A *p*-menth-1-ene-4, 7-diol (EC-1) from *Eucalyptus camaldulensis* Dnhh. triggers apoptosis and cell cycle changes in Ehrlich ascites carcinoma (EAC) cells

Running title: EC-1 inhibits cancer growth

Farhadul Islam^{a,c}, Jahan Ara Khanam^{a*}, Mahbuba Khatun^a, Natasha Zuberi^a, Laboni Khatun^a,
Syed Rashel Kabir^a, Md. Abu Reza^b, MM Ali,^c M A Rabbi^d, Vinod Gopalan^e and
Alfred King-Yin Lam^{e*}

^aDepartment of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh

^bDepartment of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh

^cDepartment of Applied Chemistry and Chemical Engineering, University of Rajshahi, Bangladesh

^dBCSIR Laboratories, Bindodpur Bazar, Rajshahi-6206, Bangladesh

^eCancer Molecular Pathology, Griffith Health Institute, Griffith University, Gold Coast, Australia

* Corresponding authors: Jahan Ara Khanam & Alfred K. Lam

E-mail addresses: Jahanara_khanam@yahoo.com (JA Khanam), a.lam@griffith.edu.au (A. Lam).

Address for sending details:

Professor Alfred Lam, School of Medicine, Griffith University Gold Coast Campus. Gold Coast, Q4222, Australia.

Phone: +61 07 56780718

E-mail: a.lam@griffith.edu.au

Abstract

Anti-cancer activities of *p*-menth-1-ene-4, 7-diol (EC-1) isolated from *Eucalyptus camaldulensis* *Dhnh* were studied on Ehrlich ascites carcinoma (EAC) cells by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. They were also analyzed in EAC-bearing mice by assessment of cancer growth inhibition, change in cancer volume, changes in life span and haematological parameters. Apoptosis was analyzed by fluorescence microscope, DNA fragmentation assay and flow-cytometry. The expression of apoptosis-related genes, *Bcl-2*, *Bcl-X*, *PARP-1*, *p53* and *Bax* were analyzed using polymerase chain reaction. EC-1 significantly inhibited proliferation of EAC cells *in vivo* and restored the altered haematological parameters of EAC-bearing mice. Cytological observation by fluorescence microscope showed apoptosis of EAC cells upon treatment with EC-1. Also, DNA fragmentation assay revealed EAC cells' apoptosis following EC-1 treatment. Increased mRNA expressions of *p53* & *Bax* genes and negative expressions of *Bcl-2* & *Bcl-X* were observed in cells treated with EC-1. These findings confirmed the induction of apoptosis by EC-1. In addition, MTT assay showed dose dependent anti-cancer activity of EC-1 against EAC cell. Cell cycle analysis revealed that EC-1 treatment caused suppression of EAC cells at S phase. To conclude, EC-1 is a novel anti-cancer compound and showed anti-proliferative and apoptotic activities in cellular and mice models.

Keywords: Apoptosis, cell cycle suppression, *Eucalyptus camaldulensis*, cancer, EAC cells.

Introduction

The genus *Eucalyptus* (family: Myrtaceae) is a group of plant that includes many species and subspecies which are native to Australia. It has a worldwide distribution including regions in Bangladesh (Hossain and Pasha, 2004; Brooker and Kleinig, 2006). The leaves and bark of these plants have been widely used as traditional medicine for the treatment of many diseases like influenza, dysentery, tonsillitis and gastrointestinal tract infections (Takasaki et al., 2000; Silva et al., 2003; Tatsuya et al., 2008). Bioactive compounds from *Eucalyptus camaldulensis*, *Eucalyptus urophylla* and other species also have cytotoxic, anti-bacterial, anti-fungal, analgesic, anti-inflammatory, antioxidant and larvicidal activities (Cimanga et al., 2002; Silva et al., 2003; Benyahia et al., 2005; Su et al., 2006; Siramon and Ohtani, 2007; Cheng et al., 2009). A plant under this family, *Eucalyptus camaldulensis* Dhn, is used traditionally for the treatment of various ailments including bronchial catarrh, fever, croup, diphtheria, whooping cough, wound, ulcer, etc. (Coelho-de-Souza et al., 2005). The plant also showed anti-bacterial, anti-nociceptive effects and cytotoxic activity against different cancer cells line both *in vivo* and *in vitro* (El-Ghorab et al., 2003; Al-Fatimi et al., 2005; Abdel-Nasser et al., 2011).

Our group has previously reported that petroleum ether and methanol extracts of this plant exhibit promising anti-cancer activity against Ehrlich ascites carcinoma (EAC) cells in Swiss albino mice in a dose dependent manner (Islam et al., 2012; Islam et al., 2014a). These results inspired us to develop compound of natural origin with anti-cancer effect. In this study, we first reported the isolation of a pure compound *p*-Menth-1-ene-4, 7-diol (designated as EC-1). In addition, the effects of this compound against carcinoma cells (EAC) *in vitro* as well as in animal model were also studied.

Materials and methods

Materials

The stem bark of *Eucalyptus camaldulensis* Dhn. (Family: Myrtaceae) was collected from the Rajshahi University campus, Rajshahi, Bangladesh in February, 2011. It was then authenticated by Professor A. T. M Naderuzzaman and Dr. Goura Pado Ghosh, of Department of Botany, University of Rajshahi, Bangladesh. A voucher specimen was conserved in the Department of Botany, University of Rajshahi, Bangladesh.

Extraction and isolation

The collected plant materials were shed-dried. Powdered stem bark (750 g) was kept immersed in 2.5 liters of methanol (Merck, Munchen, Germany) at room temperature for 10 days with occasional shaking and stirring. The contents were then pressed through a tincture press (Karlkolb, Dreieich, Germany) and concentrated with a rotary evaporator at 40 °C followed by filtration. Twenty-six gram of crude methanol extract was obtained. A portion of this crude methanol extract (~18 g) was applied on a 0.15 to 0.3 mm silica gel (Merck) and chromatographed using chloroform with a gradient of low polar solvent system to high polar solvent system up to 100 % methanol. Sixty nine fractions were collected after chromatography and fractions 39 to 45 were further subjected to preparative thin layer chromatography (PTLC). The samples were dissolved in methanol and applied to the plates. The plates were then developed with a mobile phase of chloroform/methanol in the fraction of 7 to 3. Then, the plates were allowed to dry under ultraviolet light (at wavelengths 254nm and 366 nm) and a specific active band was scraped off from each plate. The final compound was eluted from collected silica matrix in the plates by dissolving it in methanol and then removing from silica gel by

filtration. The purified compound was obtained in amorphous powder form (~180 mg) and was designated as EC-1.

Spectrometric analysis

Spectrometric analysis was performed to determine the structure of the purified EC-1 compound. Infra-red (IR) spectra were taken on FTIR-8900 spectrophotometer (Shimadzu, Kyoto, Japan) and nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz FT spectrometer DPX-400 (Burker, Karlsruhe, Germany). The analysis was performed in Analytical Research Division, Bangladesh Council of Scientific and Industrial Research (BCSIR) Laboratories, Dhaka-1205, Bangladesh.

Animals

Adult male Swiss albino mice, six to eight weeks old (25 ± 4 g body weight) were collected from animal resource branch of the International Centre for Diarrheal Disease Research, Bangladesh. Animals were housed in polypropylene cages containing sterile paddy husk as bedding material under hygienic conditions with a maximum of six animals in a cage. They were maintained under controlled conditions (12:12 h light-dark with temperature $22 \pm 5^{\circ}\text{C}$). Ethic approval was obtained from Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee for experimentations on animal, human, microbes and living natural sources from Institute of Biological Sciences, University of Rajshahi, Bangladesh (225/320-IAMEBBC/IBSc).

Cell culture

Ehrlich ascites carcinoma (EAC) cells were obtained by the courtesy of Indian Institute of Chemical Biology (IICB), Kolkata, India. The cells were maintained as mammary gland cancer cell in ascites in Swiss albino mice by intra-peritoneal inoculation (bi-weekly) of 1×10^6 cells/mouse. For *in vitro* study, EAC cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium having glucose, 2mM L-glutamine in presence of 10 % fetal calf serum and 1 % (v/v) penicillin-streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

Cytotoxicity assay

Cytotoxicity of EC-1 against EAC cell was assayed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) colorimetric technique. For this experiment 1×10^6 EAC cells in 200 µl RPMI 1640 media were plated in a 96-well plate. To assess the maximum efficiency, five different concentrations (10 µg, 20 µg, 40 µg, 80 µg and 100µg/ml) of EC-1 compound were added into each well of EAC cells. EAC cells treated solvent, dimethyl sulfoxide (DMSO), was used as control. The assays were performed in triplicates to avoid experimental errors. These cells were then incubated for 24 hours at 37 °C in CO₂ incubator. After the incubation period, aliquot were removed and 180 µl of PBS (phosphate buffered saline) and 20 µl of MTT were added to each well. After that, the plates were further incubated for 8 hours at 37 °C. Then, the aliquot was removed and 200 µl of acidic isopropanol was added and incubated again at 37 °C for 1 hour. Lastly, absorbance was taken at 570 nm in micro-titer plate reader (Optica Microplate Reader, Mikura Ltd., Horsham, UK).

Cell growth inhibition *in vivo*

To determine the cell growth inhibition properties of the compound, five groups of Swiss albino mice ($n = 6$) weighing 25 ± 4 g were used (Kabir et al., 2011). To assess the therapeutic evaluation, 1×10^6 EAC cells were inoculated into each group of mice on day 0. Treatments were started after 24 hours of tumor inoculation and continued for five days. In brief, group 1, 2 and 3 received EC-1 at the doses of 0.3, 1.0 and 2.0 mg/kg/per day respectively via intra-peritoneal injection. Group 4 received an anti-cancer drug, bleomycin (0.3 mg/kg/day) whereas group 5 was used as control receiving DMSO only. Mice of each group were sacrificed on day six and the total intra-peritoneal cancer cells were harvested by 0.98% normal saline. Viable cells were first identified with trypan blue and then counted by a haemocytometer under inverted microscope (XDS-1R, Optika, Bergamo, Italy).

Survival time and tumor weight

The survival time of the EAC-bearing mice and tumor weight were analyzed as previously published methods (Khanam et al., 2010). In summary, five groups of Swiss albino mice (6 in each group) were used. For therapeutic evaluation 1×10^6 EAC cells per mouse were inoculated to each group of mice on day 0. Treatment was started after 24 hours of tumor inoculation and continued for 10 days. Group 1-3 received EC-1 at different concentrations; group 4 was treated with bleomycin and group 5 animals used as solvent (DMSO) control. Tumor growth of the mice was monitored by recording the daily weight change. The survival time of the mice in each group was recorded and the mean survival time (in days) of each group was calculated.

Haematological parameters

Haematological profiles of the experimental mice were investigated to reflect the effects of EC-1 on the body of EAC-bearing mice after intra-peritoneal administration of the compound. They were determined by haemocytometer using cell-dilution fluids (Islam et al., 2014b).

Morphological changes and nuclear damage

EAC cells' apoptosis induced by EC-1 was studied. Morphological observation of EAC cells in both treated (2.0 mg/kg/day) and control groups were studied using a fluorescence microscope (Olympus IX71, Seoul, Korea). In short, EAC cells were collected from the experimental mice and stained with 0.1 µg/mL of Hoechst 33342 at 37°C for 20 min. Then the cells were washed and re-suspended with PBS for analyzing the morphological changes under fluorescence microscopy.

DNA fragmentation assay

DNA fragmentation assay by agarose gel electrophoresis was determined by the method described previously (Islam et al., 2014a). EAC cells were collected from mice treated with (five consecutive days at 2.0 mg/kg/day) and without the compound EC-1. The total DNA was isolated from the cells by using a commercial DNA extraction kit (Promega, Madison, WI, USA) and analyzed by electrophoresis on 1.5% agarose gel containing 0.1 µg/mL ethidium bromide and visualized under ultra-violet illuminator.

Reverse Transcription-PCR (RT-PCR)

Total RNA was extracted using TRIzol method from mice receiving EC-1 (2.0 mg/kg/day) and control EAC-bearing mice on day six of tumor implantation. cDNA was prepared 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, Ipswich, MA, USA) according to the manufacture's guidelines. Expression of five growth regulatory genes namely *Bcl-2*, *Bax*, *p53*, *PARP-1* and *Bcl-X* were examined using these cDNA as template for polymerase chain reaction (PCR), where *β-Actin* was used as control. Reaction mixture (25 µl) were prepared containing 1X of taq polymerase, 25 pmol each of forward and reverse primer, 2.5 mM of each dNTP and 0.25 U of platinum Tag polymerase (Tiangen, Beijing, China). Primer sequences and their thermal cycle conditions were listed in Table 1. BioRad (Hercules CA, USA) gradient thermal cycler was used for amplifications. All the PCR products were analyzed in 1.0 % agarose gel and GeneRuler 1 kb DNA ladder (Fermentus, Pittsburgh, PA, USA) was used as marker.

Cell cycle analysis

Cancer cells from EC-1 treated (2.0 mg/kg) and untreated mice were collected after five days of initial tumor inoculation and were fixed in 70% ethanol after washing with cold PBS. Cells in 1 ml PBS were then treated with 50 µl RNase A (1mg/ml) for 30 min at 37 °C. Finally, the cells were stained with 5 µl Propidium Iodide (1 mg/ml) in dark at 4 °C for 5 minutes. The fractions of cells in G₀/G₁, S and G₂/M phase were then analyzed by a FACS Flow cytometer (Partec CyFlow SL, Münster, Germany).

Statistical Analysis:

The percentage of cell growth inhibition, increase of animals' life span and body/tumour weight were expressed as the mean $\pm$ SEM (standard error of mean). The numerical results were then compared statistically using paired t-test to establish if the differences were statistically significant. All the data was entered into a computer database and the statistical analysis was performed using the Statistical Package for Social Sciences for Windows (version 22.0, IBM SPSS Inc., New York, NY, USA). Significance level was taken at $p < 0.05$.

Results

Purification of the compound and spectrometric analysis

Isolated and purified EC-1 was characterized by IR and NMR spectral data. The IR spectrum revealed two hydroxyl bands observed at 2941.54 cm^{-1} and 3344.68 cm^{-1} . Its ^1H -NMR spectrum exhibited one olefinic proton at δ 5.98 and a singlet of one methylene with the integration of two protons was observed at 4.37 (H-7) which indicated the presence of a hydroxylated methylene group. Moreover, signals at 2.14 ~2.46 (m), 1.80 (ddd) and 2.67 (ddd) ppm were assigned to three additional methylene groups. The ^{13}C -NMR of EC-1 also showed two olefinic carbons at δ 112.9 (C-1) and δ 148.9 (C-2). In addition, the carbon signals observed at δ 76.3 (C-4) and δ 67.2 (C-7) indicated the presence of a tertiary and a primary hydroxyl group, respectively. Based on the observations (Table 2) and comparison with published (Ishikawa et al., 2001) data, the structure of EC-1 was confirmed as *p*-Menth-1-ene-4, 7-diol (Fig. 1A). Thus, the isolation of Menth-1-ene-4, 7-diol is reported for the first time from “*Eucalyptus camaldulensis* Dhnh.” in this study.

Inhibition of cancer cell growth with EC-1 *in vitro*

The cytotoxic effect of EC-1 on EAC cell was assessed by MTT calorimetric assay. The compound inhibit cancer cell growth in a dose depended manner (Fig. 1B). EC-1 showed maximum cancer cell growth inhibition (85 %) at the concentration of $100\mu\text{g/ml}$ when compared to the growth of cancer in the control solvent ($p < 0.01$). The minimum suppression of cancer cell growth (12.5 %) was observed at $10\mu\text{g/ml}$ compared to control cells (treated with solvent only). The IC_{50} value of the compound was determined as $32\mu\text{g/ml}$ against EAC cell (Fig. 1C).

Cell growth inhibition of EC-1 *in vivo*

Effects of EC-1 and *bleomycin* on EAC cells growth after tumor inoculation are shown in figure 2A-B. Treatment with EC-1 resulted in significant reduction of cell growth *in vivo*. The maximum cell growth inhibition with EC-1 was noted at doses of 2 mg/kg and 1 mg/kg (73% and 57% respectively) compared to the control mice ($p < 0.01$). Meanwhile, the bleomycin treated mice showed cell growth inhibition by 87.62 % (0.3 mg/kg/day).

Increase of life span and reduction of tumour burden in EC-1 treated mice

There was significant increase of mean survival time as well as life span of tumour bearing mice was noted with EC-1 treatment (Fig. 2C-D). It has been observed that tumor bearing mice treated with EC-1 at doses 0.3, 1.0 and 2.0 mg/kg, resulted in significant increase of life span, which were 36, 55.8 and 66.6%, respectively, compared to that of control mice ($p < 0.01$). On the other hand, mice treated with bleomycin increased life span by 90% in comparison to the control group (treated with solvent only).

Effect of EC-1 at doses 0.3, 1.0 and 2.0 mg/kg and bleomycin (0.3 mg/kg) on tumor weight was shown in figure 2E. EAC bearing mice showed inhibition of their tumour weight following EC-1 treatment compared to control mice. In control group (EAC-bearing), the body weight increased by 68% on 20th day when compared to the normal animal (without EAC and EC-1 treatment). Mice treated with EC-1 at doses 0.3, 1.0 and 2.0 mg/kg showed increase in body weight by 36%, 24% and 23.2% respectively on the 20th day. In contrast, with the use of bleomycin as standard at the doses of 0.3 mg/kg, the body weight was increased by 17.2% on the 20th day.

Haematological parameters

The hematological parameters of both treated and non-treated mice were studied. In non-treated EAC-bearing mice, these parameters showed effect consistent with physiological deterioration as compared to the control mice due to toxic effects of EAC cells. However, these deteriorated parameters became reversed towards the normal ranges when EC-1 supplementation was given to them at the dose used in this study (Table 3).

EC-1 induced morphological changes and nuclear damage

Morphological changes of EAC cells were examined by Hoechst 33342 staining after collecting the cells from mice treated with and without EC-1 (2.0 mg/kg/day) for five days. EAC cells nuclei were round, regular and homogeneously stained with Hoechst 33342 in control group (solvent treated) as shown in figure 3A-B. EC-1 treated EAC cells showed manifest fragmented DNA in nuclei as shown in figure 3C-D, whereas the control cells showed round with regular nuclei with homogeneous Hoechst 33342 staining. Apoptotic morphologic alterations such as membrane and nuclear condensation were also noted in EC-1 treated EAC cells. These results indicated that EC-1 treatment could induce apoptosis in EAC cells.

DNA fragmentation analysis

The activation of the endogenous $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease is the most distinctive biochemical hallmark of apoptosis (Islam et al., 2014a). This activated endonuclease will mediate the cleavage of inter-nucleosomes and generate oligonucleotide fragments of about 180-200 base pairs. DNA isolated from EC-1 treated EAC cells showed ladder type DNA which

is a characteristic feature of apoptosis induction, whereas in the control group, a smear-like DNA degradation was obtained (Fig. 3E).

Altered expressions of cancer related genes in EC-1 treated cells

Reverse transcription (*RT*)-PCR was used to study the mRNA expression levels of several tumor related genes in control and EC-1 treated EAC cells (Fig. 3F). The control cells showed high expressions of *Bcl-2* and *Bcl-X* genes. Also, the *Bax* mRNA expression was not found in the control cells. When treated with EC-1, EAC cells showed reduced *Bcl-2* and *Bcl-X* mRNA expressions whereas the expression level of *Bax* gene was increased remarkably. In addition, the *p53* and *PARP-1* genes showed increased expressions in EC-1 treated cells. On the other hand, no expression of these genes was found in control mice EAC cells (Fig. 3F)

Cell cycle changes with EC-1 treatment

The effect of EC-1 on different cell cycle phases of EAC cells was studied. The untreated cells showed three phases G_0/G_1 , S and G_2/M in the cell cycle and the proportion of G_0/G_1 , S and G_2/M phases in the untreated EAC cells were calculated to be 49, 32 and 19% respectively. On the other hand, cells treated with EC-1 were changed at G_2/M (40% vs 19%) and G_0/G_1 (56% vs 49%) phase population compared to control cells. Meanwhile, the cells in S phase showed remarkable reduction in phase population (4% vs 32%) compared to control cells (Fig. 4). These results indicated that the EC-1 compound inhibits the cellular proliferation of EAC cells by suppressing the synthesis of DNA in S phase of cell cycle. The compound also attenuated the cancer cells in G_2/M phase to some extent.

Discussion

The compound EC-1 (*p*-Menth-1-ene-4, 7-diol, a simple mono-terpinoid), was isolated first time in this study from the plant *Eucalyptus camaldulensis* Dhnh. and there is no report in the literature of its anti-cancer properties. It is reported that other terpinoids from *Eucalyptus* showed anti-inflammatory, analgesic and antipyretic activities (Annalucia et al., 2008). These results scientifically support for some traditional usages of this plant in traditional medicine, although the precise mechanism needed additional research.

In the present study, we found that the EC-1 compound inhibited EAC cells proliferation *in vitro* in a dose-dependent manner (Fig. 1B-C). *In vivo* effect of the compound has been compared with the data obtained by parallel experiments using conventional anti-cancer drug, *bleomycin*. EC-1 treatment resulted in significant reduction EAC cells growth and this was reasonably comparable to that of *bleomycin* (73% vs 88 %). Also, this compound significantly increases the life span of EAC-bearing mice (Fig. 2C-D). During this study, we also found that the EC-1 compound can reduce the tumor burden of EAC-bearing mice remarkably, as the EC-1 treated mice showed only 23 % increment of body weight in comparison to control mice whereas body weight of bleomycin treated mice increased by 17% (Fig. 2E). Normal mice also treated with EC-1 at the highest dose used in this study to investigate the toxic effect (regarding behavioral, bodyweight and biochemical parameters) of the compound on host. There was no adverse effects observed (data were not presented here).

In EAC-bearing mice, anaemia occurred due to breakdown of RBC (Kabir et al., 2012). Supplementation with EC-1 in EAC-bearing mice restored all the altered haematological parameters towards normal range on day 12 of tumor inoculation (Table 3) and similar results

were also observed in EAC-bearing mice treated with bioactive compounds (Rasida et al., 2012). These findings rectify the suitability of EC-1 as an emerging natural anti-cancer agent.

Apoptosis is a self cell-suicidal mechanism that can be triggered by cytotoxic compounds and regulated by various signaling pathways (Islam et al., 2014a). Cells undergoing apoptosis are characterized morphologically by irregular cell shape, shrinkage, nuclear material condensation and apoptotic body formation and these changes caused by proteases (caspases) (Graf et al., 2007). Induction of apoptosis in EAC cells by EC-1 was confirmed by the observation of changes in nuclear material and cell shape as compared to that of untreated control EAC cells (Fig. 3). Apoptosis of EAC cell was also studied using different compounds of plant origin (Islam et al., 2014a). Ottelion A, a plant product, inhibited the EAC cell growth by apoptosis with the mediation of increased levels of p53 and CD8⁺ cells (El-Missiry et al., 2012). Oleandrin, a plant glycoside, can induce apoptosis in many cell lines by up-regulating *Fas* and tumor necrosis factor receptor 1 (Sreenivasan et al., 2006). Similarly, this study showed induction of apoptosis in EC-1 treated EAC cells by regulating many cancer related genes (Fig. 3F). Switch on/off of apoptosis is regulated by numerous growth related genes, of them *Bcl-2* (B cell lymphoma gene 2) family gene was believed to be the first one attributed in the apoptotic process (Gradilone et al., 2003). The gene was expressed in cancer and was correlated with clinical pathological parameters of patients with cancer (Yuen et al., 2001). Among the apoptosis related genes, *Bax*, *Bid*, *Bak* act as pro-apoptotic and other members *Bcl-2*, *Bcl-X*, *Bcl-W* act as anti-apoptotic class (Kabir et al., 2013). In response to anti-cancer agents, the expression levels of these genes alter in cancer cell. Decreased expression of *Bcl-X* and increased level of *Bax* and *Bak* was observed in HeLa cell with the treatment of a plant compound FRAP (Ju et al., 2012).

In the present study, we found that *Bcl-2* and *Bcl-X* expressions were observed in control mice and they were absent in mice treated with EC-1, whereas pro-apoptotic genes like *Bax*, P^{53} and *PARP 1* expressions showed opposite results. We found over-expression of these genes in EC-1 treated mice in comparison to that of control mice (Fig. 3F). Similar results were also found in different cancer cell lines treated with compounds obtained from plant materials (Zhao et al., 2010; Groc et al., 2001). Increased expression of these genes could lead to cytochrome c release from mitochondria, which in turn, stimulate the activation of caspases-9, initiating a downstream cascade leading to cell death (Antonsson, 2001). Altered expression of these pro- and anti-apoptotic genes in EAC cell upon EC-1 treatment further confirmed the induction of programmed cell death of cancer cell.

Many chemotherapeutic and DNA-damaging agents induce cancer cells' apoptosis by arresting cells at G₁, S, or G₂/M phase. Compounds of natural origin like cape aloe, ottelione A, guarana etc. caused EAC cells death by arresting cell cycle progression (El-Missiry et al., 2012; Kametani et al., 2007; Fukumasu et al., 2011). In the present study, we found that the isolated compound caused suppression of EAC cell in S phase of cell cycle (Fig. 4). It implies that treatment with EC-1 compound inhibited the synthesis of DNA in EAC cell. Also, EC-1 driven DNA damage in EAC cells have then led to apoptosis. The compound also increased the population of cells in G₂/M phase in comparison to control group. In addition, many human cancer cell lines have shown arrested at G₀/G₁ and S phase inhibition by the treatment of chemotherapeutic agents (El-Sherbiny et al., 2001).

In conclusion, the EC-1 compound (Menth-1-ene-4, 7-diol) isolated from *Eucalyptus camaldulensis* can induce apoptosis of cancer cell and reduced cells population at S phase of the cell cycle. Structural changes in cells and altered expression pattern of different genes treated

with this compound supported that EC-1 induces apoptosis in EAC cells. Further works with other cancer cell lines and with higher animals are required to develop effective anti-cancer drug in future with this compound.

Acknowledgements

The authors would like to acknowledge the International Centre for Diarrheal Disease research, Bangladesh (ICDDR'B) for providing experimental animal and laboratory facility of flow-cytometry and Bangladesh Council of Scientific and Industrial Research, Dhaka, Bangladesh for spectrometric analysis of the compound. We would like to thank Griffith University for the support of the project.

Conflict of interest

The authors have no conflict of interest

References

- Abdel-Nasser S, Nahla A, Eman AS, Olli M, Jari S, Kalevi P. 2011. Phenolic constituents of *Eucalyptus camaldulensis* Dehnh, with potential antioxidant and cytotoxic activities. *Rec Nat Prod* 5: 271-280.
- Al-Fatimi M, Friedrich U, Jenett-Siems K. 2005. Cytotoxicity of plants used in traditional medicine in Yemen. *Fitoterapia* 76: 355-358.
- Annalucia S, Paola SV, Federica A, Manuela Z, Luana M, Femmo F, Guido R, Enrico G, Pasquale P. 2008. Stimulatory effects of Eucalyptus essential oil on innate cell-mediated immune response. *BMC Immunol* 9:17.
- Antonsson B. 2001. Bax and other pro-apoptotic bcl-2 family “killer proteins” and their victim, the mitochondrion. *Cell Tissue Res* 306: 347-361.
- Benyahia S, Benayache S, Benayache F, León F, Quintana J, López M, Hernández JC, Estévez F, Bermejo J. 2005. Cladocalol, a pentacyclic 28-nor-triterpene from Eucalyptus cladocalyx with cytotoxic activity. *Phytochemistry* 66: 627-632.
- Brooker MIH, Kleinig DA. 2009. *Field Guide to Eucalyptus* (3rd edition). Bloomings, Melbourne, Australia.
- Cheng SS, Huang CG, Chen YJ, Yu JJ, Chen WJ, Chang ST. 2009. Chemical compositions and larvicidal activities of leaf essential oils from two eucalyptus species. *Bioresour Technol* 100: 452-456.
- Cimanga K, Kambu K, Tona L, Apers S, De Bruyne T, Hermans N, Totté J, Pieters L, Vlietinck AJ. 2002. Correlation between chemical composition and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. *J Ethnopharmacol* 79: 213-20.

- Coelho-de-Souza LN, Leal-Cardoso JH, de Abreu Matos FJ, Lahlou S, Magalhães PJ. 2005. Relaxant effects of the essential oil of *Eucalyptus tereticornis* and its main constituent 1, 8-cineole on guinea-pig tracheal smooth muscle. *Planta Med* 71: 1173–1175.
- El-Ghorab AH, El-Massry KF, Marx F, Fadel HM. 2003. Antioxidant activity of Egyptian *Eucalyptus camaldulensis* var. *brevirostris* leaf extracts. *Nahrung*. 47: 41-45.
- El-Missiry MA, Othman AI, Amer MA, Mohamed E. 2012. Ottelione A inhibited proliferation of Ehrlich ascites carcinoma cells in mice. *Chem Biol Interact*. 200: 119–127.
- El-Sherbiny YM, Cox MC, Ismail ZA, Shamsuddin AM, Vucenik. 2001. G0/G1 arrest and S phase inhibition of human cancer cell lines by inositol hexaphosphate (IP6). *Anticancer Res* 21: 2393-403.
- Fukumasu H, Latorre AO, Zaidan-Dagli ML. 2011. *Paullinia cupana* Mart. var. *sorbilis*, guarana, increases survival of Ehrlich ascites carcinoma (EAC) bearing mice by decreasing cyclin-D1 expression and inducing a G0/G1 cell cycle arrest in EAC cells. *Phytother Res* 25: 11–16.
- Gradilone A, Gazzaniga P, Ribuffo D, Scarpa S, Cigna E, Vasaturo F, Bottoni U, Innocenzi D, Calvieri S, Scuderi N, Frati L, Aglianò AM. 2003. Survivin, bcl-2, bax, and bcl-X gene expression in sentinel lymph nodes from melanoma patients. *J Clin Oncol* 21: 306-312.
- Graf D, Bode JG, Häussinger D. 2007. Caspases and receptor cleavage. *Arch Biochem Biophys* 462: 162–170.
- Groc L, Bezin L, Jiang H, Jackson TS, Levine RA. 2001. Bax, bcl-2, and cyclin expression and apoptosis in rat substantia nigra during development. *Neurosci Lett* 306: 198-202.
- Ishikawa T, Segi Y, Kitajima J. 2001. Water-soluble constituents of Ajowan. *Chem Pharm Bull* 49: 840-844.

Hossain MK, Pasha MK. 2004. An account of the exotic flora of Bangladesh, *J For Environ* 2: 99-115.

Islam F, Khatun H, Khatun M, Ali MM, Khanam JA. 2014a. Growth inhibition and apoptosis of Ehrlich ascites carcinoma cells by the methanol extract of *Eucalyptus camaldulensis*. *Pharm Biol* 52: 281-290.

Islam F, Ghosh S, Khanam JA. 2014b. Antiproliferative and hepatoprotective activity of metabolites from *Corynebacterium xerosis* against Ehrlich ascites carcinoma cells. *Asian Pac J Trop Biomed* 4: S284-S292.

Islam F, Khatun H, Ghosh S, Ali MM, Khanam JA. 2012. Bioassay of *Eucalyptus* extracts for anticancer activity against Ehrlich ascites carcinoma (EAC) cells in Swiss albino mice. *Asian Pac J Trop Biomed* 2: 394-398.

Ju HK, Lee HW, Chung KS, Choi JH, Cho JG, Baek NI, Chung HG, Lee KT. 2012. Standardized flavonoid-rich fraction of *Artemisia princeps* Pampanini cv. Sajabal induces apoptosis via mitochondrial pathway in human cervical cancer HeLa cells. *J Ethnopharmacol* 141: 460-468.

Kabir SR, Nabi MM, Haque A, Zaman R, Mahmud ZH, Reza AM. 2013. Pea lectin inhibits growth of Ehrlich ascites carcinoma cells by inducing apoptosis and G2/M cell cycle arrest in vivo in mice. *Phytomed* 20: 1288-1296.

Kabir SR, Islam MF, Alom MJ, Zubair MA, Absar N. 2012. Purification and characterization of a snake guard seed lectin with antitumor activity against Ehrlich ascites carcinoma cells *in vivo* in mice. *Protein Pept Lett* 19: 360-368.

Kabir SR, Zubair, MA, Nurujjaman M, Haque MA, Hasan I, Islam MF, Hossain MT, Hossain MA, Rakib MA, Alam MT, Shaha RK, Hossain MT, Kimura Y, Absar N. 2011. Purification and

characterization of a Ca^{2+} -dependent novel lectin from *Nymphaea nouchali* tuber with antiproliferative activities. ***Biosci Rep*** 31: 465-475.

Kametani S, Oikawa T, Kojima-Yuasa A, Kennedy DO, Norikura T, Honzawa M, Matsui-Yuasa I. 2007. Mechanism of growth inhibitory effect of cape aloe extract in Ehrlich ascites tumor cells. ***J Nutr Sci Vitaminol*** 53: 540–546.

Khanam JA, Islam MF, Jesmin M, Ali MM. 2010. Antineoplastic activity of acetone semicarbazone (ASC) against Ehrlich ascites carcinoma (EAC) bearing mice. ***J Natn Sci Foundation Sri Lanka*** 38: 225-231.

Rasida P, Islam F, Khanum JA, Yeasmin T. 2012. Preventive effect of ethanol extract of *Alpinia calcarata* Rose on Ehrlich's ascitic carcinoma cell induced malignant ascites in mice. ***Asian Pac J Trop Med*** 5: 121-125.

Silva J, Abebe W, Sousa SM, Duarte VG, Machado MI, Matos FJ. 2003. Analgesic and anti-inflammatory effects of essential oils of Eucalyptus. ***J Ethnopharmacol*** 89: 277-283.

Siramon P, Ohtani Y. 2007. Antioxidant and antibacterial activities of Eucalyptus camaldulensis leaf oils from Thailand. ***J Wood Sci*** 53: 498-504.

Sreenivasan Y, Raghavendra PB, Manna SK. 2006. Oleandrin mediated expression of Fas potentiates apoptosis in tumor cells. ***J Clin Immunol*** 26: 308–322.

Su YC, Ho CL, Wang EI Chang ST. 2006. Antifungal activities and chemical compositions of essentials oils leaves of four eucalyptus. ***Taiwan J For Sci*** 21: 49-61.

Takasaki M, Konoshima T, Etoh H, Pal Singh I, Tokuda H, Nishino H. 2000. Cancer chemopreventive activity of euglobal-G1 from leaves of Eucalyptus grandis. ***Cancer Lett*** 155: 61-65.

Tatsuya H, Fumihide T, Takanobu T, Masato N, Tomihisa O. 2008. Bioactive monoterpene glycosides conjugated with galic acid from the leaves of *Eucalyptus globules*. *Phytochemistry* 69: 747-753.

Yuen AP, Lam KY, Choy JT, Ho WK, Wei WI. 2001. The clinicopathological significance of bcl-2 expression in the surgical treatment of laryngeal carcinoma. *Clin Otolaryngol Allied Sci* 26: 129-133.

Zhao Q, Cao X, Zeng B, Wang C, Yan L, Xu C. 2010. *Musca domestica* larva lectin induces apoptosis in BEL-7402 cells through a mitochondria-mediated reactive oxygen species way. *Biol Pharm Bull* 33: 1274–1278.

Table1. Primer sequences and PCR protocol used for the qRT-PCR assay

Target Genes	Primers	PCR Protocol	Amplification
BCL-2	5'-GTGGAGGAGCTCTTCAGGGA-3' 5'-AGGCACCCAGGGTGATGCAA-3'	Cycling condition for initial PCR activation step of 3 min at 96 °C, followed by 35 cycles of 95 °C/1 min. For <i>β-actin</i> , <i>P⁵³</i> , <i>PARP1</i> and <i>Bcl-2</i> gene 55 °C/1 min, 72°C/1 and a final extension of 72 °C/1 min. In the case of Bax and Bcl-X the annealing temperature was 54 °C.	0.304 kb
BAX	5'-GGCCCACCAGCTCTGAGCAGA-3' 5'-GCCACGTGGGCGTCCCAAAGT-3'		0.479 kb
P53	5'-GCGTCTTAGAGACAGTTGACT-3' 5'-GGATAGGTCGGCGGTTTCATGC-3'		0.458 kb
PARP-1	5'-AGGCCCTAAAGGCTCAGAAT-3' 5'-CTAGGTTTCTGTGTCTTGAC-3'		0.27 kb
BCL-X	5'-TTGGACAATGGACTGGTTGA-3' 5'-GTAGAGTGGATGGTCAGTG-3'		0.78 kb, 0.59kb
β-actin	5'-ACCCACACTGTGCCCATCTACGA-3' 5'-CAGGAGGAGCAATGATCTTGATCTTC-3'		0.27 kb

Table 2. ^1H - and ^{13}C -NMR spectral data of EC-1

Carbon Number	EC-1	
	δ_{C}	δ_{H}
1	112.9	-
2	148.9	5.98 (1H, s, H-2)
3	33.3	2.14 ~2.46 (2H, m, H-3)
4	76.3	-
5	47.7	1.80 (2H, ddd, $J = 6, 10.5$ Hz, H-5)
6	37.2	2.67 (2H, ddd, $J = 6, 10.5$ Hz, H-6)
7	67.2	4.37 (2H, s, H-7)
8	49.5	1.89 (1H, m, H-8)
9	17.3	1.07 (3H, d, $J=7.0$, H-9)
10	17.2	1.00 (3H, d, $J=7.0$, H-10)

Table 3. Effect of EC-1 on blood parameters of tumor bearing Swiss albino mice on day 12 of tumor inoculation

Name of Exp.	RBC Cells/mL	WBC Cells/mL	% of Hb gm/dL
Normal mice	$(5.65 \pm 0.20) \times 10^9$	$(10.3 \pm 1.2) \times 10^6$	12.32 ± 0.7
Control (EAC bearing) mice	$(2.30 \pm 0.3) \times 10^9$	$(26.8 \pm 0.6) \times 10^6$	4.9 ± 0.75
EAC+ Bleomycin (0.3 mg/kg)	$(5.2 \pm 1.5) \times 10^9$	$(13.0 \pm 2.48) \times 10^6$	13.5 ± 3.5
EAC+ 0.3 mg/kg	$(2.8 \pm 0.7) \times 10^9$	$(17.2 \pm 4.2) \times 10^6$	10.0 ± 2.5
EAC+ 1.0 mg/kg	$(3.5 \pm 0.9) \times 10^9$	$(17.0 \pm 1.33) \times 10^6$	12.3 ± 1.7
EAC+ 2.0 mg/kg	$(4.25 \pm 0.5) \times 10^9$	$(14.50 \pm 1.8) \times 10^6$	12.5 ± 3.3

The results are shown in mean $\pm$ SEM.

Haematological parameters were studied in 6 groups of mice, of them four groups were EAC bearing mice treated with EC-1 at the doses of 0.3, 1, 2 mg/kg/day and bleomycin (0.3 mg/kg) for 10 consecutive days. On day 12 blood were collected and analyzed. Remaining two groups used as EAC-bearing control and Normal mice (without any treatment). Number of mice per group was 6.

Figure Legends

Fig. 1. Structure and *in vitro* cytotoxicity of EC-1 (p-menth-1-ene-4, 7-diol). A) Structure of the compound. B) The EAC cells were treated with various doses of EC-1 for 24 hours in RPMI-1640 medium. The growth inhibition was measured by MTT assay (n = 3, mean $\pm$ SEM). C) IC₅₀ value of EC-1 was calculated from the dose-response curve. Level of significance *p<0.05 and **p<0.01 when compared with that of control group.

Fig. 2. *In vivo* anti-cancer activities of EC-1. A) Number of EAC cells in experimental mice, counted by inverted microscope in presence and absence (control) of EC-1 at different dose on day 6 of tumour inoculation. B) Percentage of cell growth inhibition at different doses in comparison to control mice. C) Mean survival time of experimental mice D) Percentage of life span increase with the treatment and E) Reduction of tumour burden at different doses. Data are expressed as mean $\pm$ SEM (n=6). Level of significance *p<0.05 and **p<0.01 when compared with that of control group.

Fig. 3. Apoptosis of EAC cell by the treatment of EC-1. Cells were collected from non-treated EAC-bearing mice and treated EAC-bearing mice and stained with Hoechst 33342 and observed by fluorescence microscopy. Control cells in under fluorescence (A) and optical (B) microscope. EC-1 treated cell under fluorescence (C) and optical (D) microscope; Arrows indicating apoptotic features (condensed chromatin, apoptotic bodies and nuclear fragmentation). (E) DNA was extracted from EC-1 treated and control mice and run in (1.5 %) agarose gel electrophoresis. Lane 1) DNA from EC-1 treated EAC cell, lane 2) Ladder and lane 3) DNA from control EAC-bearing mice (DNA fragmentation was observed in EC-1 treated mice). (F) RNA was extracted

from the experimental mice on day 6 and level of mRNA expression of anti-apoptotic genes (*Bcl-2*, *Bcl-X*) and pro-apoptotic genes (*p53*, *Bax*, *PARP1*) were studied. In EC-1 treated mice, all the three pro-apoptotic genes expression increased remarkably and the expression of *Bcl-2* and *Bcl-X* was not observed. In control group opposite results was observed.

Fig. 4. Effect of EC-1 on cell cycle phases. (A) Bar diagram showing the percentages of cell in each phase, were evaluated by flow cytometry. Data are presented as mean values obtained from three independent experiments and the results are expressed as means $\pm$ SEM. Histogram representing flow cytometry results (B) control and (C) effects of EC-1 on the cell cycle phases. The x-axis (FL3) is representing the intensity of the propidium iodide (PI) staining which is directly proportional to the amount of DNA in cells and y-axis representing the cell number.