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Antioxidant and antineoplastic activities of roots of *Hibiscus sabdariffa* Linn. Against Ehrlich ascites carcinoma cells

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Abstract

Background: The goal of this study was to explore the inherent antioxidant and antineoplastic activities of methanolic extract of the roots of *Hibiscus sabdariffa* (MEHSR).

Methods: The dried coarse powder of roots of *Hibiscus sabdariffa* was subjected to methanolic extraction. Here in vitro methods were used to determine the various types of phytochemical content and antioxidant activity of MEHSR as well as its cytotoxic effect against Ehrlich ascites carcinoma (EAC) cells. In vivo, antineoplastic activity of MEHSR against EAC cells was also evaluated by determining the viable tumor cell count, survival time, body weight gain, hematological profiles of experimental mice along with observing morphological changes of EAC cells by fluorescence microscope. Analysis of the chemical composition of MEHSR was carried out using GC-MS.

Results: Total phenolic and flavonoid contents of MEHSR were found to be 143.36 and 82.81 mg/g of extract in terms of gallic acid and catechin equivalent, respectively. The MEHSR exhibited very good scavenging property on DPPH (IC_{50}: 13.37 μg/mL) and ABTS (IC_{50}: 18.88 μg/mL) radicals in respect to nitric oxide (IC_{50}: 72.82 μg/mL) radical and lipid peroxidation (IC_{50}: 75.78 μg/mL) inhibition. MEHSR was found to induce Ehrlich ascites carcinoma (EAC) cell death at a dose dependent fashion. At dose 10 mg/kg, MEHSR significantly inhibited tumor cell growth rate (62.24%; $p < 0.05$), decreased tumor weight (57.81%; $p < 0.05$), increased life span (38.97%) compared to the untreated control mice. MEHSR also restored all hematological parameters of EAC-bearing mice towards normal level. Furthermore, administration of MEHSR induced apoptosis of EAC cells as observed in Hoechst 33342 stained cells under fluorescence microscope. Arachidic acid (49.18%), oleic acid (36.36%) and octadecanoic acid (14.47%) were identified as the major components of MEHSR by GC-MS analysis.

Conclusion: In a nutshell, our findings proposed that MEHSR may possess promising antioxidant and antineoplastic efficacy against Ehrlich ascites carcinoma cells by induction of cell apoptosis. Therefore, it might be a potent and novel candidate for anticancer therapy.

Keywords: *Hibiscus sabdariffa*, Root, Antioxidant, Anticancer, GC-MS analysis

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Introduction

In our everyday lives, we are continuously exposed to various chemical and physical offenses like environmental pollutants, food additives, ultraviolet and ionizing radiation and so on. In addition to these external stresses, free radicals and reactive oxygen species (ROS) are produced as byproducts of both physiological and pathological cellular processes occurring in the mitochondria, peroxisomes and endoplasmic reticulum (ER) [1]. These free radicals and ROS play a critical role in the initiation and progression of various types of cancers. The different signaling pathways that control many cellular processes, including cell proliferation are affected by ROS [2]. These alterations induced by ROS stimulate the uncontrolled growth of cells which encourages the development of tumors [3]. However, previously it was assumed that antioxidants from natural sources exerted their health benefits by reacting with ROS as well as free radicals and destroying them [4]. But many studies have refuted the hypothesis where reaction of ROS and free radicals with antioxidants is considered the main mechanism of neutralization [5]. Instead, several polyphenolic compounds of plant origin help cells to counteract the detrimental effects of ROS and free radicals through the activation of nuclear factor E2-related factor 2 (Nrf2) [6–8]. As a regulator of endogenous cellular defense mechanisms, Nrf2 binds to antioxidant response elements (AREs) that are commonly found in the promoter region of antioxidant and detoxification genes thereby controlling their expression [9, 10]. Thus phytochemicals with antioxidant properties can overcome the incidence of cancer by alleviating free radical born cell injury without showing any destructive effects [11, 12]. Moreover, an interesting finding revealed by many scientists is the induction of apoptosis during the treatment of cancer cells with extracts produced from medicinal plants [13]. That's why, antioxidant compounds generating plants has grown an increasing interest among scientists.

The plant Roselle (*Hibiscus sabdariffa* L.), popularly known as "Lal-mesta", belongs to the Malvaceae family and widely distributed in Bangladesh [14, 15]. It has a long history of use in traditional system of medicine. From the ancient times, the fruits and leaves of *Hibiscus sabdariffa* have been used for ethnomedicine in the management of abscesses, cancer, cough, debility, fever, heart ailments. Previous studies have shown that the extracts of different parts of *Hibiscus sabdariffa* plant possesses antihypercholesterolemic, antinociceptive and antipyretic activities [16]. The above overall information from literature indicates that the roots of *Hibiscus sabdariffa* may have anticancer effect. As far we know, the antioxidant and anticancer activities of roots of *Hibiscus sabdariffa* were not studied. Therefore, the objectives of this investigation was set to evaluate antineoplastic potential of methanol extract of *Hibiscus sabdariffa* roots (MEHSR) using Ehrlich ascites carcinoma (EAC) cell line. In addition, this study also focused on the antioxidant properties and chemical composition of MEHSR.

Materials and methods

Chemicals and reagents

2,2′-diphenyl-1-picrylhydrazyl (DPPH•), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS•+), catechin, sulphanilamide, hydroxylamine hydrochloride, Hoechst 33342, RPMI-1640 medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA) whereas N-(1-naphthyl) ethylenediamine dihydrochloride, aluminum chloride ($AlCl_3{\cdot}6H_2O$), gallic acid, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), trypan blue, isopropanol and RNA extraction kit (TRIzol) were from Carl Roth GmbH (Karlsruhe, Germany). Penicillin-streptomycin and fetal calf serum were obtained from country distributer of Invitrogen (USA) and Folin Ciocalteu (FC) reagent was supplied from Sisco Research Laboratory, Mumbai, India. Methanol and other solvents were purchased from E-Mark (Germany) and all the chemicals used here were of analytical and HPLC grade with 99.9% purity.

Plant materials and extraction

Fresh samples of *Hibiscus sabdariffa* roots were collected from the relevant area of Rajshahi, Bangladesh and authenticated by Professor Dr. A. H. M. Mahbubur Rahman (a taxonomist), Department of Botany, University of Rajshahi. A sample specimen was preserved (No. 12) in the herbarium of this department for further reference. After shade drying, the clollected plant materials were reduced to coarse powder. An amount of 250 g of the powdered sample was extracted by maceration with 500 mL methanol for 15 days at room temperature. After filtration, the solvent was completely removed by rotary vacuum evaporator to yield 5 g methanol extract of *Hibiscus sabdariffa* roots (designated as MEHSR).

GC-MS analysis of chemical constituents

Separation and identification of the constituents of MEHSR were performed by GC–MS agilent 6890 N gas chromatography hooked to agilent 5973 N mass selective detector. They equipped with a flame ionization detector and capillary column with HP-5MS (30 m × 0.25 mm × 0.25 μm). In GC settings: the initial oven temperature was set at 60 °C for 1 min and ramped at 10 °C min^{-1} to 180 °C for 1 min and then ramped at 20 °C min^{-1} to 280 °C for 15 min. The temperature of the injector was controlled at 270 °C. The samples (1 μl) were injected neat, with a split ratio of 1: 10. Helium was used as the

carrier gas at a flow rate of 1.0 ml min $^{-1}$. Spectra were scanned from 20 to 550 *m/z* at 2 scans s^{-1}. Identification of most constituents by gas chromatography was done by comparing their retention indices with those reported in the literature or with those of authentic components available in database.

Estimation of total phenolic and flavonoid content

Total phenolic contents of MEHSR were estimated according to the Folin-ciocalteu reagent (FCR) method and gallic acid was used for making calibration curve [17]. 1 ml methanolic solution of gallic acid of different concentration and MEHSR was mixed with 5 ml FCR (diluted tenfold). The each mixture was shaken and left to stand for 5 min before the addition 1 ml of 5% (w/v) sodium carbonate (Na_2CO_3) solution. Then 3 ml distilled water was added to make the total solution 10 ml. After incubation of the mixture at room temperature for 60 min, the absorbance was taken at 760 nm using a UV-Visible spectrophotometer. Total phenolic contents of MEHSR was calculated and expressed in terms of gallic acid equivalent (mg)/g of extract ($y = 0.117x + 0.051$, $R2 = 0.998$).

Total flavonoid content was measured by the aluminum chloride colorimetric assay and catechin was used as a standard [18]. 1 ml of catechin of different concentration and MEHSR was taken in different test tubes and mixed with an equal volume of 2% (w/v) $AlCl_3{\cdot}6H_2O$ solution in methanol. The mixture was vigorously shaken and after 10 mins of incubation, the absorbance was taken at 430 nm. Calibration curve ($y = 0.005x + 0.047$, $R2 = 0.998$) for catechin was drawn and the total flavonoid content (TFC) of MEHSR was calculated by the same way as described in the estimation of total phenolic content and expressed as in mg of catechin (CAT) equivalent/g of the extract.

DPPH and ABTS radical scavenging assay

DPPH radical scavenging activity of MEHSR was measured using the procedure reported previously [18]. A solution of 0.1 mM DPPH was prepared in methanol and 3 ml of this solution was mixed with 1 ml of extract/catechin (standard) in methanol at different concentrations. The mixture was then vortexes thoroughly and kept in the dark place for 30 min at room temperature. The absorbance of the mixture was recorded at 517 nm and the percentage scavenging activity was calculated using the following formula:

SA% = [(Absorbance of control− Absorbance of sample) /Absorbance of control] × 100.

Finally percentage DPPH radical scavenging activity was plotted against concentration and IC_{50} was calculated from the graph.

Free radical scavenging activity of MEHSR was also determined by ABTS radical cation decolorization assay [18]. ABTS● + was generated by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate. The mixture was allowed to stand in the dark place for 12–16 h at room temperature. The ABTS● + solution was then diluted with water to obtain an absorbance of 0.70 ± 0.02 at 734 nm. ABTS● + solution (3 ml) was added to 0.1 ml of MESMS and catechin with various concentrations and mixed vigorously. The absorbance was measured at 734 nm after standing for 6 min. The percentage (%) scavenging activity for ABTS● + and IC_{50} were calculated by the similar way as described in DPPH$^{\bullet}$ assay.

Nitric oxide scavenging activity

Determination of nitric oxide scavenging was carried out as reported by Shukla et al. [19]. 2 ml of 10 mM sodium nitroprusside was dissolved in 0.5 ml phosphate buffer saline (pH 7.4) and then it was mixed with 0.5 ml of MEHSR and catechin (positive control) at different concentrations. After incubation at 25 °C for 150 min the samples were added to 0.5 ml of greiss reagent containing 1% (w/v) sulphanilamide, 2% (v/v) H_3PO_4 and 0.1% (w/v) napthylethylenediamine dihydrochloride. After incubation at room temperature for 30 min, absorbance was measured at 546 nm. Then percentage of scavenging activity and IC_{50} were measured similar to that of DPPH assay .

Lipid peroxidation inhibition assay

This assay system demonstrates the ability of phytoconstituents to induce lipid peroxidation that was measured by the method of Jelili et al. [20]. Briefly, 1 ml of reaction solution containing 0.5 ml liver homogenate, 100 μl of 10 mM $FeSO_4$, 100 μl of 0.1 mM acetic acid and 0.3 ml of MEHSR at distinct concentrations was incubated at 37 °C for 20 min. Then the mixtures were further heated at 100 °C for 15 min followed by the addition of 28% TCA and 1.5 ml of 1% TBA. After cooling at room temperature the absorbance was measured at 532 nm. The percentage of inhibition and IC_{50} were calculated by the similar equation used in DPPH assay. Catechin was used as positive control.

Animals and ethical clearance

Swiss albino mice of either sex, 3–4 weeks of age, weighting between 20 and 25 g were collected from the animal research branch of the international center for diarrheal Diseases and Research, Bangladesh (ICCDRB). They were maintained under standard laboratory conditions (temperature 22-28 °C, humidity 55–3%) with 12 h day-night cycle and also provided with standard dry pellet diet and water ad libitum.

Cell culture

Ehrlich ascites carcinoma cells were collected from Institute of chemical Biology, India and maintained by weekly (i.p.) inoculation of 1×10^6 cells/mouse under standard laboratory conditions. For in vitro cytotoxic assay, EAC cells were cultured in RPMI-1640 media provided with 10% fetal calf serum and 1% (v/v) penicillin-streptomycin in a CO_2 incubator.

In vitro cell viability test by MTT colorimetric assay

MTT colorimetric assay was performed to measure the cytotoxicity of MEHSR against EAC cells [21]. In brief, EAC cells (2.5×10^5 in 200 μl RPMI 1640 media) along with MEHSR at different concentrations (15.625–500 μg/ml) were plated in each well of a culture plate (containing 96-well bottom), followed by incubation in CO_2 incubator at 37 °C for 24 h. After the incubation period, aliquot was aspirated. Then 180 μl of PBS and 20 μl of MTT were added and further incubated at 37 °C for 8 h. Subsequently, the aliquot was removed; 200 μl acidic isopropanol was added and again incubated at 37 °C for 1 h. Finally, the absorbance was taken at 570 nm in micro-titer plate reader. Cell proliferation inhibition ratio was calculated by the following equation:

Proliferation inhibition ratio (%) = {(A − B)/A} × 100.

Where A is the OD_{570} nm of the cellular homogenate without MEHSR (control) and B is the OD_{570} nm of the cellular homogenate with MEHSR.

Studies on in vivo EAC cell growth

To study the effect of MEHSR on EAC cell growth, 1.5×10^6 cancer cells were inoculated (i.p.) into four groups of mice (6 in each) on day zero. After 24 h of tumor cells inoculation, MEHSR at doses of 5 and 10 mg/kg per day were given to every mouse of group 1–2, respectively. Bleomycin at the dose of 0.3 mg/kg was given to group-3 while group-4 was used as untreated control mice. Treatment was continued for 5 days and on day 6 after EAC cell inoculation, animals were sacrificed. EAC cells were collected by repeated washing with 0.9% saline and viable EAC cells per mouse of the treated groups were compared with untreated control [22].

Studies on weight gain and survival time

These parameters were measured under similar experimental conditions as stated in the previous experiment [15]. Briefly, four groups of Swiss albino mice (six in each group) were used. For therapeutic evaluation, 1.5×10^6 EAC cells per mouse were inoculated to each group of mice on day zero. Treatment was given after 24 h of inoculation and continued for 10 days. Groups 1 & 2 received MEHSR at different concentrations; group 3 was treated with bleomycin and group 4 was used as control. The average weight gain of each group was noted on 10th day after EAC cell inoculation and survival period of each mouse of each group was recorded to determine mean survival time [22].

Studies on hematological parameters

To assess the haemotological parameters, Swiss Albino mice were divided into four groups ($n = 6$). All the animals were injected with EAC cells (2×10^6 cells/mouse) intraperitoneally except for the normal group. This was taken as day zero. Group 1 served as the normal control and group 2 served as the untreated EAC control. Group 3 and 4 were treated with MEHSR at 5 and 10 mg/kg doses, respectively. On the 12th day, after tumor transplantation, tail vein blood was collected which was used to measure hematological profile [22].

Studies on morphological appearance of EAC cells

Morphological change of EAC cells in MEHSR-treated (10 mg/kg) and untreated control mice were investigated using a fluorescence microscope (Olympus iX71, Korea). Briefly, the EAC cells from both groups of mice were collected and stained with Hoechst 33342 at 37 °C for 10 min in the dark and subsequently, washed with phosphate buffered saline (PBS). Finally, fluorescence microscopic observation was carried out to determine morphological changes [22].

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

Chemical profile of MEHSR analyzed by GC-MS

The chemical profile of MEHSR that was identified by GC-MS spectrum (Fig. 1), are summarized in Table 1. A total of three components as arachidic acid (49.18%), oleic acid (36.36%) and octadecanoic acid (14.47%) were identified in MEHSR, accounting for 100% of the extract.

Total phenolic and flavonoid contents

The total phenolic and flavonoid contents of MEHSR were found to be 143.36 ± 4.13 mg of gallic acid equivalent/g of dry extract and 82.81 ± 3.96 mg of catechin equivalent/g of dry extract, respectively (Table 2).

Antioxidant activity assay

The scavenging activity of MEHSR was found to be increased with increasing concentrations in DPPH, ABTS,

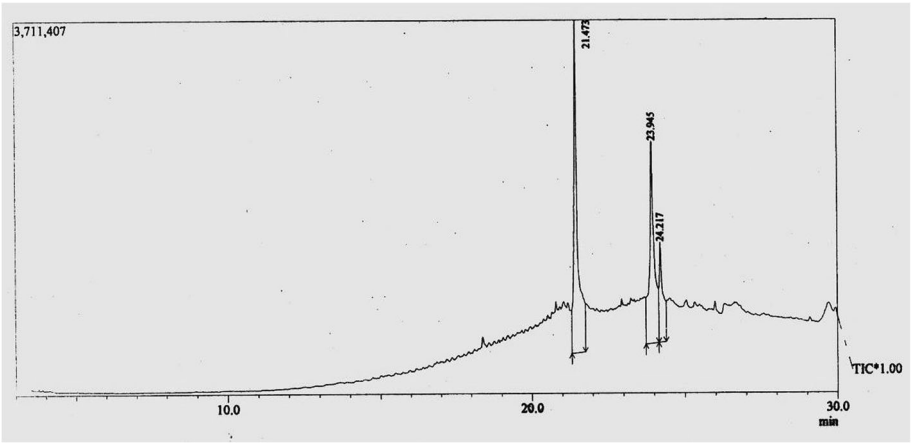

Fig. 1 Gas chromatography–mass spectrometry chromatogram of MEHSR

nitric oxide and lipid peroxidation scavenging assays. As shown in Table 2, the IC_{50} values of MEHSR were 13.37 ± 1.06, 18.88 ± 1.72, 72.82 ± 2.26 and 75.78 ± 2.94 μg/ml in case of DPPH, ABTS, nitric oxide and lipid peroxidation activities, respectively and it was comparable to catechin used as standard (Table 2).

In vitro inhibition of EAC cells by MEHSR

In vitro cytotoxicity assay, MEHSR reduced EAC cell viability in a dose dependent fashion (Fig. 2a). A decrease of viable cells was observed at a concentration of MEHSR as low as 15.625 μg/mL and loss of cell viability was found to be increased with the increasing concentrations of MEHSR (Fig. 2a). The IC_{50} value of the MEHSR was determined as 156.20 μg/mL against EAC cell (Fig. 2b).

In vivo the effect of MEHSR on EAC cell proliferation

Mice treated with MEHSR showed a significant ($P < 0.05$) decrease in viable EAC cells. The average number of viable EAC cells per mouse of untreated EAC control group was found to be $(3.64 \pm 0.33) \times 10^7$ cells/ml which was significantly reduced to $(2.01 \pm 0.06) \times 10^7$ and $(1.37 \pm 0.14) \times 10^7$ cells/ml when mice were treated with MEHSR at the doses of 5 and 10 mg/kg body weight, respectively (Table 3). MEHSR at 5 mg/kg and 10 mg/kg doses induced 44.42 and 62.24% inhibition of EAC cell growth, respectively whereas it was 90.29% for the standard bleomycin (0.3 mg/kg) (Table 3).

Table 1 Chemical constituents of MEHSR identified by its GC-MS spectrum

Peak#	Name of compound	Retention Time	(%) percentage composition
1	Arachidic acid	21.473	49.18
2	Oleic acid	23.945	36.36
3	Octadecanoic acid	24.217	14.47

Effect of MEHSR on survival time and weight gain of EAC bearing mice

In this study the mean survival time (MST) of untreated EAC cell bearing mice was 20.25 ± 1.70 days whereas it was 23.75 ± 1.25, 28.00 ± 0.81 and 39.25 ± 0.79 days for the mice treated with MEHSR (5 and 10 mg/kg body weight) and bleomycin (0.03 mg/kg body weight), respectively (Table 3). The life span of EAC cell bearing mice treated with MEHSR was found to be increased markedly (17.37% % and 38.97% at 5 and 10 mg/kg, respectively), which was comparable to bleomycin (93.82%) (Table 3). The average weight gain of untreated control group was 16 ± 0.75 g whereas it was significantly ($P < 0.05$) reduced by treatment of MEHSR and bleomycin (Table 3).

Effect of MEHSR on cell morphological change

As shown in Fig. 3, the induction of EAC cell death by MEHSR was accompanied by chromatin condensation and nuclear fragmentation, a notable hallmark of apoptosis whereas intact cell nuclei were observed in untreated control.

Effect of MEHSR on hematological parameters of EAC cell bearing mice

From the results regarding the effect of MEHSR on the hematological parameters of both treated and untreated mice, it was found that hemoglobin, total RBC and total WBC, were found to be significantly altered as compared to those of the normal mice (Table 4). However, these deteriorated parameters became reversed toward the

Table 2 Phytochemical analysis and antioxidant effect of MEHSR

Sample	TPC (mg of gallic acid equivalent/g of extract)	TFC (mg of catechin equivalent/g of extract	DPPH radical scavenging activity (IC_{50} values in μg/ml)	ABTS radical scavenging activity (IC_{50} values in μg/ml)	Nitric oxide scavenging activity (IC_{50} values in μg/ml)	Lipid peroxidation inhibition activity (IC_{50} values in μg/ml)
MEHSR	143.36 ± 4.13	82.81 ± 3.96	13.37 ± 1.06	18.88 ± 1.72	72.82 ± 2.26	75.78 ± 2.94
Catechin	–	–	2.65 ± 0.98	4.59 ± 1.13	3.23 ± 2.68	7.45 ± 2.00

Data are expressed as mean ± SD;
TPC Total Phenolic Content; *TFC* Total Flavonoid Content

normal ranges when MEHSR supplementation was given to them at the dose used in this study (Table 4).

Discussion

In human body, excessive accumulation of ROS or depletion of intermediates with antioxidant capacity alters the redox homeostasis and leads to oxidative stress [23]. An integrated cellular enzymatic redox system consisted of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and peroxiredoxins (PRXs) is a cellular defence mechanism that maintains the oxidative equilibrium [24]. Expressions of these detoxifying and antioxidant enzymes are modulated by a key transcription factor Nrf2 which can be activated by ROS at cellular level. Nrf2 translocates into nucleus and regulates antioxidant response elements (AREs) mediated transcriptions of various genes encoding the above mentioned antioxidant enzymes [25, 26]. However, this mechanism is deficient under oxidative stress from excess ROS [23]. Consumption or supplementation of polyphenolic and flavonoid type compounds have been shown to be able to restore the redox homeostasis and prevent systemic or localized inflammation by enhancing activities of the antioxidant enzymes SOD, CAT, GPx and GR [25, 27]. Dietary polyphenols and flavonoids, are also capable of triggering Nrf2 translocation to induce subsequent activation of the endogenous antioxidant actions through ligand interaction with cytosolic aryl hydrocarbon receptor (AhR) [28]. Flavonoids and their derivatives have shown agonistic potential to regulate AhR- mediated signalling in cells [23]. Thus effects on the cross-link between AhR and Nrf2 signalling pathways are the key molecular mechanism underlying the ability of polyphenols in promoting endogenous antioxidant defensive system based on SOD, CAT, GPx and GR, to restore the cellular redox homeostasis [28].

In addition, polyphenols can also suppress oxidative stress by reducing inflammatory responses via interfering with nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) controlled inflammatory signalling cascades [29]. Activation of these cellular processes leads to innate magnification of regulatory immune responses. As a result, pro-inflammatory cytokines, including interleukin (IL)-1b, IL-6, IL-8, tumour necrosis factor (TNF)-α and interferon (IFN)-γ are released into the circulation system which, if not properly regulated, can trigger irreversible systemic inflammation and disrupted immune homeostasis [29]. Thus polyphenolic and flavonoid type compounds have significant modulatory effect on cellular biomarkers related to oxidative stress and inflammation, which lead to reduced

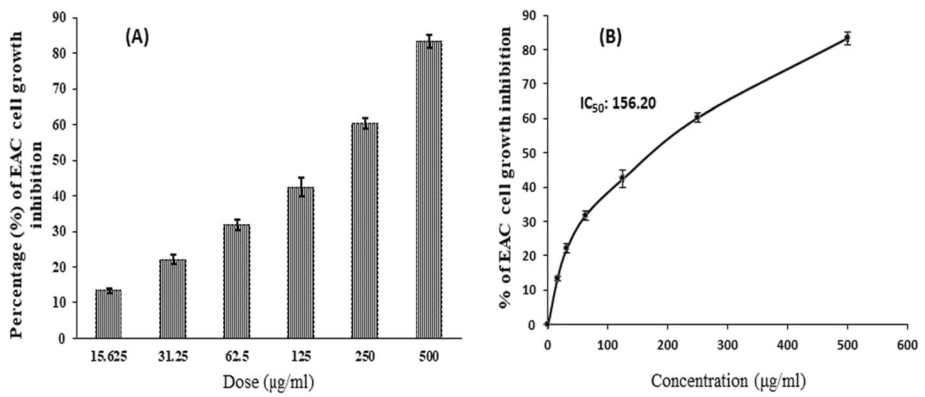

Fig. 2 In vitro the effect of MEHSR on the viability of EAC cells as determined by by the MTT assay. **a** Growth inhibition of EAC cells by MEHSR when EAC cells were treated with various doses of MEHSR for 24 h ($n = 3$, Mean ± SD). **b** IC_{50} value of MEHSR calculated from the dose–response curve

Table 3 Effect of MEHSR on cell growth inhibition, survival time and body weight gain of Ehrlich ascites carcinoma (EAC) cell bearing mice

Treatment	Viable EAC cells on day 6 after inoculation ($\times 10^7$ cells/ml)	Percentage (%) cell growth inhibition	MST (in days)	%ILS	Body weight gain (g) after 15 days
EAC + Control	3.64 ± 0.33	–	20.25 ± 1.70	–	16.00 ± 1.41
EAC + MEHSR (5 mg/kg)	2.01 ± 0.06*	44.42 ± 3.07	23.75 ± 1.25*	17.37 ± 3.41	8.75 ± 1.50*
EAC + MEHSR (10 mg/kg)	1.37 ± 0.14*	62.24 ± 2.71	28.00 ± 0.81*	38.97 ± 4.94	6.75 ± 0.95*
EAC + Bleomycin (0.3 mg/kg)	0.39 ± 0.09*	90.29 ± 1.14	39.25 ± .79*	93.82 ± 1.25	3.60 ± 0.45*

Data are expressed as mean ± SD for six animals in each group. $P < 0.05$: against EAC control group. *MST* Mean survival time; *% ILS* Percentage increase of life span

risk of many chronic diseases like cancer. In the present study, we found MEHSR as a rich source of phenolic and flavonoid compounds and it acts as a strong free radical scavenger (Table 2). With addressing the molecular mechanisms of the antioxidant and antiinflammatory effects of the phenolic and flavonoid compounds, these results indicate that rich amount of polyphenolic and flavonoid type compounds in MEHSR are capable to reinforce the endogenous antioxidant defense from ROS ravage and restore redox homeostasis thereby focusing their probable antineoplastic efficacy.

Having concern with the facts as described above, here in vitro MTT-based cytotoxic assay was also used to evaluate the inhibitory efficiency of MEHSR against the growth of Ehrlich ascites carcinoma (EAC) cell lines. Ehrlich ascites carcinoma (EAC) is a swiftly growing experimental tumor with very aggressive behavior and resembles human tumors [30]. In vitro, exposure of EAC cells to various concentrations of MEHSR for 24 h triggered significant cell death in a dose dependent fashion (Fig. 2a) and the results obtained from previous studies sustain the findings of this study [21], indicating the potentials of this plant extract as an anticancer agent.

EAC cells are also expedient for anticancer drug test due to their suitability to study in mice model. Therefore, we were interested to examine the antineoplastic activity of MEHSR using Ehrlich ascites carcinoma (EAC) in Swiss albino mice. From the results of this experiment, we found that MEHSR (at dose 5 and 10 mg/kg) can significantly inhibit the EAC cells growth, decrease body weight gain and enhance survival time of EAC cells bearing mice (Table 3). Administration of MEHSR in EAC cell-bearing mice also reversed back all the altered hematological parameters more or less to normal level thereby suggesting its protective action on the hemopoetic system (Table 4). Moreover, EAC cells treated with MEHSR showed characteristics features of apoptosis (cell membrane blebbing, nuclear condensation and fragmentation) whereas regular and round shape was found in EAC cells of untreated control. So characteristics of apoptotic death were observed in MEHSR treated EAC cells and similar type of observations were also reported by using different plant extracts [21, 31]. All these are considered as imperative and promising characteristics of an antineoplastic agent [32].

We further conducted an analysis on the chemical composition of MEHSR and its GC-MS profile confirmed the presence of arachidic acid, oleic acid and octadecanoic acid. Among the identified components, oleic acid and octadecanoic acid showed antitumor

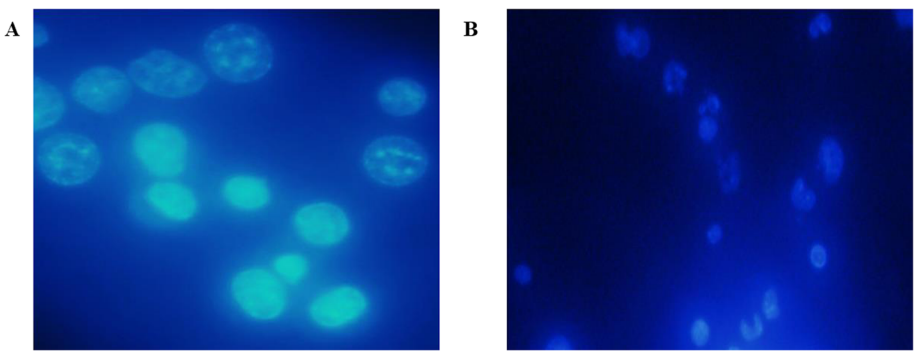

Fig. 3 Fluorescence microscopic view of EAC cells collected from the mice of untreated EAC control (**a**) and MEHSR treated (**b**) groups and stained with Hoechst 33342. Note that apoptotic characteristics e.g. nuclear condensation and fragmentation are seen in (**b**)

Table 4 Effect of MEHSR on blood parameters of tumor bearing Swiss albino mice

Name of Exp.	RBC Cells ($\times 10^9$) /ml	WBC Cells ($\times 10^6$) /ml	% of Hb gm/dl
Normal	6.67 ± 0.23	8.75 ± 0.53	14.48 ± 0.52
EAC + Control	2.00 ± 0.18^{t}	42.00 ± 5.71^{t}	9.50 ± 0.73^{t}
EAC + MEHSR (5 mg/kg)	2.52 ± 0.21*	24.00 ± 2.16*	11.75 ± 0.52*
EAC + MEHSR (10 mg/kg)	3.50 ± 0.20*	15.50 ± 2.64*	13.10 ± 0.41*

Data are expressed as mean ± SD for six animals in each group
$^{t}P < 0.05$: when compared with that of Normal group
$^{*}P < 0.05$: when compared with that of EAC control group

activity in previous studies [33, 34]. Moreover, previous scientific evidences also explored that as a major constitute, oleic acid played a key role in the antioxidative activity of *Camellia tenuifolia* and involved transcription factor DAF-16/FOXO to mediate oxidative stress resistance in *Caenorhabditis elegans* [35]. So, the observed antineoplastic activity of MEHSR are thought to be a result of the synergistic action of these compounds with antioxidative property.

Conclusion

In conclusion, the overall novel outcomes from this study concluded that MEHSR was effective in inhibiting the growth of EAC in vitro and *vivo* conditions. The possible mechanism of this antineoplastic effect may be due to the rich polyphenolic and flavonoid content of MEHSR and its antioxidant properties. However, our future studies will be carried out to identify the compounds involved in antioxidant and antineoplastic activities.

Abbreviations

CA: Catechin; EAC: Ehrlich ascites carcinoma; FCR: Folin-Ciocalteu reagent; GA: Gallic acid; MEHSR: Methanol extract of roots of *Hibiscus sabdariffa*; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

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Declarations

All authors have approved this manuscript. The content of this manuscript or any portion there of, has not been published or submitted for publication elsewhere.

Authors' contributions

RYH is primary author of this manuscript, conducted most of experiments, data analysis with aid of HA, MI, RH and TY. RYH and TY designed this study. MAS contributed to GC-MS analysis. They also critically revised this manuscript. All authors have approved this final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Protocol used in this study for the use of mice as a animal model for cancer research was approved by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes and Living Natural Sources, (225/320-IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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