvaceae family and widely distributed in Bangladesh [5, 6]. 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 [7]. The leaves have antiscorbutic, emollient, cooling and sedating properties. 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 to enhance digestive and kidney function. Experimentally, an infusion decreases the viscosity of blood, reduces blood pressure and stimulates intestinal peristalsis. The plant is also reported to have antiseptic, aphrodisiac, demulcent purgative properties [7]. It is used as folk remedy in treatment of abscesses, cancer, cough, debility, fever, heart ailments. Commercially, the calyx was found to be the most important part of this plant and is used to treat fever, high blood pressure and liver disease. The extract of this part of the plant was found to have antihypercholesterolemic, antinociceptive, and antipyretic activities [7]. The above overall information suggests that the calyx of *Hibiscus sabdariffa* may have an antineoplastic effect. But we are unable to find literature regarding the antineoplastic effects of calyx of

*Hibiscus sabdariffa* extract against Ehrlich ascites carcinoma (EAC) cells. Therefore, in the current study we studied the antineoplastic activity of the calyx of *Hibiscus sabdariffa* against EAC cells. We also analyzed the chemical composition as well as antioxidant activity of *Hibiscus sabdariffa* calyx.

## Materials and Methods

### Collection of plant materials and authentication

Calyx of *Hibiscus sabdariffa* L. was collected from the campus area of Rajshahi University, Bangladesh. Authentication of the plant material was done by a taxonomist at the Department of Botany, University of Rajshahi. The voucher sample (No. 12) of this collection was deposited for further reference.

### Extraction of plant materials

Powdered plant material was dissolved in methanol (250 g powder in 500 mL methanol) at room temperature and it was stored for several days with occasional shaking. After filtration, the filtrate was evaporated under reduced pressure at 40°C using a rotary evaporator to yield 19.7 gm of methanol extract (about 8% yield) of *Hibiscus sabdariffa* calyx (MEHSC.)

### Chemicals and reagents

Hoechst 33342, RPMI 1640-medium and MTT were obtained from Sigma-Aldrich (USA). Penicillin-streptomycin and fetal calf serum were obtained from Invitrogen (USA). Trypan blue and all other chemicals/reagents that were used in this study, were of analytical grade.

### Analysis of phytochemicals

The method of Folin-Ciocalteu method was used for estimation of total phenolic content of MEHSC, where gallic acid was used as the standard compound [8]. Absorbance was recorded at 760 nm. Total phenolic content of the extract was calculated and expressed in terms of gallic acid equivalent (mg)/g of extract ( $y = 0.117x + 0.051$ ,  $R^2 = 0.998$ ).

The method of Dewanto et al. was used to estimate the total flavonoid content of MEHSC with catechin used as a standard [9]. Absorbance was recorded at 510 nm. Results were calculated and expressed in terms of catechin equivalent (mg)/g of extract ( $y = 0.005x + 0.047$ ,  $R^2 = 0.998$ ).

### Free radical (DPPH, ABTS and Nitric oxide) scavenging assays

We used to a previously described method to determine the 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt (ABTS•+) and nitric oxide radical scavenging activity of MEHSC and standard (catechin) [10-12]. After completing the procedure, absorbance of the resulting mixtures was measured at 517 nm (for DPPH), 734 nm (for ABTS•+) and 546 nm (for nitric oxide). Each assay was repeated three times. The percentage scavenging activity was calculated using the following formula:

$$\text{Percentage (\%)} \text{ scavenging of radical} = [(1 - A_s/A_c)] \times 100$$

Where  $A_s$  = Absorbance of sample and  $A_c$  = Absorbance of Control (Catechin)

$EC_{50}$  values ( $\mu\text{g/mL}$ ), the effective concentration of the sample needed to scavenge each radical by 50%, was determined from the plots showing the scavenging activity as a function of the concentration of the extract.

### Determination of Lipid peroxidation inhibition activity assay

The method reported by Liu and Ng was used to determine the lipid peroxidation inhibition activity of the extract and the standard (catechin) with some modification [13]. After harvesting the livers, the liposomes were obtained by homogenizing the tissue in buffer and centrifuging it. Then 100  $\mu\text{L}$  10 mM  $\text{FeSO}_4$ , 100  $\mu\text{L}$  0.1 mM ascorbic acid and 0.3 mL of extract/standard (catechin) were added at various concentrations to 0.5 mL of supernatant and the mixture was incubated for 20 minutes at 37°C. After incubation, 1 mL of 28% TCA and 1.5 mL of 1% TBA was added immediately and then the mixture was again heated at 100°C for 15 minutes and cooled at room temperature. This experiment was repeated three times. After recording the absorbance at 532 nm, the percentage inhibition of lipid peroxidation was calculated by the following equation:

$$\text{Percentage (\%)} \text{ inhibition of lipid peroxidation} = [(A_{b2} - A_{b1})/A_{b2}] \times 100$$

where  $A_{b2}$  is the absorbance of the control (without sample), and  $A_{b1}$  is the absorbance of the samples (extract or standard). Then percent of inhibition was plotted against concentration, and half maximal inhibitory concentration ( $IC_{50}$ ) was calculated from the graph.

### Tumor cells

The Indian Institute for Chemical Biology (IICB), Kolkata, India, provided Ehrlich ascites carcinoma cells and these cancer cells were maintained in the laboratory by weekly intraperitoneal inoculation ( $10^6$  cells/mouse).

### Animals and ethical clearance

Swiss-albino mice of both genders, weighing between 28-32 gm were collected from International Center for Diarrheal Disease Research, in Bangladesh (ICDDR'B). These animals were kept in standard laboratory conditions (temperature  $25 \pm 2^\circ\text{C}$ ; humidity  $55 \pm 5\%$ ) with 12h dark/light cycle) throughout whole study. Ethical approval and permission for using mice in our research was obtained from the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee, Institute of Biological Sciences (225/320-IAMEBBC/IBSc), University of Rajshahi, Bangladesh.

### Cell Culture

For *in vitro* assay, RPMI-1640 medium supplemented with 10 % fetal calf serum, and 1 % (v/v) penicillin–streptomycin, was used to culture EAC cells in a humidified atmosphere of 5 %  $\text{CO}_2$  at 37 °C.

### Cell viability test by MTT colorimetric assay

MTT cell viability assay was used to determine EAC cells proliferation as described by Kabir et al. [14]. 200  $\mu\text{L}$  of EAC cells ( $2.5 \times 10^5$  cells) cultured in RPMI-1640 media, was plated in the 96-well culture plate in the presence of different concentrations (3.125-100  $\mu\text{g/mL}$ ) of extract. Then the culture plate was placed in  $\text{CO}_2$  incubator at 37°C for 1 day followed by the removal of the supernatant. After addition of 180  $\mu\text{L}$  of PBS and 20  $\mu\text{L}$  of MTT to each well of the culture plate, they were incubated at 37°C for an additional 8 hours. The supernatant was removed again and 200  $\mu\text{L}$  of acidic isopropanol was added into each well followed by incubation at 37°C for 1 hour. Finally, absorbance was recorded at 570 nm wave length using titer plate reader. This process was repeated 3 times. The following equation was used to calculate the cell proliferation inhibition ratio: Proliferation inhibition ratio (%) =  $[(A - B)/A] \times 100$  where A is the  $OD_{570\text{nm}}$  of the cellular homogenate (control) without extract and B is the  $OD_{570\text{nm}}$  of the cellular homogenate with extract.

### Cell growth inhibition *in vivo*

Four groups of Swiss albino mice ( $n=6$ ) weighing  $26\pm 4$  gm were used to study the cell growth inhibition properties of MEHSC [15]. For this study,  $1.5\times 10^6$  EAC cells were inoculated into each group of mice on day zero. After 24 hours of inoculation, treatment was started and continued for five days. Mice of Groups 1 and 2 were treated with MEHSC at the doses of 5 and 10 mg/kg/per day, respectively, via intraperitoneal injection. Group 3 received an anticancer drug, bleomycin (0.3 mg/kg/day), whereas group 4 was an untreated control. After 5 days of treatment, tumor cells were harvested and then counted with a haemocytometer using an inverted microscope (XDS-1R, Optika, Bergamo, Italy).

### Survival time and tumor weight

Survival time of the EAC-bearing mice and tumor weight were determined using the method reported by Khanam et al. [16]. Briefly, four groups of Swiss albino mice (six in each group) were used. For therapeutic evaluation, each mouse receiving EAC was inoculated with  $1.5\times 10^6$  EAC cells on day zero. Treatment was given after 24 hours of inoculation and continued for 10 days. Groups 1–2 received MEHSC at the doses of 5 and 10 mg/kg/per day, respectively, whereas group 3 was treated with bleomycin. Group 4 was used as untreated control. The daily weight change of each mouse was recorded throughout 10 days of treatment and finally the mean survival time and percent increase in life span (%ILS) was determined using the previously described formula [16].

### Studies on Hematological Parameters

The effect of MEHSC on hematological characteristics of EAC cell bearing were also studied using the method of Mukherjee [17]. Five groups were designed (6 mice in each group) and mice of four groups were injected with EAC cells ( $1.5\times 10^6$  cells/mouse) intraperitoneally except group 1 which served as the normal group. MEHSC at 5 and 10 mg/kg/mouse/day doses were administered in groups 3 and 4, respectively for 10 days, whereas the mice in group 2 were kept untreated control. Group 5 received bleomycin at 0.3 mg/kg/mouse/day dose for 10 days. Hematological parameters were measured on 12<sup>th</sup> day after tumor inoculation from the freely-flowing tail vein blood of each mouse in each group.

### Cell morphologic change and nuclear damage

In cell growth inhibition assay, EAC cells were collected from MEHSC-treated (10 mg/kg/mouse/day) mice and untreated control groups after five days treatment and collected cells were stained with 1 mg/mL of Hoechst 33342 at 37 °C for 10 min in the dark. The cells were then washed with purified bovine serum (PBS). After washing again, the nuclear morphological changes of these cells were investigated using a fluorescence microscopy (Olympus iX71, South Korea) [14].

### Reverse transcription polymerase chain reaction

The TRIzol method was used to extract total RNA from EAC cells which were received from mice of untreated and MEHSC (10 mg/kg/day) treated groups on day six after EAC cell inoculation [14]. The previously described method was applied here to prepare cDNA using 3 µg RNA in a final volume of 20 µL having 100 pmol random hexamer and 50 U of MuLV reverse transcriptase (New England Biolab) [14]. Expression of apoptosis related gene *p53* was examined using these cDNA as template and the reaction mixture containing deoxyribonucleotide triphosphate (dNTP), primer (forward and reverse) and platinum Taq polymerase (Tiangen, Beijing, China) for polymerase chain reaction (PCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as control. Primer sequences and their thermal cycle conditions are presented in Table 1. Amplification reactions were carried out by BioRad (Hercules CA, USA) gradient thermal cycler. All the PCR products were analyzed in 1.5% agarose gel and Gene Ruler 1kb DNA ladder (Tiangen Biotech, Beijing, China) was used as a marker [14].

### GC-MS analysis of bioactive molecule

Separation and identification of the components of MEHSC were performed by Gas chromatography–mass spectrometry (GC-MS) using an Agilent 6890N gas chromatograph connected to a Agilent 5973 N mass selective detector. This instrument was equipped with a flame ionization detector and HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm). The temperature program for the gas chromatography (GC) was as follows: the initial oven temperature was set at 60°C for 1 min and ramped at 10° C min<sup>-1</sup> to 180° C for 1 min and then ramped at 20° C min<sup>-1</sup> to 280 °C for 15 min. The temperature of the injector was maintained at 270°C whereas the injected volume of samples was 1 µL with a

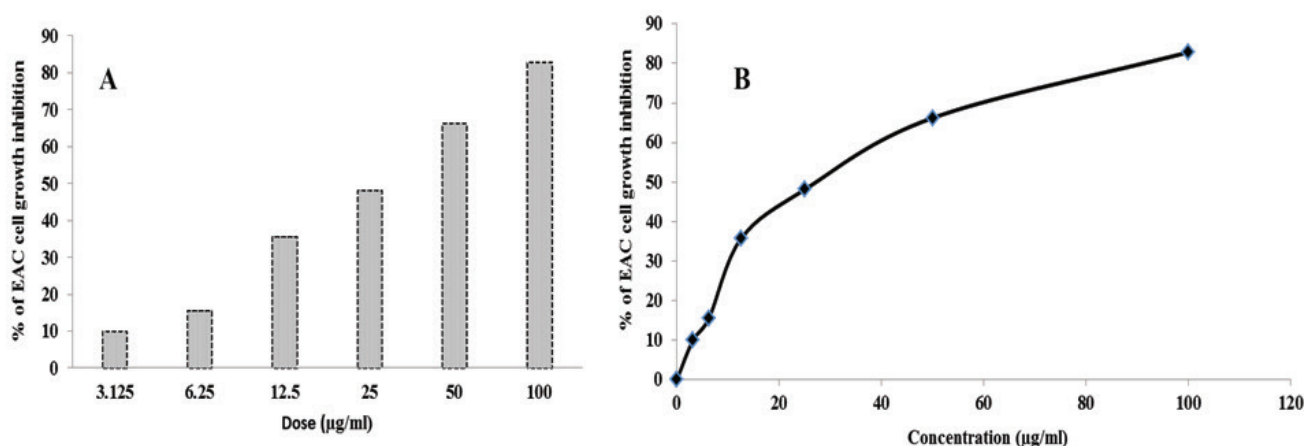
**Table 1.** Primer sequences and PCR protocol used for the RT-PCR assay

Target genes	Primers	PCR protocol	Amplification (kb)
<i>p53</i>	5'-GCGCTTAGAGACAGTTGACT-3'	Cycling condition for initial PCR activation step of 5 min at 95 °C, followed by 35 cycles of 95 °C/1 min. For <i>p53</i> , caspase-8 gene 55°C/1 min, 72 °C/1, and a final extension of 72 °C/10 min. In the case of GAPDH, the annealing temperature was 52 °C.	0.450
	5'-GGATAGGTCGGCGGTTTCATGC-3'		
<i>GAPDH</i>	5'-GTGGAAGGACTCATGCCACAG-3'		0.350
	5'-CTGGTGCTCAGTGTAGCCAG-3'		

**Table 2.** Phytochemical analysis and antioxidant effect of MEHSC

Parameters	MEHSC	Catechin
TPC (mg of gallic acid equivalent/g of extract)	18.41 ± 0.25	-
TFC (mg of catechin equivalent/g of extract)	104.44 ± 1.50	-
DPPH radical scavenging activity (EC <sub>50</sub> values in µg/mL)	5.59 ± 0.45	2.65 ± 0.11
ABTS radical scavenging activity (EC <sub>50</sub> values in µg/mL)	13.93 ± 1.87	3.74 ± 0.27
Nitric oxide scavenging activity (EC <sub>50</sub> values in µg/mL)	47.61 ± 2.14	3.23 ± 0.18
Lipid peroxidation inhibition activity (EC <sub>50</sub> values in µg/mL)	22.60 ± 1.09	7.52 ± 0.89

Data are expressed as mean ± SD for three replications.



**Figure 1.** A: Growth inhibition of EAC cells by MEHSC when EAC cells were treated with various doses of MEHSC for 24 h. The inhibition ratios were measured by the MTT assay (n = 3, Mean±SD). B: IC<sub>50</sub> value of MEHSC was calculated from the dose–response curve.

split ratio of 1: 10. As carrier gas, Helium was used at a flow rate of 1.0 ml/min. For spectra, scanning was performed from 20 to 550 m/z at 2 scans /sec.

### Statistical analysis

The data were analyzed by one-way ANOVA (analysis of variance) followed by multiple comparisons using Dunnett's post hoc test using SPSS software of 16 version. All results were represented

as mean ± standard deviation (SD). Differences at p<0.05 level were considered to be statistically significant.

## Results

### Total phenolic and flavonoid contents and antioxidant activity

Table 2 shows the total content of polyphenols in the MEHSC. The

total phenolic and flavonoid content of MEHSC were  $18.41 \pm 0.25$  mg of gallic acid equivalent/gm of extract and  $104.44 \pm 1.50$  mg of catechin equivalent/gm of extract, respectively.

The antioxidant efficacy of MEHSC as determined by the DPPH, ABTS, nitric oxide and lipid peroxidation inhibition assay techniques, is shown in Table 2. In each radical scavenging assay, the radical scavenging activity of MEHSC was found to increase with increasing MEHSC concentration and the  $IC_{50}$  values for MEHSC were found to be 5.59, 13.93, 47.61 and 22.60  $\mu\text{g}/\text{mL}$  in DPPH, ABTS, nitric oxide and lipid peroxidation inhibition assays, respectively. Here, catechin showed the lowest  $IC_{50}$  values in each assay.

### Cell growth inhibition *in vitro*

*In vitro* the anticancer activity of MEHSC against EAC cells was evaluated by MTT colorimetric assay and as a result, it was found that MEHSC inhibited EAC cell growth in a dose dependent manner. Maximum (82.85%) and minimum (9.92%) cell growth inhibition was recorded at the concentration of 100 and 3.125  $\mu\text{g}/\text{mL}$ , respectively (Fig. 1A). The  $IC_{50}$  value of MEHSC was found to be 28.16  $\mu\text{g}/\text{mL}$  against EAC cells (Fig. 1B).

### Cell growth inhibition *in vivo*

We used EAC cell-bearing mice to observe the effects of MEHSC on EAC cell growth inhibition at MEHSC doses of 5 mg/kg/day and 10 mg/kg/day, compared to bleomycin at 0.3 mg/kg/day which was used as standard. Maximum anticancer activity (66.91% growth inhibition) was found at the dose of 10 mg/kg, which was comparable to 91.82% inhibition found with the standard drug at the dose of 0.3 mg/kg (Table 3).

### Average tumor weight and survival time

EAC cell-bearing mice were treated with MEHSC at the doses of 5 and 10 mg/kg/day for 10 days and their body weight gain

due to tumor burden, was calculated. It was observed that the MEHSC and bleomycin reduces body weight gain approximately the same (Table 4). It was also noticed that treatment of tumor-bearing mice with MEHSC at doses 5 and 10 mg/kg/day resulted in significant increase of life span, which were 27.36% and 55.04%, respectively, compared with that of control mice ( $p < 0.05$ ) (Table 4).

### Hematological studies

Results regarding to the effects of MEHSC on the hematological profile of the EAC cell-bearing mice, are presented in Table 5. Inoculation of EAC cells resulted in the reduction of Hb content and RBC counts, whereas an increase in WBC counts was observed. Interestingly, MEHSC restored these altered hematological parameters (Table 5).

### Cell morphological examination

After staining with Hoechst 33342, the fluorescence microscopy of MEHSC-treated EAC cells showed apoptotic morphological changes (e.g. chromatin condensation, fragmentation, membrane blebbing) characteristic of cell death (Fig. 2B). Under fluorescence microscopy of untreated EAC, it was found that these cells exhibited a round and homogeneously stained nucleus (Fig. 2A). So, the above findings confirmed the MEHSC-induced apoptosis in EAC cells.

### Effect of MEHSC on gene expression (RT-PCR)

An amplification reaction using GAPDH primers was performed to test the suitability of the purified RNAs and they were found to be suitable for RT-PCR. In the present study, the *p53* gene showed increased expression in MEHSC-treated cells. On the other hand, no expression of this gene was found in control mice EAC cells (Fig. 3)

**Table 3.** Effect of MEHSC on Ehrlich ascites carcinoma (EAC) cell growth inhibition

Group No.	Treatment	Viable EAC cells on day 6 after inoculation ( $\times 10^7$ cells/mL)	Percentage (%) cell growth inhibition
1	EAC + Control	$4.77 \pm 0.44$	-
2	EAC + MEHSC (5 mg/kg)	$2.16 \pm 0.31^*$	$54.78 \pm 5.44$
3	EAC + MEHSC (10 mg/kg)	$1.57 \pm 0.08^*$	$66.91 \pm 1.64$
4	EAC + Bleomycin (0.3 mg/kg)	$0.39 \pm 0.09^*$	$91.82 \pm 1.14$

Data are expressed as mean  $\pm$  SD for six animals in each group. \* $p < 0.05$ : against EAC control group.

**Table 4.** Effect of MEHSC on survival time and body weight gain of EAC cell bearing mice

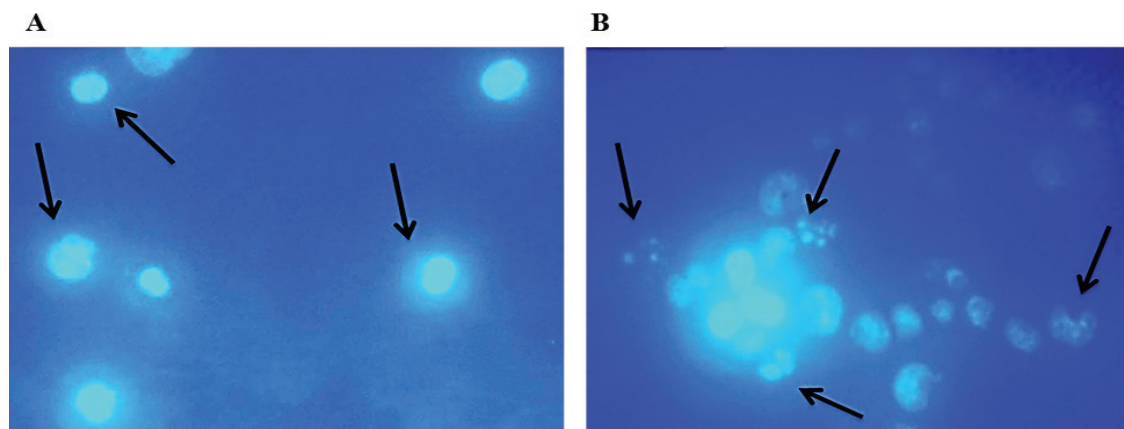
Group No.	Treatment	MST (in days)	%ILS	Body weight gain (g) after 15 days
1	EAC + Control	20.25±1.70	-	16.00±1.41
2	EAC + MEHSC (5 mg/kg)	25.5±1.29*	27.36±4.79	6.61±0.51*
3	EAC + MEHSC (10 mg/kg)	31.25±1.5*	55.04±5.07	4.75±0.95*
4	EAC + Bleomycin (0.3 mg/kg)	39.25±0.79*	93.82±3.45	3.62±0.47*

Data are expressed as mean ± SD for six animals in each group. P<0.05: against EAC control group.

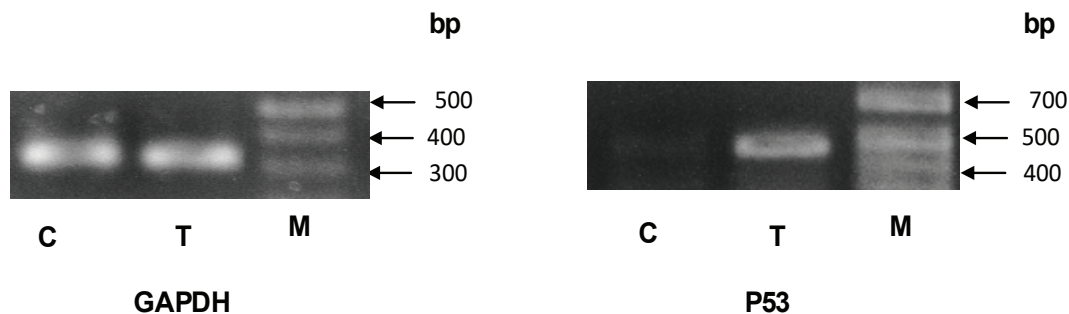
**Table 5.** Effect of MEHSC on blood parameters of tumor bearing and normal Swiss albino mice

Parameters	Normal	EAC+ Control	EAC + MEHSC (5mg/kg)	EAC + MEHSC (10 mg/kg)	EAC + Bleomycin (0.3 mg/kg)
Hgb (g/dL)	14.48 ± 0.52	10.5±1.08	12.65±0.65*	14.05±0.80*	14.32±0.22*
RBC(x10 <sup>9</sup> cells/mL)	6.67 ± 0.23	1.59±0.12	2.82±0.31*	4.86±0.29*	5.89±0.11*
WBC(x10 <sup>6</sup> cells/mL)	8.75 ± 0.53	39.5±4.4	28.5±3.10*	11.00±1.85*	9.32±0.67*

Data are expressed as mean ± SD for six animals in each group. \*P<0.05: against EAC control group.



**Figure 2.** MEHSC-induced apoptosis in Ehrlich ascites carcinoma (EAC) cell. EAC cells collected from the treated and non-treated EAC-bearing mice were stained with Hoechst 33342 and then examined by fluorescence microscopy. Left panel A: indicates control and right panel B: indicates MEHSC (10 mg/kg/day) noted that apoptotic characteristics e.g. nuclear condensation and fragmentation are seen in figure B.



**Figure 3.** RNA was extracted from the experimental mice on day 6 and level of mRNA expression of proapoptotic gene (*p53*) was studied. In MEHSC-treated mice, *p53* gene expression increased remarkably whereas opposite result was observed in control group. M represents 1 kb DNA ladder; T, RNA from EAC cells of MEHSC treated mice; C, RNA from EAC cells of MEHSC untreated mice.

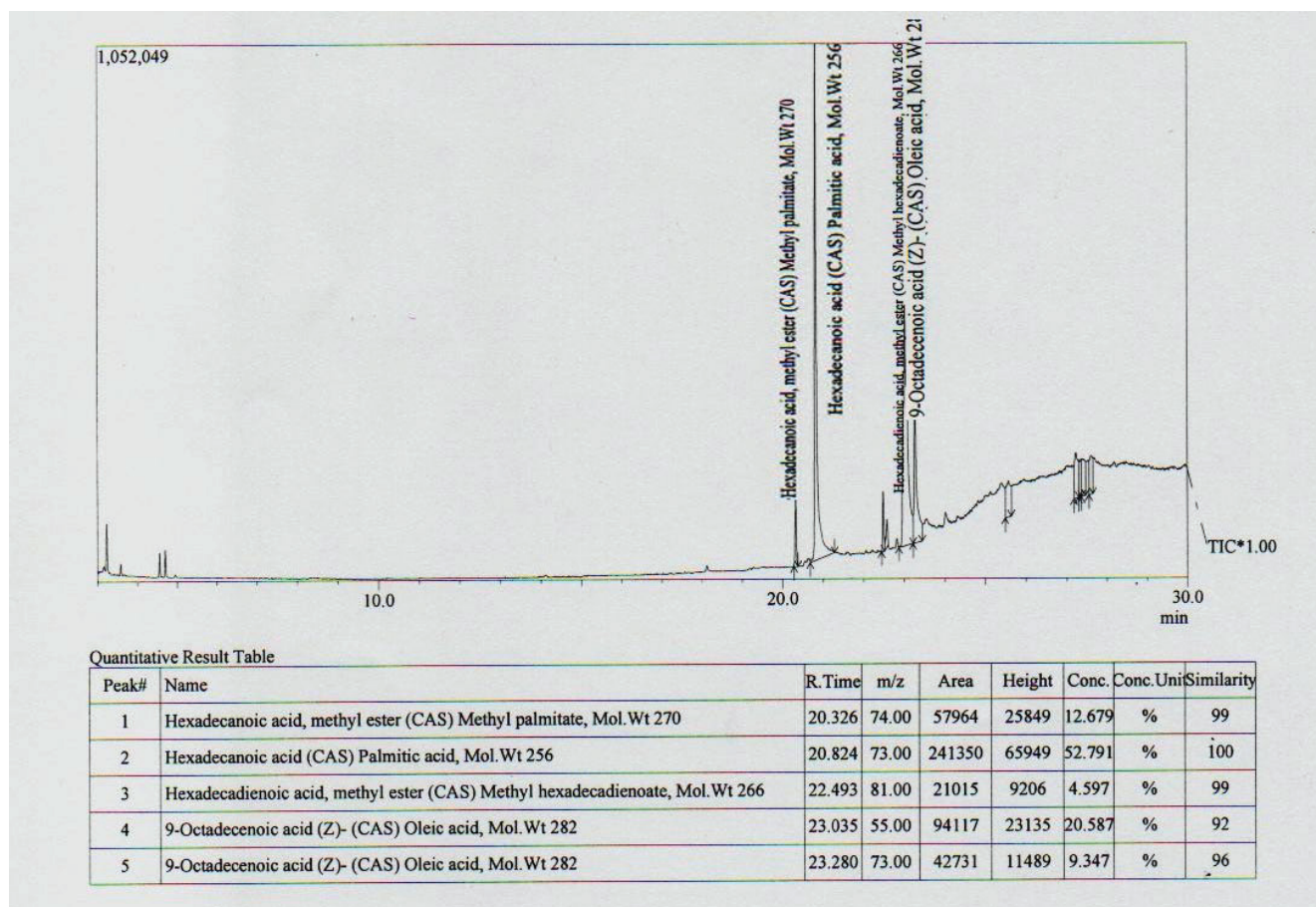


Figure 4. Gas chromatography–mass spectrometry chromatogram of methanol extract of *Hibiscus sabdariffa* calyx showing the peaks of the main active compounds.

Table 6. Chemical constituents of methanol extract of *Hibiscus sabdariffa* calyx

Peak#	Name of compound	Retention Time (minutes)	(%) percentage composition
1	Hexadecanoic acid, methyl ester	20.326	12.679
2	Hexadecanoic acid	20.824	52.791
3	Methyl hexadecadienoate	22.493	4.597
4	Oleic acid	23.035 23.280	29.934

### Chemical profile of MEHSC analyzed by GC-MS

The chemical profile of MEHSC extract that was identified by GC-MS (Fig. 4), is summarized in Table 6. A total of four components as hexadecanoic acid, methyl ester (12.68%), hexadecanoic acid (52.79%), methyl hexadecadienoate (4.60%) and oleic acid (29.93%) were identified in the methanol extract, accounting for 100% of the extract.

### Discussion

Extensive research has been done on the ability of antioxidant compounds to prevent various human cancers [18]. Flavonoids are the most abundant antioxidant found in the plant kingdom, a number of plant species rich in flavonoids reportedly have high therapeutic efficiency for the treatment of cancer [19, 20]. In



addition, phenolic contents of plant kingdom have also produced remarkable effects in the prevention of numerous oxidative stress associated diseases including cancer due to their strong antioxidant activities [21, 22]. In this investigation, we found MEHSC as a rich source flavonoid and phenolic compounds.

Furthermore, we employed several *in vitro* assay models to assess the antioxidant activity of MEHSC. In DPPH assay, the potential of a plant extract as a free radical scavenger is monitored as DPPH free radicals accept hydrogen or electrons from an antioxidant extract [23]. For measuring the total antioxidant potential of plant extracts, the ABTS assay is also a widely used method [23]. In addition, excessive nitric oxide (NO) undergoes reaction with oxygen to generate toxic free radicals like nitrite and peroxy nitrite anions, although at normal levels NO plays important role in many physiological processes and regulation of cell mediated toxicity [24, 25]. Lipid peroxidation is an harmful process in living cells in which polyunsaturated fatty acids undergo conversion into lipid peroxides and various carbonyl products. In lipid peroxidation inhibition assay,  $FeCl_3$  was used to induce lipid peroxidation in rat liver homogenate where thiobarbituric acid reactive substances (TBARS), a pink chromogen, are produced as by-products of lipid peroxidation. A decline in the production of this pink chromogen in the pre-treated reaction is considered as inhibition of lipid peroxidation [26]. This study demonstrated the potent antioxidant activity of MEHSC in DPPH, ABTS, nitric oxide and lipid peroxidation inhibition assays, which suggests its probable use in the treatment of various oxidative stress related diseases including cancer.

EAC cells offer special benefits for anticancer drug testing due to their suitability for study in almost any mouse host; hence they were used in the present investigation. Moreover, these cells lack H-2 histocompatibility antigens, which is the probable reason for their quick proliferation [14].

We assessed the *in vitro* anticancer property of MEHSC against EAC cells by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay and found significant cell growth inhibition (82.85%) at the concentration of 100  $\mu$ g/ml. Numerous studies performed MTT assay previously to evaluate the *in vitro* capacity of an extract or a compound to inhibit the growth of cancer cell and the results obtained from those studies support the findings of the present study [14] indicating the probable use of the present extract as an

anticancer agent.

*In vivo* anticancer activity of the present extract was evaluated by using EAC cell-bearing mouse model and the results were compared with the data obtained by performing parallel experiments with a reference standard drug, bleomycin (0.3 mg/kg). In this study, untreated EAC cell-bearing mice gained body weight rapidly due to rapid tumor growth but the treatment of the EAC-cell bearing mice with MEHSC reduced body weight gain significantly, inhibited cell growth significantly, and increased life span remarkably comparable to that of bleomycin (0.3 mg/kg).

Reduction of average tumor weight, cell growth inhibition and enhancement of life span of tumor bearing mice are measured to judge the potential of compounds as anticancer agents. These data from this study are also consistent with those reported in the literature [27, 16] suggesting that MEHSC possesses potent anticancer activity.

Further evaluation of anticancer activity of this methanol extract was carried out by examining the change in hematological parameters. EAC-cell bearing mice were found to gradually have reduced hemoglobin concentrations and RBC counts, the major problems in cancer bearing mice. Administration of the methanol extract in EAC cell-bearing mice reversed all the altered hematological parameters more or less to normal level and this result is similar with that of previous studies where EAC cell-bearing mice were treated with bioactive compounds [27, 16] suggesting the protective action of MEHSC on the hemopoietic system.

Apoptosis, a cell-suicidal mechanism, is characterized by the change of morphological features, such as nuclear fragmentation, blebbing, cell shrinkage and chromatin condensation [14]. Capability of selectively inducing apoptosis in cancer cells is a highly desired feature of an anticancer drug since cancer or malignant cells are selectively removed through this process without damaging normal cells. After staining with Hoechst 33342 (a blue fluorescing dye that stains), fluorescence microscopy enabled us to observe cell morphological features related to apoptosis such as nuclear fragmentation, chromatin condensation etc. [28]. In our present study, when EAC cells were treated with MEHSC, cell shape was changed and the nucleus was fragmented and condensed; these findings were comparable with the control EAC cells suggesting that MEHSC can play significant role in cancer prevention by inducing

apoptosis. Induction of apoptosis in EAC cells by different plant extracts has been identified in several previous studies [28].

Dysregulation of apoptosis is usually considered as a major cancer hall mark since 50% of all human tumors bear the functionally mutant of *p53* gene which is an important contributor to the induction of apoptosis derived by various cellular stresses [29]. A large number of studies have produced evidence that many chemotherapeutic agents act by inducing apoptosis in cancer cells [30, 31]. In our study, after five days treatment, expressions of *p53* were found in MEHSC-treated mice but not untreated control mice. This finding indicates that apoptosis induced in MEHSC-treated EAC cells was probably mediated by *p53*-dependent pathway.

GC-MS analysis of MEHSC revealed the presence of four compounds one of which (oleic acid) have been reported previously to possess antitumor activity [32] suggesting that the potent anticancer activity of MEHSC could be attributed to these active components. Hexadecanoic acid was also found to have potential antioxidant activity [33].

Our study has some limitations. We did not identify the main components that are responsible for the antineoplastic effect of calyx of *Hibiscus sabdariffa*. In addition, this study did not confirm the detail molecular mechanism underlying the inhibition of EAC cells by MEHSC. Additional research will be carried out to purify the main active compounds from the crude extract and to elucidate the molecular mechanism of inhibition.

## Conclusion

We conclude that calyx of *Hibiscus sabdariffa* possesses potential antioxidant and anticancer activity, and therefore, might be considered as a promising agent in cancer chemotherapy.

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