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Original Article

In Vitro Cytotoxicity and Apoptosis-inducing Activity of *Quercus infectoria* Extracts in HeLa Cells

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ABSTRACT

Background: *Quercus infectoria galls* (QI) extracts were previously reported to have cytotoxicity effects towards human cervical cancer cells, HeLa. However, the underlying molecular mechanisms of the extracts have been poorly determined. **Objective:** The present study was undertaken to examine the effect of ethyl acetate extracts of QI (EAQI) on cell cytotoxicity and induction of apoptosis in HeLa cells. **Materials and Method:** The *in vitro* cytotoxicity was investigated by using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay and the OD values were read at 570 nm. Meanwhile the induction of apoptosis was measured by using acridine orange and propidium iodide (AO/PI) staining, flow cytometry analysis of annexin V/PI staining and cell cycle distribution. **Results:** MTT assay showed that EAQI exhibited cytotoxicity effect on HeLa cells with IC_{50} of 11.50 ± 0.50 µg/ml. HeLa cells underwent apoptosis in response to EAQI treatment, demonstrated by an increase in the percentage of apoptotic cell stained with AOPI from 1.00% to 10.33% compared to untreated cell population ($p<0.05$) at 72 hours of treatment. The evidence of early apoptosis in treated cells were also observed in annexin V/PI staining. Furthermore, an increase of cell population in sub G0/G1 phase revealed that apoptosis as the mode of cell death in HeLa cells treated with EAQI. **Conclusion:** These findings indicated that EAQI significantly inhibits HeLa cell growth through induction of apoptosis. Further studies are needed to confirm the mechanism of cell death by expression of apoptotic cascade in HeLa cells treated with EAQI.
Key words: *Quercus infectoria*, HeLa cells, Cytotoxicity, Apoptosis, Cell cycle.

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INTRODUCTION

Cervical cancer is one of the most common cancers of the reproductive system in women worldwide. Cancer treatments including chemotherapy, radiation and surgery remains the most common treatment for metastatic cancers.[1] The aim of cancer therapy is to promote the death of cancer cells without harming the normal cells by induction of apoptosis.[2] Apoptosis induction is one of the most important cytotoxicity markers of antitumor agents.[3]

Apoptosis is a form of programmed cell death characterized by chromatin condensation and nuclear fragmentation (pyknosis), plasma membrane blebbing and cell shrinkage. Eventually, the cells break up into small membrane-surrounded fragments known as apoptotic bodies which are cleared efficiently by phagocytosis without inducing inflammatory response.[4] However, the increase in resistance as well as adverse effects of radiotherapy and chemotherapy have lead biomedical researchers to investigate novel cancer chemoprevention from herbal resources which could be used for effective treatment of cancer diseases.[5,6] Many reports have revealed strong evidence that medicinal plant could be a reliable sources for development new therapeutic agents of cancer treatment.[7-11]

Quercus infectoria (QI) is one of the herbal plant that is locally known as "manjakani" in Malaysia and "Majuphal" in Indian traditional medicine.[12] The QI trees usually grow in Greece and Iran and then spread to Asia Minor, Europe and Northern Africa.[13] It grows to a height of 4–6 feet with smooth and bright, acorn long, narrow, scaly, and downy leaves. The galls are greyish-brown to brownish-black in colour externally and are yellow internally. It is globular (2 inch), uneven, porous with size of 1.4–2.3 cm in length and 1–1.5 cm in diameter. The powder of galls is coarse, creamish brown with no odour but bitter taste.[14] The gall arise in the branches is resulting from the deposition of eggs by gall wasp *Cynips gallae tinctoriae*.[13] The galls have been reported to contain large amount of bioactive constituents such as tannins (50-70%), small amount of free gallic acid and ellagic acid. [5] In Malaysia, it has been regularly consumed as herbal beverages to restore postpartum uterine elasticity and also stimulate the contraction of vaginal muscles.[16] In Indian traditional medicine, it is a constituent of toothpowder or toothpaste for treatment of oral cavity diseases.[12]

Pharmacologically, QI extract has been reported to have antibacterial activity,[17] antiviral activity[18] and antifungal activity.[19] The QI extract also possesses numerous other beneficial functions, including anti-diabetic[20], anti-inflammatory[21], anti-amoebic[22] and wound healing.[23] It was also reported that methanol, ethanol and aqueous extract of QI inhibited the growth of cancer cell lines including HeLa (human cervical cancer cell line)[7]. In addition, ethyl acetate

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extract of QI demonstrated higher cytotoxicity effect towards HeLa cells compared to other solvents extracts.[24] However, the potential of QI as an anticancer agents and its underlying molecular mechanism still remain to be elucidated yet. Therefore, the current study was designed to evaluate the effect of ethyl acetate extracts of QI (EAQI) on cell cytotoxicity and apoptosis induction in human cervical cancer cell line (HeLa).

MATERIALS AND METHODS

Preparation of extracts

QI galls were purchased from Chinese herbal shop in Kota Bharu, Kelantan. The voucher specimen (number: UniSZA 00423) was authenticated by a botanist (Prof Madya Dr Khamsah Suryati Mohd) and was deposited at the Herbarium of Faculty of Bioresourses and Food Industry, UniSZA. The galls were ground using a grinder to be powder form. About 500 g of powdered galls were soaked with ethyl acetate (2.5L) for 72 hours using shaker at 120 rpm as previously described by Trusheva et al. (2007)[25]. The samples were filtered through whatman no.1 filter paper fitted with a Buchner funnel using suction pressure. The soaking process was repeated for three times. The extracts were evaporated under reduced pressure below 40°C to give 90.23 g of EAQI. The crude extracts were stored at 4°C until use. The yield of extracted samples was calculated using the following equation:

$$\text{Percent of yield extraction} = \frac{\text{Final weight (g)} \times 100}{\text{Initial weight (g)}}$$

Cell culture

HeLa (human cervical cancer cells) and Vero (African Green Monkey kidney cells), a type of normal cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Both cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin 1% (v/v) (Gibco, Grand Island, NY). The cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere.

In vitro cytotoxicity assay

Cytotoxicity activity of EAQI was carried out by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium reduction assay.[26] The EAQI extract was diluted in DMSO (Sigma, St. Louis, MO) to obtain a stock solution of 10.00 mg/mL. Cells (3 x 10^4cells/mL) were treated with various concentration of extract in 96-well culture plates for 72 hours. After 72 hours of treatment, each well was then added with 20 µL of MTT solution (5 mg/mL in PBS) and the cells were further incubated for 4 hours at 37°C. After incubation, the medium was discarded and the insoluble formazan crystals were dissolved by adding 100 µL of DMSO. The plates were shaken and the optical density was measured by a microplate reader at 570 nm. (Thermo Scientific Multiskan Spectrum, Thermo Fisher Scientific, Inc., Waltham, MA). The IC_{50} value of EAQI extract was determined by using non liner regression analysis (percent inhibition versus concentration) and used in subsequent experiments. In this study, cisplatin was used as a positive control.

Cell morphology analysis

Morphological detection of apoptosis were examined using acridine orange and propidium iodide (AO/PI) staining.[27] HeLa cells at a concentration of 5 x 10^4cells/mL were treated with EAQI for 24, 48 and 72 hours at their IC_{50} concentrations in triplicates. The treated cells were trypsinised and harvested with 1 mL cold phosphate buffered saline (PBS), followed by centrifuged at 1500 rpm for 10 minutes at 4°C. This process was repeated twice. The cell suspension was mixed with 20 µL of AO/PI solution (1:1) and the mixture (10 mL) was transferred onto a slide and covered with a cover slip. Viable, apoptotic and necrotic cells were quantified in a population of 200 cells using a fluorescence microscope equipped with B-2A filter (Nikon TE2000-U, Japan). Untreated HeLa cells served as a control negative and cisplatin was used as a positive control.

Determination of phosphatidylserine (PS) externalization

Annexin V-FITC Apoptosis Detection Kit 1 (Becton Dickinson, USA) was used in determining of phosphatidylserine (PS) externalization. The kit contains Annexin V conjugated to the fluorochrome FITC, propidium iodide and binding buffer. Briefly, HeLa cells (5 x 10^4cells/mL) were treated with IC_{50} value of the EAQI extract and cisplatin at 3, 6 and 9 hours. The cells were collected, washed trice with cold PBS and 100 µL of binding buffer was added into the tubes. A 3 µL of FITC-conjugated Annexin V (Annexin V-FITC) and 3 µL of propidium iodide (PI) were then added and incubated at room temperature in the dark for 15 minutes. The stained cells were diluted by the binding buffer (400 µL) and immediately analysed with a CytoFlex flowcytometer (Beckman Coulter, USA). About 10000 events were accumulated per sample. The results were generated in a quadrant graph with four different populations of cells represents a viable cells (Annexin V-FITC and PI are negative), early apoptosis (Annexin V-FITC positive and PI are negative), late apoptosis (Annexin V-FITC and PI are positive) and necrosis (Annexin V-FITC negative and PI are positive).

Cell cycle analysis

Cell cycle analysis was performed according to the protocol in CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson, USA). HeLa cells (5 x 10^4cells/mL) were cultured overnight in a 6-well plate and treated with EAQI for 24, 48 and 72 hours at their IC50 concentrations in triplicates. The cells were trypsinised and harvested in a similar manner with AO/PI staining protocol. The cell pellets were resuspended in 250 µL of buffer solution A. The cells were then incubated for 10 minutes at room temperature. A 200 µL of solution B was added and the mixture was further incubated for 10 minutes at room temperature. A 200 µL of solution C containing propidium iodide was added and the mixture was incubated in a dark place at 4°C for a further 10 minutes. The cellular DNA content was measured by using CytoFlex flowcytometer (Beckman Coulter, USA). Untreated cells were used as a negative control and cells treated with cisplatin were utilized as a positive control.

Statistical analysis

Results were obtained from three independent experiments. Data were expressed as the mean ± standard error of the mean (SEM) and analysed by A one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test. The statistical software SPSS (version 22) was used and $p<0.05$ was defined as statistical significance compared to control.

RESULTS

Cytotoxic effects of EAQI on the cervical cancer cell lines (HeLa)

Cytotoxic effect of EAQI extract was tested against HeLa and Vero cells using calorimetric method MTT assay. Cisplatin was used as a positive control drug to compare with EAQI tested against HeLa and Vero cells. Bioactivity of EAQI and cisplatin was determined based on the concentration that induced 50% inhibition on the growth of the treated cells in triplicate from three independent experiments. EAQI extract and Cisplatin showed toxicity effect towards cancer cell lines and induced significant cell death in HeLa cells with an IC_{50} value of 11.50 ± 0.50µg/mL and 1.85 ± 0.15µg/mL respectively at 72 hours of treatment. However treatment of EAQI towards Vero cells exhibited no cytotoxic effect (Figure 1 and Table 1).

Table 1: IC_{50} **of EAQI and cisplatin on HeLa and Vero cells.**

	IC_{50}(µg/mL)	
	HeLa	Vero
EAQI extracts	11.50 ± 0.50	>100
Cisplatin	1.85 ± 0.15	14.33 ± 1.53

Data are expressed as mean ± S.E.M from three independent experiments.

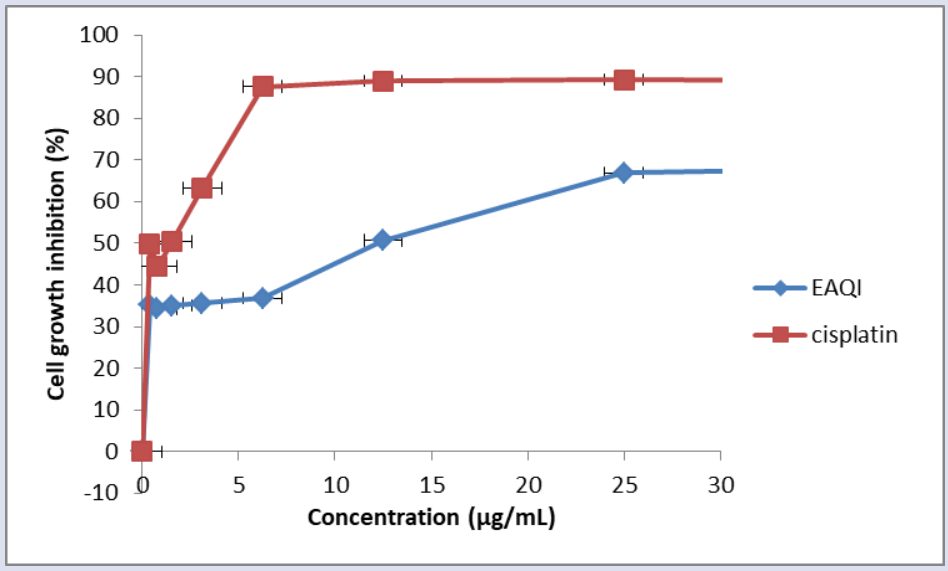

Figure 1: Cytotoxic effect of EAQI and cisplatin on HeLa cell line.

Morphology of apoptosis by AO/PI staining

AO/PI staining was performed to determine the mode of cell death of HeLa cells induced by EAQI and cisplatin. Figure 2 showed that the HeLa cells stained with AO/PI for untreated cells (a), cells treated with IC_{50} value of EAQI (b) and cells treated with IC_{50} value of cisplatin (c). Viable cells (V) with intact nucleus structure were stained green. Early apoptotic cells (E) were identified as green, condensed nuclear structure, cell shrinkage and formation of apoptotic bodies. Meanwhile late apoptotic cells (L) have bright orange areas of condensed chromatin in the nucleus and necrotic cells (N) have a uniform red colour. HeLa cells which were treated with EAQI and cisplatin for 24, 48 and 72 hours showed apoptotic event with membrane blebbing, cell shrinkage and the presence of apoptotic bodies compared to untreated (UT) cells. AO/PI analysis also revealed that the percentage of apoptotic cell population in treated cells increased significantly ($p<0.05$) in a time-dependent manner compared to control (UT). EAQI and cisplatin treatment significantly increased the apoptotic cell population from 0.33% to 7.67% (EAQI) and 7.33% (cisplatin) at 24 hours of treatment. At 48 hours of treatment, a significant increase in the apoptotic cell population was observed from 1.33% to 8.67% (EAQI) and 21.33% (cisplatin). Additionally, at 72 hours of EAQI and cisplatin treatment, the population of apoptotic cell increased from 1.00% to 10.33% and 22.67% respectively compared with the untreated cell population ($p<0.05$) (Figure 3).

Phosphatidylserine (PS) externalization and apoptotic induction

Consistent with the AO/PI staining result, the flowcytometry annexin V/PI analysis demonstrated that apoptosis was induced in a time dependent manner (Figure 4). The plots showing the distribution of HeLa cells within four quadrants that were labelled as Q1 (An-, PI+), Q2 (An+, PI+), Q3 (An-, PI-) and Q4 (An+, PI-). For the untreated cells, the percentages of viable, early apoptotic, late apoptotic, and necrotic cells were 99.38 ± 0.15%, 0.18 ± 0.09%, 0.14 ± 0.08 % and 0.25 ± 0.07%, respectively. However, after 3, 6, and 12 hours of treatment with EAQI and cisplatin (CIS), the number of cells increased significantly ($p<0.05$) compared to untreated (UT) cells in all three quadrants (Q1, Q2 and Q4) except for Q3. For EAQI treatment, the population of early apoptotic cells (Q4) increased to 1.77 ± 0.06%, 1.95 ± 0.01%, and 2.48 ± 0.02% after 3, 6, and 12 hours, respectively. Similar to early apoptosis, the percentage of late apoptotic cells gradually increased with longer treatment time to 1.54 ± 0.04%, 1.95 ± 0.01% and 2.92 ± 0.03% cells for 3, 6, and 12 hours, respectively. Meanwhile, the percentage of viable population (Q3) for EAQI treated cells decreased significantly ($p<0.05$) from 99.38 ± 0.15%, to 94.53 ± 0.35%, 93.47 ± 0.12% and 92.31 ± 0.21% after 3, 6, and 12 hours of treatment, respectively. Hence, the overall shift in the population of cells revealed the EAQI capability to cause apoptotic cell death in HeLa cells at the early stage of apoptosis.

Cell cycle analysis

Cell cycle distribution of HeLa cells treated with IC_{50} value of EAQI and cisplatin (CIS) at 24, 48 and 72 hours was evaluated by flow cytometric analysis (Figure 5). Results indicated that the sub G0 population in the treated cells (EAQI and cisplatin) were significantly increased ($p<0.05$) compared to untreated cells (UT) in a time-dependent manner (Figure 6). At 24 hours treatment of EAQI, the sub G0 population increased from 4.48 ± 0.06% to 7.71 ± 0.17%. After exposure for 48 hours, the sub G0 population increased markedly from 7.75 ± 0.18% to 18.79 ± 0.53%. The sub G0 population increased further from 8.54 ± 0.11% to 32.04 ± 0.25% after 72 hours of EAQI treatment. A similar increased of sub G0 cell population were also observed in cisplatin treated cell. The increase in sub G0 population reveals the induction of apoptosis, as sub G0 peak is reported to be a quantitative apoptosis marker.

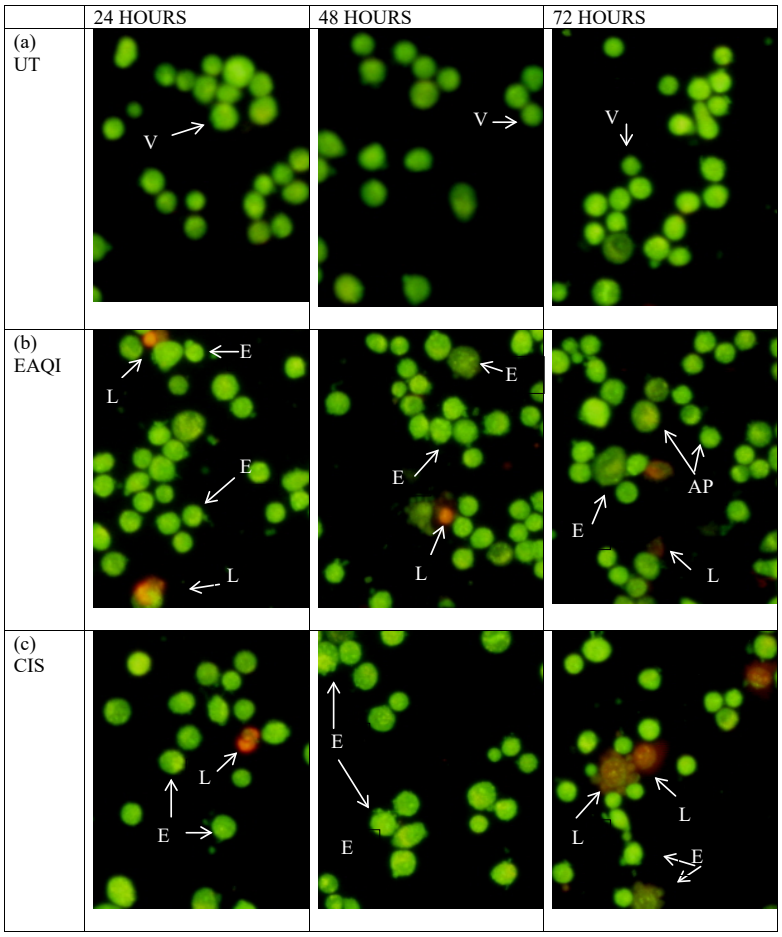

Figure 2: AO/PI staining of HeLa cells were viewed under a fluorescence microscope at 24, 48 and 72 hours. The cells incubated separately as the untreated (a), 11.50µg/mL EAQI, (b) and 1.85 µg/mL cisplatin, (c). V, viable cell; E, early apoptotic cell; L, late apoptotic cell. The magnification was 100 X.

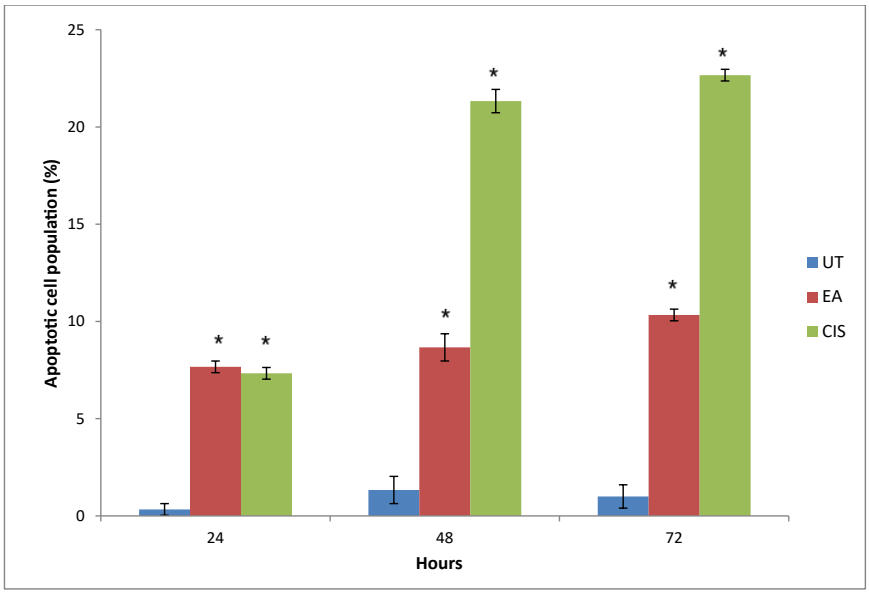

Figure 3: Frequency of apoptotic cell population after treatment with EAQI (EA) and cisplatin (CIS) at 24, 48 and 72 hours of treatment. Values are mean ± S.E.M (n=3). Asterisk (*) indicates a significant value ($p<0.05$) as compared with untreated group (UT).

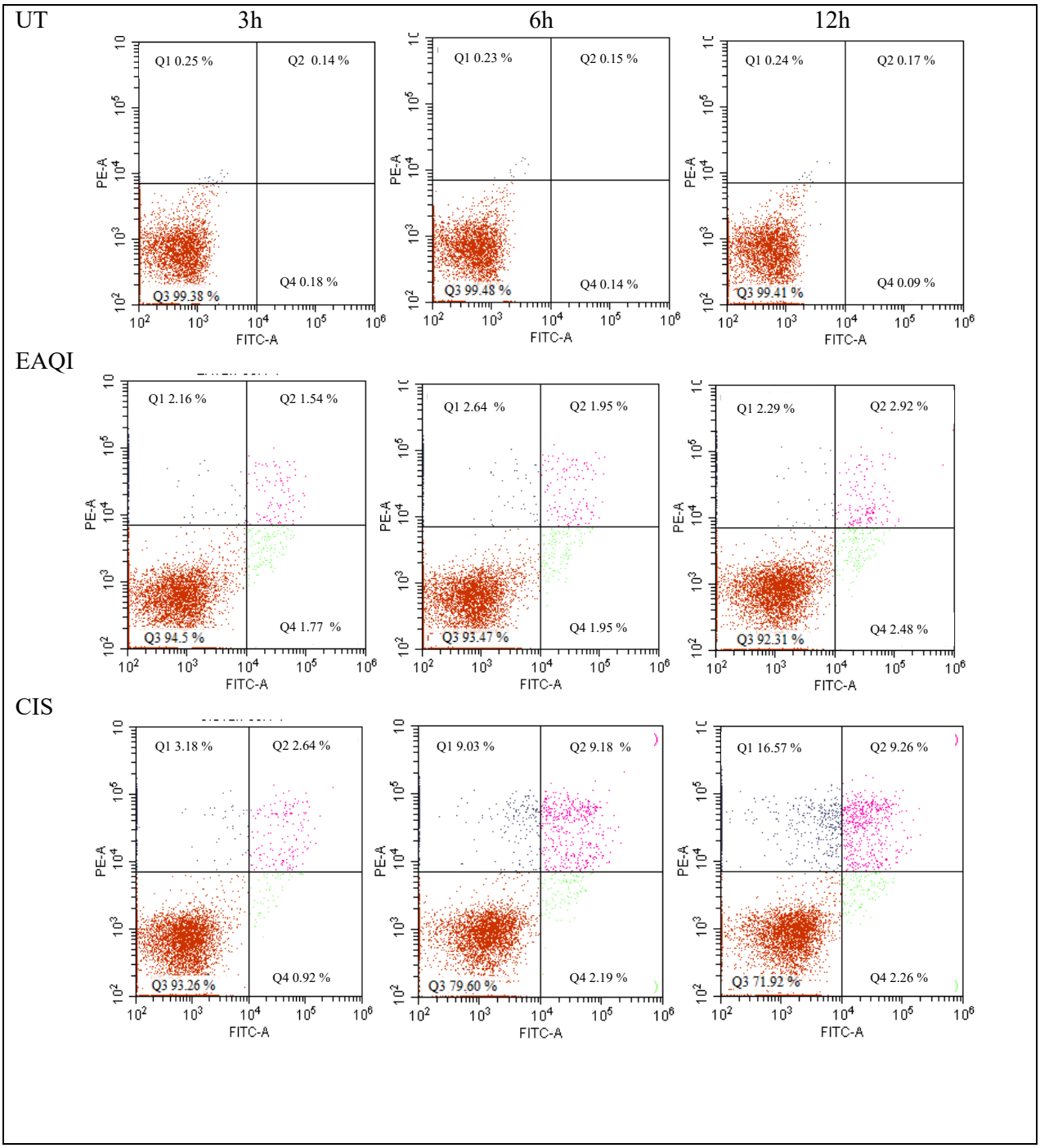

Figure 4: Annexin V(AV) / propidium iodide (PI) staining of HeLa cells treated with EAQI (EA), cisplatin (CIS) as control positive and untreated (UT) group as control negative at 24, 48 and 72 hours of treatment. The Y-axis indicates the PI-labelled population, whereas the X-axis indicates the FITC-labelled Annexin V positive cells. The lower left quadrant of the fluorocytogram (An-, PI-) shows viable cells (Q3), whereas the lower right quadrant (An+, PI-) shows early apoptotic cells (Q4). The upper right quadrant (An+, PI+) shows late apoptotic cells (Q2) and the upper left quadrant (An-, PI+) shows the necrotic cells (Q1).

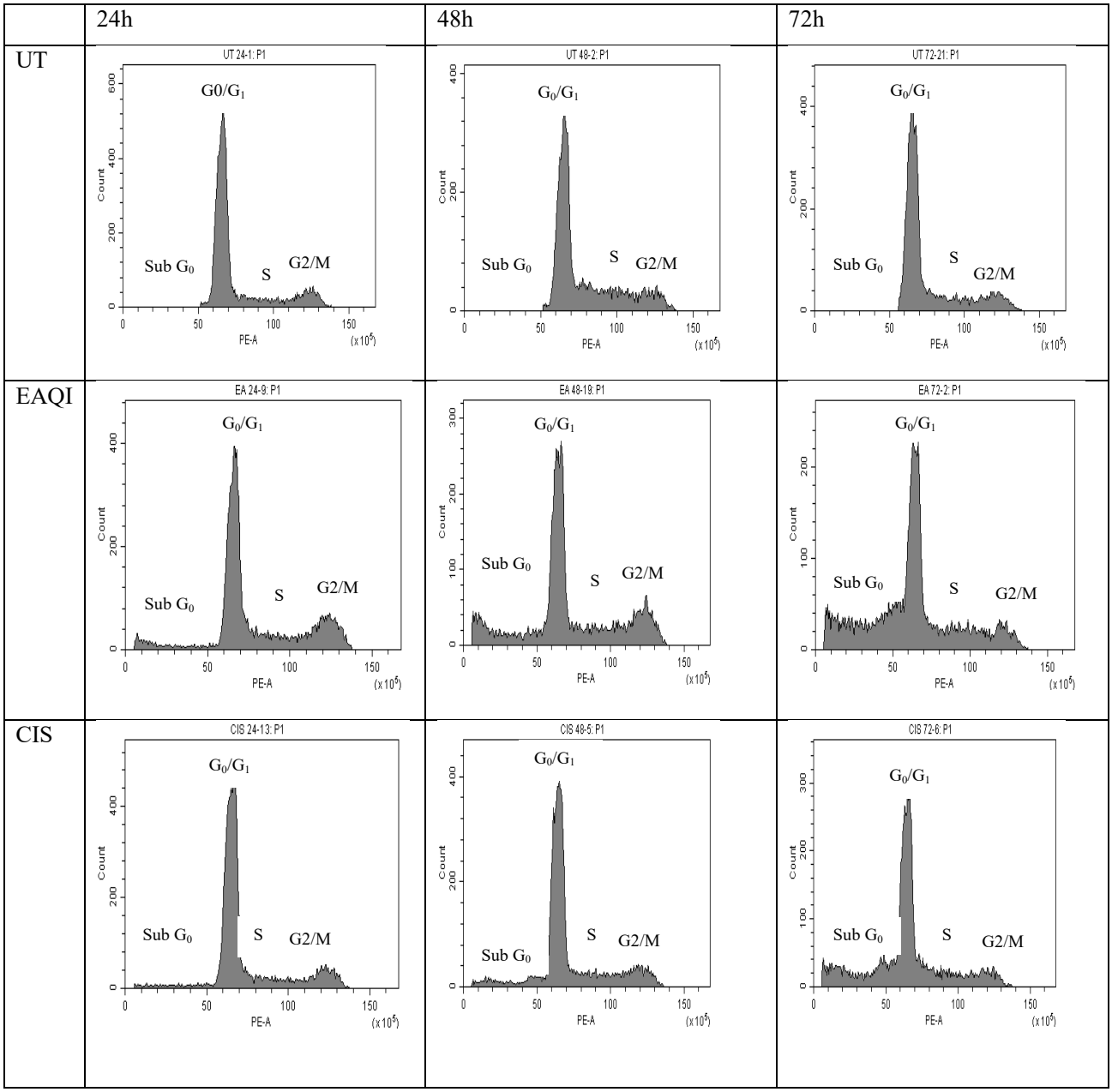

Figure 5: Cell cycle distribution of HeLa cells treated with EAQI, cisplatin (CIS) as control positive and untreated (UT) group as control negative, at 24, 48 and 72 hours of treatment.

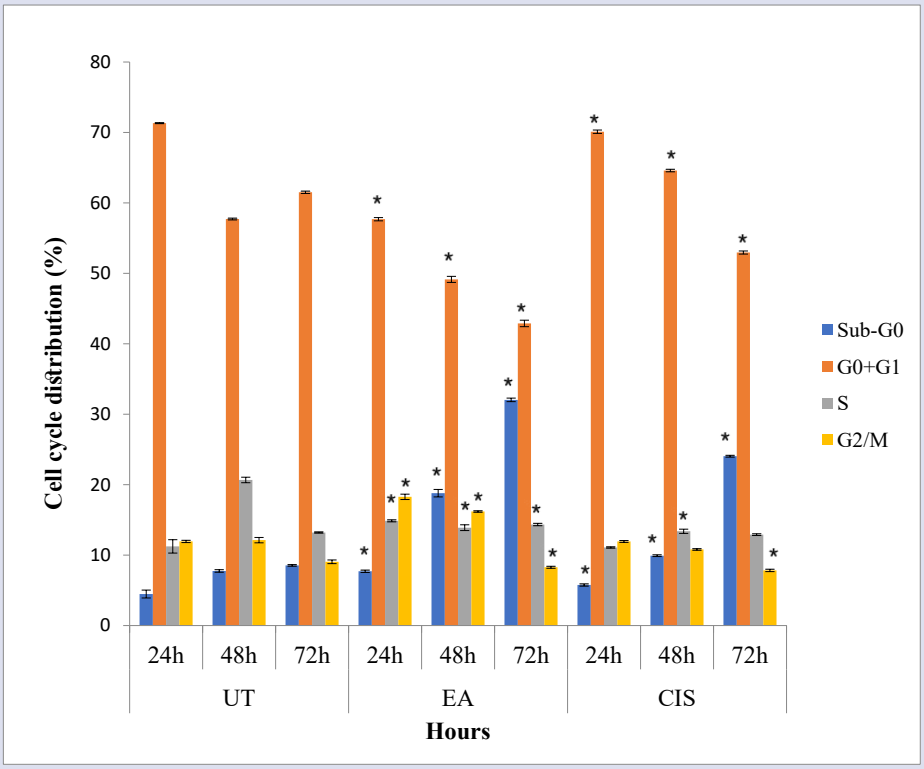

Figure 6: Percentage of cell cycle distribution of HeLa cells treated with EAQI, cisplatin (CIS) as control positive and untreated (UT) group as control negative, at 24, 48 and 72 hours of treatment. Values are expressed as mean ± S.E.M of triplicate. *p<0.05 as compared to the control group.

DISCUSSION

Natural products have been reported to have potent anti-cancer activities and promising candidates as anti-cancer agents.[28] In Asian, QI has been used as a medicinal plant for years since it was stated to contain large amounts of bioactive compounds.[20] However, the number of current studies conducted on QI anticancer properties is surprisingly low. The present study was designed to investigate the effects of EAQI on cytotoxicity and to elucidate the mechanism of apoptotic effects in HeLa cells.

This study revealed that ethyl acetate extract of *Quercus infectoria* (EAQI) showed cytotoxicity effect and caused cell death towards HeLa cell line. According to Umachigi et al. (2008) the antiproliferative activity of *Quercus infectoria* might be due to the presence of tannins, gallic acid and ellagic acid.[23] Furthermore, these bioactive compounds have demonstrated to possess anticancer activity on many types of cancer cells [29–31]. On the other hand, EAQI did show the capability to act as a cytoselective anticancer agent since this extract was not cytotoxic to normal kidney epithelial cell line (Vero). Therefore, the effects of EAQI were specific for cancer cells. However, cisplatin, a standard therapeutic drug demonstrated its cytotoxic effect towards both cancer and normal cell lines. The finding from this study were similar to Sakinah et al. (2007) who reported that cisplatin showed potent cytotoxic effect towards normal Chang liver cells and Vero cells line.[32] Based on MTT assay, EAQI can thus be suggested as a promising cytotoxic agent that might be related to apoptosis induction with cytoselective effect against normal cells.

Apoptosis induction is one of the important approaches in cancer chemotherapy[33]. To determine if the cytotoxicity of extracts involved apoptosis induction treated HeLa cells were analysed using AO/PI staining, determination of phosphatidylserine (PS) externalization and cell cycle analysis. Based on AO/PI staining, the nuclear DNA appears in green fluorescence as AO passes through the intact cell membrane, while PI emits a red-orange fluorescence in the nucleic DNA of damaged cells.[34] In this study, after 24 hours of treatment with EAQI and cisplatin, signs of apoptosis in the treated cells were began to be observed. The number of cells undergoing apoptosis (early and late apoptosis) was found to have increased with increasing the treatment duration. The treated cells demonstrated clear morphological changes including loss of cell integrity, cell nuclei fragmentation, cell shrinkage, membrane blebbing and apoptotic body formation compared to untreated cells. These findings are consistent with the study of Wan Yusof & Abdullah (2020) in which HeLa cells treated with EAQI using Hoechst stain exhibited similar apoptotic events.[24]

A significant feature of chemopreventive agents is the capability to induce cell death through apoptosis rather than necrosis.[35] During apoptosis process, phosphatidylserine (PS) is translocated and externalized on the outer surface of cell membrane and being accessible for annexin V binding. Meanwhile the propidium iodide stains late-apoptotic or necrotic cells with impaired cell membrane integrity.[10] Thus, annexin V-FITC assay was used to further evaluate the apoptotic potential of EAQI. Flow cytometric analysis revealed that EAQI extract induced cell death through early apoptosis induction. Significant increases in the percentages of early and late apoptotic cell populations in HeLa treated cells without significantly increasing the percentage of necrotic cells were consistent with the results of AO/PI staining assay that showed strong apoptotic morphological changes. Meanwhile, cisplatin incubation of HeLa cells resulted in a significant increase in the number of both apoptotic and necrotic cells. The finding indicated that cisplatin induced cell death through apoptosis and necrosis pathway.[36]

Another significant feature of a potential anticancer agent is its ability to induce cell cycle arrest. The molecular mechanism underlying

a compound's cytotoxicity can be understood from the cell cycle analysis [37]. Based on the cell cycle analysis, cell cycle arrest at sub G0 was demonstrated in HeLa cells in response to EAQI and cisplatin. Available evidence indicated DNA fragmentation and decreased of DNA content.[38] Cell death caused by fragmentation of DNA produce apoptotic cells with less DNA than healthy cells, resulting in a sub G0 peak in the cell population profile. [39] Thus, the increase in sub G0 population in this result indicates apoptosis induction as the sub G0 peak is known as an indication of quantitative apoptosis.

CONCLUSION

These findings indicate that EAQI significantly inhibits the HeLa cell growth through induction of apoptosis. Further studies are needed to confirm the mechanism of cell death by expression of apoptotic signalling pathway of HeLa cells treated with EAQI.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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