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Full Length Research Paper

Cytotoxicity of *Sambucus ebulus* on cancer cell lines and protective effects of vitamins C and E against its cytotoxicity on normal cell lines

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Isolation and identification of potent anti-tumor compounds from medicinal plants, has motivated researchers to screen plant species for anti-tumor effects. Regarding the traditional utilization of *Sambucus ebulus*, Iranian native botany and its active ingredients (e.g. ebulitin and ebulin 1), cytotoxicity of ethyl acetate extract from *S. ebulus* (SEE) on HepG2 and CT26 cancer cell lines was investigated. Also, protective effects of vitamins C and E against SEE-induced cytotoxicity on normal cell lines were studied. Cytotoxicity of SEE on cancer (HepG2 and CT26) and normal (CHO and rat fibroblast) cell lines was evaluated by MTT assay. IC_{50} of SEE on the cell lines was assessed. Furthermore, IC_{50} of SEE on normal cell lines with exposure to vitamins C, E and C+E was studied. SEE possessed lower IC_{50} in cancer cell lines compared with normal cell lines. It manifested high cytotoxicity that can act as anticancer compound. Also, cytotoxicity of SEE on normal cell lines in presence of vitamins C+E, E and C decreased. The results demonstrate that SEE is an effective cytotoxic agent on liver and colon cancer cells and suggest that vitamins C and E may protect normal cells, when SEE were used in cancer therapy in future.

Key words: *Sambucus ebulus*, cytotoxicity, IC50, MTT assay, cell line, vitamins C and E.

INTRODUCTION

At present time, utilization of pharmaceutical plants (especially native plants) in treatment of diseases has been of specific importance. Isolation and identification of some potent anti-tumor compounds, such as colchicine, Vinca alkaloids and taxol, as natural anticancer compounds, has encouraged scientists to screen different parts of plant species against cancer cell lines (Shokrzadeh and Saeedi Saravi, 2008; Jafarian-Dehkordi, 2004; Prasain, 2001; Van Uden, 1992; Huang, 1986).

In Iranian folk medicine, the leaves and rhizomes of *Sambucus ebulus* have been used topically for curing inflammatory related diseases. *S. ebulus* (Elderberry) from the family Adoxaceae, extensively grows in the Northern regions of Iran and consists of several active ingredients, such as flavonoids, steroid substances, car-diac glycosides, tannins, caffeic acid derivatives and other isolated substances (Shokrzadeh and Saeedi Saravi, 2010; Ahmadiani et al., 1998; Ghannadi and Ghassemi-Dehkordi, 1997; de Benito et al., 1995; Pribela et al., 1992; Petkov, 1986). Also, *S. ebulus* consists of ebulitin (RIP-I, ribosome inactivating protein) and ebulin1 (RIP-II), which have selective cytotoxicity on mammalian, fungal and bacterial cells. Ebulitin and ebulin 1 inactivate translation via depurination of 28S-rRNA and as a result, inhibit protein synthesis and cause cell necrosis (Benitez et al., 2005; De

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Abbreviations: SEE, *Sambucus ebulus* extract; **HepG2,** human hepatocarcinoma; **CT26,** human colon carcinoma; **CHO,** Chinese hamster ovary; **IC_{50},** inhibition concentration of half of cells; **MTT,** 3 -(4, 5 -dimethylthiazol- 2 -yl)- 2, 5 –diphenyltetrazolium bromide; **RIP,** ribosome inactivating protein.

Benito et al., 1995; Barbieri et al., 1993).

There are several reports concerning pharmacological properties of the plant *S. ebulus*, such as the anti-inflammatory, antinociceptive, anti hemorrhoid, anti *Helicobacter pylori* and anti-rheumatic effects in traditional medicine (Saeedi Saravi and Shokrzadeh, 2009a, b; Ebrahimzadeh et al., 2007; Tuzlaci and Tolon, 2000; Mirhaydar, 1994; Yesilada et al., 1999a, 1999b; Guarrera, 1999; Petkov, 1986; Samsamshariat et al., 1981; Zargari, 1981; Ognyanov et al., 1979).

Here, we address whether *S. ebulus* extract (SEE) has cytotoxic effect on cancer cell lines. On the other hand, the evaluation of role of vitamins C and E, as anti-oxidant compounds on prevention of SEE-induced cytotoxicity on normal cell lines was also performed.

MATERIALS AND METHODS

Preparation of extract from *Sambucus ebulus*

Fruits of *S. ebulus* were collected from 15th km of Farah Abad road, Sari, Iranian province Mazandaran in August and September 2007. Botanical identification was confirmed by department of Pharmacognosy. A voucher specimen (No. 74) has been deposited in Sari faculty of Pharmacy.

Fruits were dried at room temperature and coarsely ground before extraction. The weighed amount of dried and powdered sample (135 g) was fractionated by successive solvent extraction at room temperature by percolation with hexane, then ethyl acetate. The resulting ethyl acetate extract was separately concentrated over a rotary vacuum evaporator until a solid extract sample was obtained. The decoction was filtered, freeze-dried and stored at 4°C.

Reagents

MTT and DMEM/F12 medium culture were purchased from DEHean-Riedal (Germany) and GIBCO BRL, respectively. α-tocopherol (vitamin E) and Fresh Bovine Serum (FBS) were acquired from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of the highest available commercial grade.

Cell culture

Human hepatocarcinoma HepG2 cells, human colon carcinoma CT26 cells, Chinese hamster ovary (CHO) cells and rat fibroblast cells were cultured at 37°C in DMEM/F12 (Gibco, BRL) supplemented with 10% fetal bovine serum (Gibco, BRL), 1 µl/ml amphotricin B (Welcome, USA), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere containing 5% CO_2/95% air. To assess the cell cytotoxicity, following cell counting via MTT assay, the cells were seeded on 96-well culture plates at a density of 1×10^5 cells/well and adapted for 12 h. The cells were then incubated for 72 h, after which the cells were assayed for cell cytotoxicity via MTT assay method, as follows.

Cytotoxicity assay

MTT assay

In MTT assay, cytotoxicity and cell viability was evaluated by measuring the mitochondrial-dependent reduction of colourless 3 - (4, 5 -dimethylthiazol 2 -yl)- 2, 5 -diphenyltetrazolium bromide (MTT) to a coloured blue formazan (Fotakis and Timbrell, 2006).

50 µL of DMEM/F12 including 1 □□10^5 cells were added to 3 wells for each concentration of SEE and Etoposide. Then, the cells were incubated for 72 h. Then, the cell lines were treated with 50 µL of each 0 (solvent), 5, 25, 50, 100, 150, 250, 500, 1000 and 1500 µg/ml of SEE and 50 µL of 0 (solvent), 5, 12.5, 25, 50, 100 µg/ml of Etoposide for 72 h and then incubated for 4 h in 30 µL of MTT for each well. After washing with sterile PBS, the intracellular formazan product was dissolved in dimethyl sulfoxide and the absorbance of each sample was spectrophotometrically measured at 490 and 630 nm with a Bio-Rad 680 microplate reader. Following this procedure, IC_{50} of SEE on all cell lines was assesses via Prism ver.3 software.

Furthermore, 50 µL of DMEM/F12 including 1 □ 10^5 cells were added to 3 wells for determined concentration of SEE and Etoposide with exposure to vitamins C, E and C+E. The cell lines were treated with 50 µL of vitamins C (AC, DC), E (AE, DE) and C+E (AEC, DEC) for 48 h. Then, 50 µL of selected concentrations of SEE and Etoposide were added to each 3 vitamin-exposured wells for 72 h. The procedure was continued similar to the mentioned MTT assay.

The specific doses of vitamin C, E, C+E are presented below:

1. AC: 0.031 g vitamin C powder in 10 ml DDW (17.6 mM).
2. DC: 2 ml of AC + 6 ml of PBS (2.2 mM).
3. AE: 1 ml vitamin E in 4 ml of DMSO (465.11 mM).
4. DE: 2 ml of AE + 6 ml of PBS (85.12 mM).
5. AEC: 50 µL of AC + 50 µL of AE.
6. DEC: 50 µL of DC + 50 µL of DE.

Statistical analysis

All data and statistical significance were analyzed descriptively by means of one way-ANOVA, followed by Tukey-post test with $p < 0.05$. All statistical analyses were done by Prism software ver.3.

RESULTS

Comparative analysis of MTT method to assess cytotoxicity in four different cell types treated with SEE

Cellular damage induced by ethyl acetate extract of *S. ebulus* was assayed via MTT reduction activity and IC_{50} of SEE on cancer and normal cell lines was assessed. A lower IC_{50} value is representative of the higher ability of a cytotoxic compound to cause cell death or inhibit cell growth.

Exposure to SEE resulted in cell death toward apoptosis and necrosis in human hepatocarcinoma (HepG2) cells and in human colon carcinoma (CT26) cells. Apoptotic and necrotic cell death in HepG2 and CT26 cancer cell lines, simply clarify in comparison with the SEE-untreated control cells.

The results showed that the lowest and highest IC_{50} of the extract was evaluated on HepG2 (97.03 ± 1.52 µg/ml) and CHO (346.2 ± 3.02 µg/ml) cell lines; also, IC_{50} of the extract and Etoposide on normal cell lines was higher than that in cancer cell lines.

The results showed that the extract of *S. ebulus* possesses higher IC_{50} in comparison with Etoposide on all 4 normal and cancer cell lines ($P < 0.05$). Measured IC_{50} of SEE and Etoposide on the cancer and normal cell lines

Table 1. The evaluated IC_{50} (µg/ml) of ethyl acetate extract of *Sambucus ebulus* and Etoposide on normal and cancer cell lines.

Substance	Cell line			
	Cancer cell line		Normal cell line	
	HepG2 (1C50*±SD)**	CT26 (1C50*±SD)**	CHO (1C50*±SD)**	Fibroblast (1C50*±SD)**
Sambucus ebulus (Ethyl acetate extract of fruits)	97.03±1.52	152.70±3.36	346.2±3.02	312.29±2.01
Etoposide	2.80±2.64	3.89±4.46	4.375±3.76	37.5±6.58

*µg/ml; **P< 0.05.

are prepared in Table 1. Also, Figure 1 shows the cell viability and IC_{50} of SEE and Etoposide on four different cell lines.

Sensitivity to SEE decreased according to the rank order of cells HepG2 > CT26 > Fibroblast > CHO. Otherwise, IC_{50} of SEE in the 4 cell lines increased according to the rank order of cells CHO > Fibroblast > CT26 > HepG2. On the other hand, IC_{50} of Etoposide in the 4 cell lines increased according to the rank order of cells Fibroblast > CHO > CT26 > HepG2.

Comparative analysis of protective effects of vitamins C and E on SEE-treated normal cell lines

Regarding the results, marked decrease of cytotoxic activities of SEE and Etoposide was observed in the treated normal cells. It is well known that vitamin E plays an important role to amplify antioxidative defense system. To understand the underlying protective effect of the vitamins, we examined the effect of different concentrations of vitamins C, E and C+E, as described. The results showed that ascorbic acid (vitamin C), as water-soluble antioxidant, did not significantly inhibit cell death caused by SEE and Etoposide ($P > 0.05$) (Table 2*),* whereas the lipid-soluble antioxidant α-tocopherol (vitamin E) significantly blocked cell death. Almost complete protection of cells was also observed when α-tocopherol and ascorbic acid were added to SEE-treated normal cells ($P < 0.001$). The IC_{50} values for SEE on CHO and fibroblast normal cells, which were exposure to combination of vitamins C and E (AEC, DEC), were higher than those of the corresponding vitamins E (AE, DE) and C (AC, DC), suggesting that AEC and DEC are more potent inhibitors. The protective effects of α- tocopherol and ascorbic acid in the 4 cell lines increased according to the rank order of vitamins AEC > DEC > AE > DE > AC > DC. As a result, IC_{50} values for SEE were significantly increased on CHO and fibroblast normal cells, but not significantly increase in IC_{50} of SEE on HepG2 and CT26 cancer cells was observed, in comparison with control group which were exposure to SEE, only (Figure 2).

DISCUSSION

This study provides evidence that SEE acts as a cytotoxic agent in cancer cell lines. In cell culture model, SEE markedly reduced viable cancer cell count and caused cell death. This was supported by observed cytotoxicity in HepG2 and CT26 cells. Results from our study demonstrate that IC_{50} of SEE on cancer cell lines was lower than that on normal cell lines. The higher IC_{50} of SEE and Etoposide on normal cells compared with cancer cells can be resulted from dysfunction of cellular organisms following cancer incidence, which cause higher rate of proliferation and increased cellular intake. Also, disorders in defensive systems of cancer cells and effusion insufficiency to escape toxic substances, can lead to inhibition of the growth of cancer cells in compa-rison with normal cells, via lower amounts of cytotoxic compounds (Hultberg et al., 1999; Van Haaften et al., 2000). Few substances established as cytotoxic agent can be approved as anticancer drug; but regarding complex development of cancer, formulation of novel anticancer pharmaceuticals is difficult. So, compounds with cytotoxic effect are selected as first-ranked candi-date to discover anticancer drugs (Mongelli et al., 2000; Suffness and Pezzuto, 1991). The higher IC_{50} of Etoposide compared with SEE can be described regarding to pure active ingredient of Etoposide (epi-podophylotoxin). But, SEE is a fractionated crude extract, which consists of a variety of pharmaceutical components with different medical effects and is not used as a pure compound. So, purification of SEE to reach ebulin 1 and ebulitin can resulted in high anti tumor potency. These findings suggest that SEE may be an important therapeutic strategy for the treatment of a variety of cancers in future.

According to the results, it is noteworthy that radical-scavenging antioxidants, such as α-tocopherol, completely blocked the cell death caused by SEE. α-Tocopherol and ascorbic acid-supplemented fibroblast and CHO normal cells showed a low loss of cell viability. α-Tocopherol was more effective than the corresponding ascorbic acid to decrease cytotoxicity of SEE. It may be ascribed primarily, if not solely, to the differences in the rate of cellular uptake. A higher uptake of α-tocopherol than ascorbic acid into culture cells has been reported (Saeedi Saravi and Shokrzadeh, 2008 a,b; Noguchi 2003; Sen et al., 2000). As a result, IC_{50} values for SEE and Etoposide, as positive control compound, significantly increased on normal cell lines which were treated with vitamins C and E. Also, combination of vitamins C and E (AEC) showed highest protective effects on the normal cell lines, in comparison

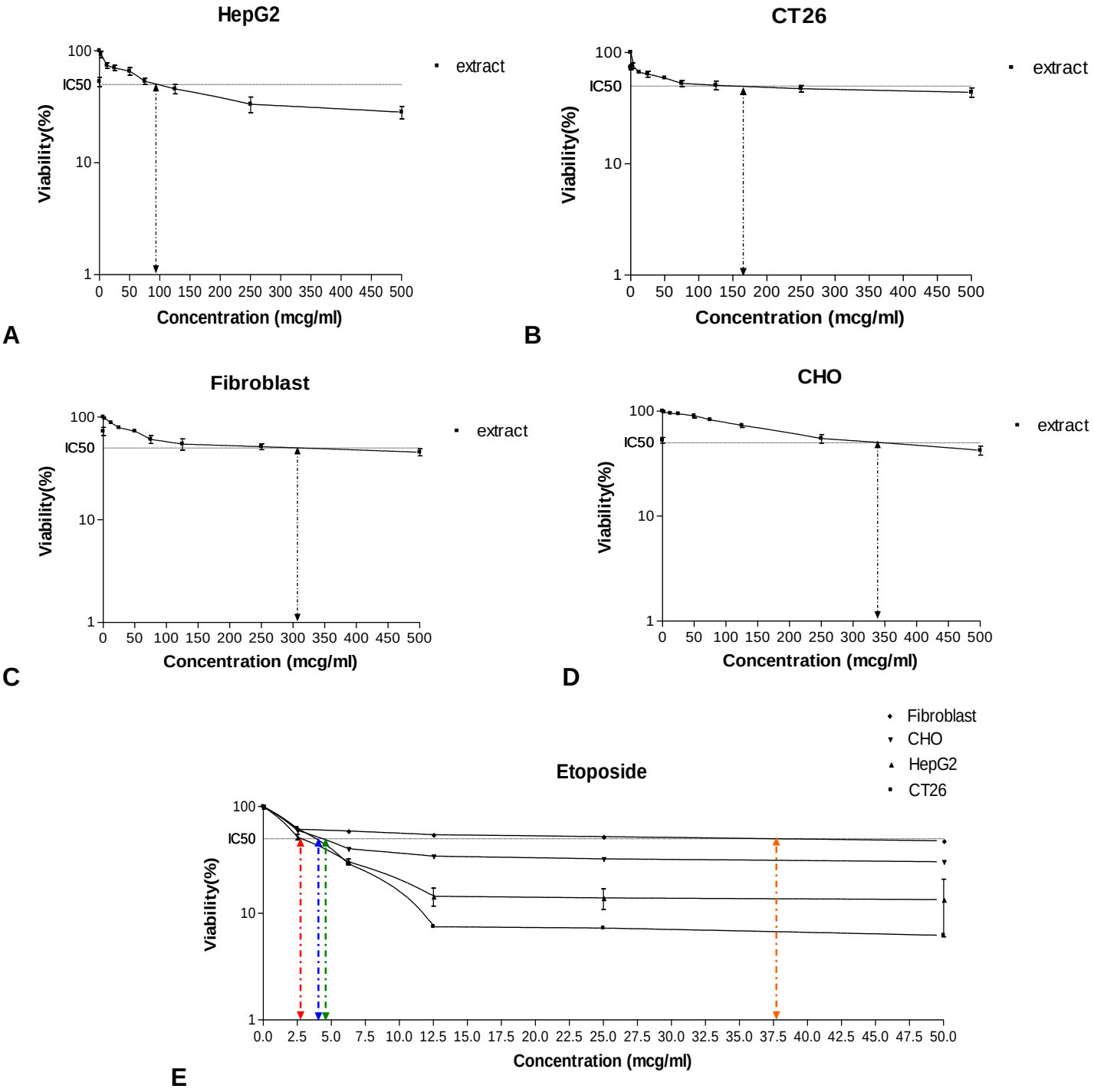

Figure 1. The evaluated IC_{50} (µg/ml) and viability of the cancer and normal cells exposure to ethyl acetate extract of *Sambucus ebulus* (SEE) and Etoposide. IC_{50} of SEE and cell viability of HepG2 cell line (A), CT26 cell line (B), Fibroblast cell line (C), CHO cell line (D), were measured via MTT assay. Also, IC_{50} of Etoposide and cell viability of HepG2, CT26, Fibroblast and CHO cell lines (E) were measured via MTT assay.

with vitamins E (AE, DE) or vitamins C (AC, DC). The present study clearly shows that cell death was inhibited by various types of antioxidants with different functions, such as tocopherols and ascorbic acid, which inhibit lipid peroxidation in the membranes, respectively (Saeedi Saravi and Shokrzadeh, 2009b). In conclusion, vitamins C and E were suggested for prevention of oxidative effects of the ethyl acetate extract of *S. ebulus*, when

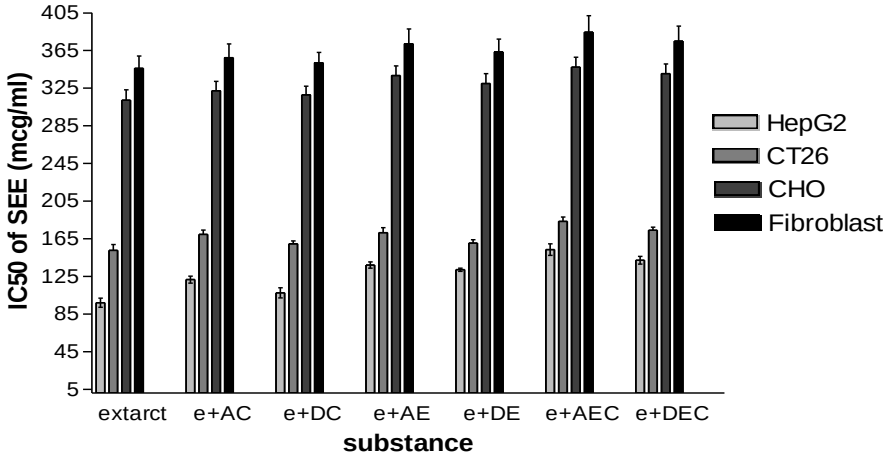

Figure 2. The evaluated IC_{50} (µg/ml) ethyl acetate extract of *Sambucus ebulus* (SEE) and Etoposide on HepG2, CT26, Fibroblast and CHO cell lines, which were treated with vitamins C, E and C+E (AC, DC, AE, DE, AEC and DEC).

Table 2. The evaluated IC_{50} (µg/ml) of ethyl acetate extract of *Sambucus ebulus* and Etoposide on normal and cancer cell lines, which were exposure to vitamins C, E and C+E.

Vitamin	Cell line			
	IC_{50} in cancer cell lines		IC_{50} in normal cell lines	
	HepG2 (µg/ml)*	CHO (µg/ml)*	Fibroblast (µg/ml)*	CT26 (µg/ml)*
Control	97.03 ± 8.3	346.2 ± 22.65	312.29 ± 18.93	152.7 ± 10.86
AC	121.57 ± 6.47	357.36 ± 25.43	322.38 ± 17.12	169.7 ± 8.09
DC	107.6 ± 9.2	352.1± 18.92	317.89 ± 16.03	159.65 ± 5.2
AE	137.12 ± 5.73	372.39 ± 26.75	338.47 ± 17.74	171.49 ± 9.34
DE	132.09 ± 2.87	363.48 ± 23.54	330.09 ± 17.83	160.36 ± 6.27
AEC	153.46 ± 10.72	384.66 ± 30.1	347.38 ± 18.09	183.54 ± 7.81
DEC	142.25 ± 7.1	375.24 ± 27.36	340.48 ± 17.63	174.14 ± 5.35

*µg/ml; **P< 0.05
AC: ascorbic acid (17.6 mM); DC: ascorbic acid (2.2 mM); AE: α-Tocopherol (465.11 mM); DE: α-Tocopherol (85.12 mM); AEC: 50 µl of AC + 50 µl of AE; DEC: 50 µl of DC + 50 µl of DE.

used in chemotherapy in future.

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