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Full Paper

Anti-proliferative and apoptosis-inducing activities of *Derris elliptica* (Roxb.) Benth. leaf extract on three human cancer cell lines

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Abstract: This study investigates selective cytotoxicity of the ethanol extract of *Derris elliptica* leaves (DEE) and its apoptosis-inducing activities against three different human cancer cell lines, namely HepG2 (hepatocyte carcinoma), Jurkat (leukemic cell line) and MCF-7 (breast adenocarcinoma) cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used for determining cell proliferation. Nuclear morphological changes and DNA fragmentation were examined by Hoechst 33258 staining and agarose gel electrophoresis respectively. DEE shows anti-proliferative effect against cancer cells with a more pronounced activity in Jurkat than MCF-7 and HepG2, but is less toxic to human normal peripheral blood mononuclear cells. DEE also induces chromatin condensation, apoptotic bodies and DNA fragmentation in all cell lines tested with the highest apoptosis induction towards Jurkat cells.

Keywords: *Derris elliptica*, anti-proliferative activity, apoptosis, human cancer cell

Maejo Int. J. Sci. Technol. **2019**, *13*(01), 62-71

INTRODUCTION

Cancer remains one of the leading causes of mortality globally. It is the second leading cause of death, and accounted for an estimated 9.6 million deaths in 2018 [1]. Cancer is a group of diseases characterised by the uncontrolled growth and spread of abnormal cells. The goal of cancer therapy is to promote the death of cancer cells without causing too much damage to normal cells. Apoptosis is a programmed cell death that plays an important role in regulating the cell number and eliminating damaged cells. Targeting apoptosis is one of the major strategies for cancer therapy. Chemotherapy can induce cancer cell death with morphological features of apoptosis [2, 3]. However, many chemotherapeutic drugs also exert toxicity to normal cells that are actively growing and dividing, such as blood cells in the bone marrow. Natural therapies by plant-derived compounds in cancer treatment may reduce these adverse and toxic side effects. Plants synthesise hundreds of chemical compounds, most being in four major biochemical classes: alkaloids, glycosides, polyphenols and terpenoids. The use of plants as medicines has a long history in the treatment of various diseases including cancer. In addition, many studies have shown that regulating the consumption of plants can reduce the risk of acquiring a specific cancer [4]. For example, the extracts of *Zingiber officinale*, *Urtica dioica* and *Olea europae* not only present apoptotic induction but also show antioxidant activities and anti-proliferative effects on human cancer cells HepG2, MCF-7 and Jurkat [5, 6, 7].

Belonging to the family Leguminocae, *Derris elliptica* (Roxb.) Benth. (DE) is a shrub grown and distributed widely in South-east Asian countries. It is traditionally used as a fish poison and effective pesticide or insecticide. Rotenone can be extracted from its root and is known to be toxic to cold-blooded animals but less toxic to warm-blooded ones [8]. DE also contains phytochemical compounds such as tannins, terpenoids, cardiac glycosides and flavonoids [9]. It possesses biological activities such as antioxidant [10] and antimicrobial activities [11]. Fayad et al. [12] found that the extract of DE shows antioxidant activity and high cytotoxicity against human cancer cells. To our knowledge, however, the differential sensitivity of cancer cells as compared to normal cells to DE-induced cytotoxicity and the apoptosis-inducing property of DE are currently unexplored. Therefore, this study aims to investigate the anti-proliferative effect of DE extract (DEE) against three different human cancer cell lines, namely HepG2, Jurkat and MCF-7, compared to human normal peripheral blood mononuclear cells (PBMCs). The apoptosis-inducing activities of DEE on HepG2, Jurkat and MCF-7 were also evaluated.

MATERIALS AND METHODS

Chemicals and Reagents

Chemicals used in the present study were obtained from different sources: agarose A from Bio Basic Inc. (Markham Ontario, Canada); Biocoll separating solution (density 1.077 g/mL) isotonic from Biochrome (Cambridge, UK); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Fluka Chemie GmbH (Buchs, Switzerland); dimethyl sulfoxide (DMSO) from Amresco Inc. (Solon, USA); Roswell Park Memorial Institute medium-1640 (RPMI-1640), Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin from Gibco Invitrogen (Grand Island, USA); Wizard genomic DNA purification kit from Promega Corporation (Madison, USA). All other reagents were purchased from Sigma-Aldrich Co.

Maejo Int. J. Sci. Technol. **2019**, *13*(01), 62-71

Plant Material and Plant Extract Preparation

Fresh leaves of DE were collected from the local home garden in Ban Nong No, Tambon Siao, Amphoe Pho Si Suwan, Sisaket province, Thailand. The plant was identified and authenticated by a taxonomist. A voucher specimen was deposited at a local herbarium, Department of Biology, Faculty of Science, Ubon Ratchathani Rajabhat University.

Fresh leaves of DE were cut into small pieces and blended in 95% ethanol with a ratio of 1: 4 (g : mL). This suspension was allowed to stand at room temperature for 30 min. and then filtered through several layers of gauze. The pooled extract was filtered through Whatman No. 1 filter paper. The ethanolic filtrate was concentrated using a vacuum rotary evaporator at 60°C and lyophilised to obtain DEE, which was stored at -20°C. It was dissolved in DMSO when used in the experiments.

Cell Culture

HepG2 (human hepatocyte carcinoma cell line) and MCF-7 (human breast adenocarcinoma cell line) were obtained from American Type Culture Collection (ATCC: Manassas, USA). Jurkat leukemic cell line was obtained from Cell Line Services (CLS: Eppelheim, Germany). Human normal peripheral blood mononuclear cells (PBMCs) were obtained from buffy coat of blood blank. HepG2 and MCF-7 cell lines were cultured in DMEM with low and high glucose respectively. Jurkat cell line and PBMCs were cultured in RPMI-1640. All cell lines were cultured at 37°C, 5% CO_2 supplemented with 10% heat-inactivated FBS and 100 U/mL penicillin-streptomycin.

Isolation of Normal Human PBMCs

Buffy coat was isolated by Biocoll separating solution, density 1.077 g/mL. Briefly, 7 mL of the solution was added into a 15-mL conical tube. Then an equal volume of buffy coat diluted in phosphate buffered saline (PBS) at a ratio of 1:1 was carefully laid onto the solution in a conical tube. This solution was centrifuged at 400*xg* for exactly 30 min. at 25°C. The layer of PBMCs between the plasma and Biocoll separating solution was collected. Then the PBMCs were washed twice with PBS by centrifuging at 400*xg* for 5 min. before being used in the next experiment.

***In Vitro* Cytotoxic Test (MTT Assay)**

The cytotoxic effect of DEE on cell proliferation was determined by MTT assay [13]. Briefly, HepG2 or MCF-7 cells were seeded at a density of $2x10^4$ cells/well in a 96-well plate and incubated overnight at 37°C under 5% CO_2. After incubation, the cells were treated with different concentrations (50, 100 or 200 µg/mL) of DEE for 24 hr. Jurkat cells and PBMCs were seeded at a density of $2.5x10^4$ cells per well and $3x10^5$ cells per well respectively, before being treated with different concentrations (50, 100 or 200 µg/mL) of DEE. After incubation for 24 hr, DEE was removed by centrifuging at 400*xg*, 4°C for 5 min. MTT dye solution (0.5 mg/mL) was added in each well of a 96-well plate and the plate was incubated at 37°C, 5% CO_2 for 4 hr. MTT dye solution was removed by centrifuging at 400*xg*, 4°C for 5 min. Then DMSO was added to each well of the 96-well plate to dissolve formazan crystals giving a uniform dark purple colour that was measured at 540 nm by a microplate reader (Bio-Rad Laboratories, Inc., Hercules, USA). The percentage of cell viability was calculated as follows: the percentage of cell viability = (absorbance of the test group / absorbance of the control group) x 100.

Maejo Int. J. Sci. Technol. **2019**, *13*(01), 62-71

Hoechst 33258 Staining

HepG2 and MCF-7 cells were seeded at a density of $2x10^4$ cells/well in each 6-well plate and incubated overnight. Jurkat cells were seeded at a density of $2.5x10^4$ cells/well in another 6-well plate. Both HepG2 and MCF-7 cells were treated with positive control etoposide (5 µg/mL for HepG2 and 400 µg/mL for MCF-7) and different concentrations of DEE (50, 100 and 200 µg/mL) and left for 24 hr. Jurkat cells were treated with positive control etoposide (40 µg/mL) and different concentrations of DEE (25, 50 and 100 µg/mL) and incubated for 24 hr. After treatment, cells were harvested, centrifuged at 400*xg* for 5 min. and washed twice with PBS. The cells were fixed with 200 µL of paraformaldehyde (4% v/v) for 20 min. and washed twice with PBS. Then the cells were stained with Hoechst 33258 dye (10 µg/mL) for 30 min. at room temperature in the dark. After incubation, the stained cells were washed with PBS. Then the blue-stained DNA of normal cells and those with apoptotic morphology (fragmented and shrunken or condensed nuclei) were investigated. The numbers of condensed and fragmented nuclei were counted in four different fields under an inverted fluorescence microscope (Olympus IX51: Olympus Corporation, Tokyo, Japan). The percentage of condensed and fragmented nuclei in each field was calculated as follows: percentage of condensed and fragmented nuclei = [number of condensed and fragmented nuclei / total number of nuclei (200 nuclei/field)] x 100.

DNA Fragmentation

HepG2 and MCF-7 cells were seeded in each 100-mm^2 culture dish at $3.8x10^6$ cells/dish and incubated overnight at 37°C, 5% CO_2. At the same time, Jurkat cells were seeded in another culture dish at $1.875x10^6$ cells/dish. Both HepG2 and MCF-7 cells were treated with positive control etoposide (5 µg/mL for HepG2 and 400 µg/mL for MCF-7) and different concentrations of DEE (50, 100 and 200 µg/mL). Jurkat cells were treated with positive control etoposide (40 µg/mL) and different concentrations of DEE (25, 50 and 100 µg/mL). After 24-hr incubation, the cells were collected and centrifuged at 400*xg* for 5 min. Cell pellets were then washed twice with PBS and centrifuged at 400*xg* for 5 min. DNA was extracted from the cell pellets using Wizard genomic DNA purification kit and the isolated DNA was quantified using a Nanodrop ND-1000 spectrophotometer. DNA samples (5 µg for HepG2 and MCF-7 cells and 3 µg for Jurkat cells) were loaded in 1.5% agarose gel. The gel was run at 70 volts for 1.50 hr and stained with 0.5 ug/mL of ethidium bromide for 20 min. Then the DNA fragment was visualised under ultraviolet light (Wealtec Corp., Sparks, USA).

Statistical Analysis

All statistical significances were determined using one-way analysis of variance with *post hoc* Tukey's analysis (GraphPad Prism5: San Diego, USA) to determine differences between treatments and control group. Values were considered statistically significant when $p < 0.05$. Data are representative of three independent experiments and values are expressed as mean ± SEM (n = 3).

Maejo Int. J. Sci. Technol. **2019**, *13*(01), 62-71

RESULTS AND DISCUSSION

Anti-proliferative Effects of DEE

The anti-proliferative effect of DEE on three different human cancer cell lines, viz. HepG2, Jurkat and MCF-7 was evaluated by *in vitro* cytotoxicity MTT assay. All cancer cell lines including human normal PBMCs exhibit different susceptibility to DEE in a dose-dependent manner (Figure 1). The highest concentration (200 μg/mL) of DEE displays more than 50% toxicity to all cancer cells, with the cytotoxic percentages of 79.43 ± 0.73% for HepG2 cells, 89.66 ± 0.85% for Jurkat cells and 81.02 ± 0.50% for MCF-7 cells, while it displays only 61.06 ± 1.71% toxicity to normal PBMCs, which is less than that to all cancer cells.

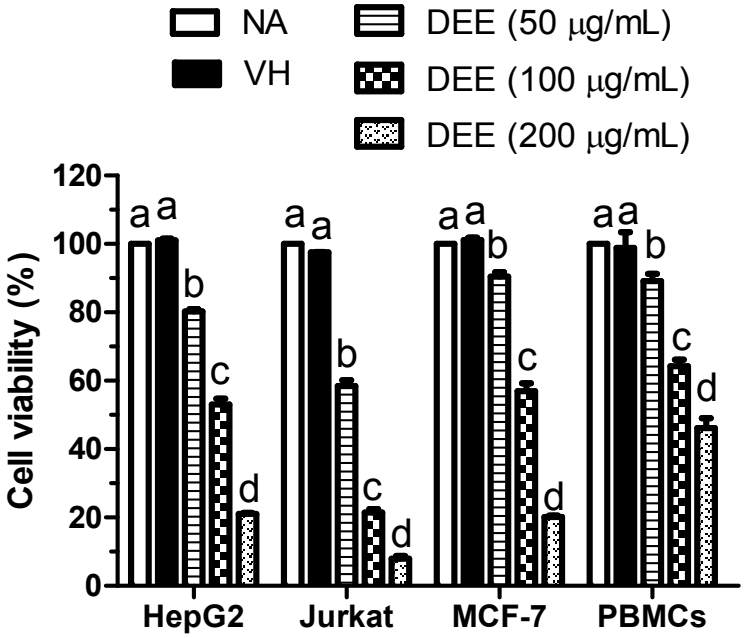

Figure 1. Effect of DEE on cell viability of cancer cell lines and normal cells (PBMCs). Bars marked with different letters within the group are significantly different at $p < 0.05$. NA and VH controls are Naïve (cells alone in media) and Vehicle (cells in 0.2% v/v of DMSO diluted in media) respectively.

The half maximal inhibitory concentration (IC_{50}) values of DEE against all cancer and normal cells were calculated from the linear regression equation of the per cent inhibition graph of each of the cancer and normal cells (Table 1). The IC_{50} for Jurkat (60.41 ± 2.49 μg/mL) is significantly ($p < 0.05$) lower than that for HepG2 (118.12 ± 2.16 μg/mL) and MCF-7 (109.58 ± 2.53 μg/mL). Therefore, DEE displays higher toxicity to Jurkat than to HepG2 and MCF-7 cells. DEE also exhibits antioxidant activities as detected by FRAP and DPPH assays (data not shown). The results are in accordance with the study of Fayad et al. [12], who found that the extract of dried leaves of DE in methanol showed antioxidant activities and high cytotoxicity to different human cancer cells: A545 (lung carcinoma), HCT-16 (colorectal carcinoma), HepG2 and MCF-7. The highest selectivity of DEE is observed in Jurkat cells with a selective index value of 2.69. This result suggests that DEE is twice more cytotoxic to Jurkat cells than normal PBMCs. The selective cytotoxicity of DEE on Jurkat cells might be due to either the sensitivity of the cell line to active compounds in the extract or to tissue specific response [14]. DEE is considered as a good source of polyphenol compounds. The major polyphenol compounds present in this extract are tannic acid

Maejo Int. J. Sci. Technol. **2019**, *13*(01), 62-71

and gallic acid, followed by isoquercetin, rutin, catechin, quercetin and apigenin respectively (data not shown). Importantly, the key property of a chemotherapeutic agents is its ability to selectively kill cancer cells with less cytotoxic effect on normal cells in order to avoid the undesired side effects [15]. These findings suggest that DEE could be suitable for the development of an anticancer drug for treating acute lymphoid leukemia.

Table 1. IC_{50} of DEE against human cancer cells and normal cells (PBMCs)

Type of cell	IC_{50} (µg/mL)	Selectivity index
HepG2	118.12 ± 2.16^{b}	1.37
Jurkat	60.41 ± 2.49^{a}	2.69
MCF-7	109.58 ± 2.53^{b}	1.48
PBMCs	162.35 ± 3.77^{c}	-

Note: Different letters within the same column are significantly different at $p < 0.05$. Selectivity index is calculated as the average of IC_{50} value for normal cell lines divided by IC_{50} value for cancer cell lines.

Apoptotic Induction by Hoechst 33258 Staining

The apoptotic cells are characterised by cell shrinkage, nuclear condensation or nuclear fragmentation. To evaluate the apoptosis induction of DEE on HepG2, Jurkat and MCF-7 cells, condensed and fragmented nuclei were determined by Hoechst 33258 staining. Morphological evidence of apoptosis, notably condensed and fragmented nuclei within the nucleus (Figure 2), is indicated with red arrows. The condensed and fragmented nuclei are clearly observed in all three different human cancer cells after treatment with DEE or etoposide, but are not observed in untreated cells, i.e. cells alone in media (NA) and cells in 0.2% v/v DMSO diluted in media (VH). The results show that treatment of HepG2 cells with 50, 100 or 200 µg/mL of DEE for 24 hr leads to 1%, 3.5% and 19.14% increases in condensed and fragmented nuclei respectively. Also, Jurkat cells after treatment with DEE at 25, 50 or 100 µg/mL for 24 hr show 0.4%, 9.7% and 27.48% increases in apoptotic nuclei respectively.

These results indicate that DEE can cause observable changes in the percentage of apoptotic nuclei in HepG2 and Jurkat cells in a dose-dependent manner, with a fragment effect on the latter. On the other hand, the exposure of DEE at 100 and 200 µg/mL results in the clumping of MCF-7 cells, which leads to the difficulty of observing the condensed and fragmented nuclei. However, apoptotic nuclei are observed in the MCF-7 cells after treating with DEE at 200 µg/mL. Together, these results suggest that DEE shows the strongest apoptotic inducing activity against Jurkat cancer cells by causing chromatin condensation together with DNA fragmentation, which is the key feature of apoptosis.

Maejo Int. J. Sci. Technol. **2019**, *13*(01), 62-71

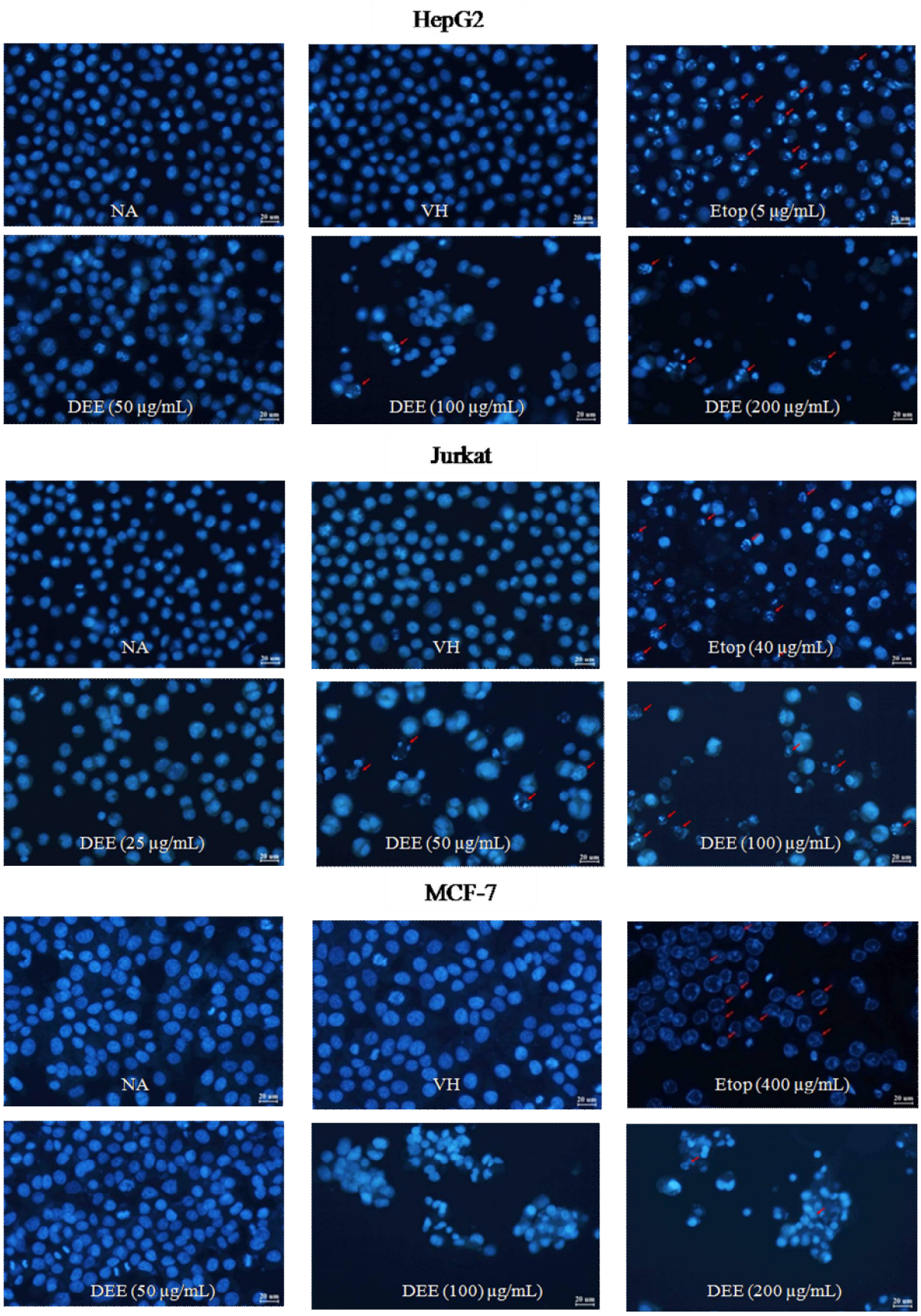

Figure 2. Apoptotic induction in different cancer cells observed under fluorescence microscope. Cells are treated with positive control etoposide (Etop) or DEE at indicated concentrations. Apoptotic bodies are indicated by red arrows.

Maejo Int. J. Sci. Technol. **2019**, *13*(01), 62-71

DNA Fragmentation in Human Cancer Cells

A distinctive biochemical feature of apoptosis is the fragmentation of DNA, which is the cleavage of chromosomal DNA into internucleosomal fragments of roughly 180-200 bp and appears as a DNA ladder when run on agarose gel [16]. DNA fragments extracted from the cells treated with different concentrations of DEE or positive control etoposide for 24 hr were separated using 1.5% gel electrophoresis. The results reveal that DEE treatment leads to DNA fragmentation in HepG2, Jurkat and MCF-7 cells in a dose-dependent manner in comparison with DNA from untreated cells (VH) (Figure 3). No DNA fragmentation is observed in the untreated group in both NA and VH. As expected, the DNA ladder formation is clearly observed in the positive control group at 24 hr of exposure. Surprisingly, the laddering pattern of DNA fragmentation in MCF-7 cells treated with DEE at 200 µg/mL is more pronounced than that treated with 400 µg/mL etoposide (Figure 3C). The lowest concentration of DEE necessary to elicit this effect is 50 µg/mL, which is observed only in Jurkat cell line (Figure 3B). Therefore, the results suggest that DEE exhibits the highest selectivity in inducing apoptosis towards Jurkat cells, with apoptotic DNA fragments corresponding to the highest percentages of condensed and fragmented nuclei as detected by Hoechst staining.

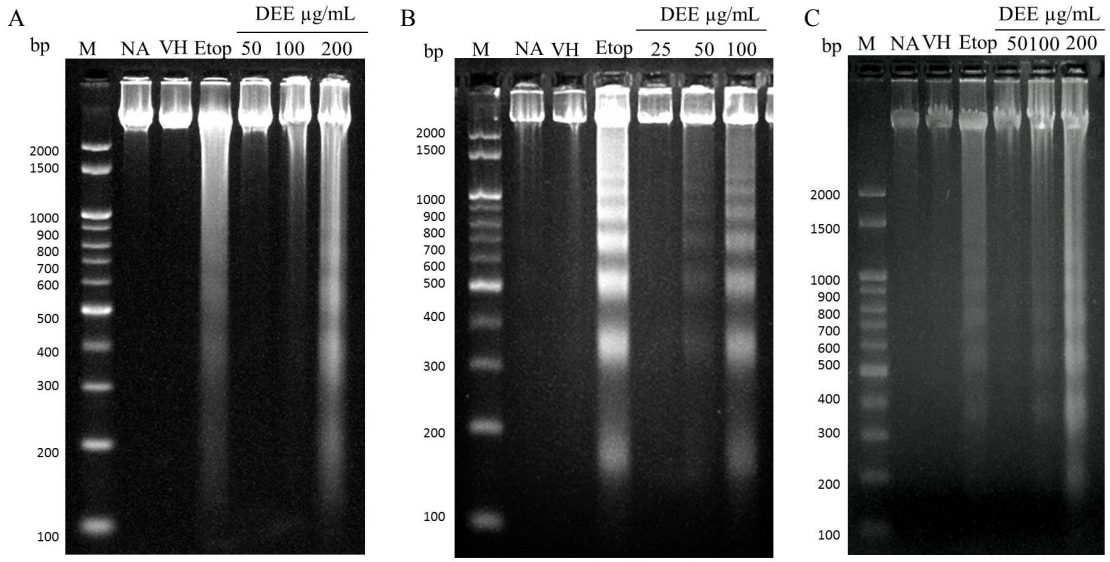

Figure 3. DNA fragmentation of cancer cells: (A) HepG2, (B) Jurkat, (C) MCF-7. The cells are treated with DEE at indicated concentrations. HepG2, Jurkat and MCF-7 cells are treated with positive control etoposide (Etop) at 5, 40 and 400 µg/mL respectively. M is marker.

CONCLUSIONS

DEE exerts selective cytotoxicity against tumour cell lines, namely HepG2, Jurkat and MCF-7, Jurkat cell being the most sensitive to the lethal effect of DEE. DEE is less toxic to normal PBMCs. The anti-proliferative effect of DEE is due to its ability to induce apoptosis, as evidenced by apoptotic body formation and DNA fragmentation. Therefore, DEE may be a potential source of anticancer drugs that causes fewer side effects on normal tissues and cells.

Maejo Int. J. Sci. Technol. **2019**, *13*(01), 62-71

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