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Original article

Induction of Apoptosis in HeLa Cervical Cancer Cells Treated with Aqueous and Supercritical Fluid Extracts of *Quercus infectoria*

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Abstract

Background and objectives: The anticancer properties of extracts from *Quercus infectoria* galls have been demonstrated in a range of cancer cells, including human cervical cancer cells. This study aimed to elucidate the cell death mechanisms of *Q. infectoria* aqueous and supercritical fluid extracts on cervical cancer cells, HeLa. **Methods:** In vitro cytotoxicity was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, whereas apoptosis induction was assessed using acridine orange/propidium iodide staining. Flow cytometry was used to analyse phosphatidylserine externalization, cell cycle distribution, and caspase activity. Meanwhile, anti-Bax, anti-Bcl-2, and anti-p53 antibody were used to examine the expression of p53, Bax, and Bcl-2. **Results:** MTT assay revealed the cytotoxic effects of the aqueous and supercritical fluid extracts on HeLa cells with IC_{50} values of 12.33±0.35 µg/mL and 14.33±0.67 µg/mL respectively. Acridine orange/propidium iodide analysis revealed morphological changes with apoptotic features in the treated cells. Cell population increase in sub G0 phase showed induction of apoptosis in the treated HeLa cells. Moreover, the activation of caspases in the treated cells revealed the execution of apoptosis. In addition, the expression of p53 and Bax proteins in the treated cells were observed whereas there was no difference in the expression of Bcl-2 in the treated cells compared to untreated control cells. **Conclusion:** Both aqueous and supercritical fluid extracts inhibited the growth of HeLa cells through induction of cell apoptosis by activation of caspases-8 and caspase-9.

Keywords: caspase; cytotoxicity; *Quercus infectoria* extract; supercritical fluid extract

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Introduction

Cervical cancer is one of the most common malignancies found in female worldwide. The new invasive cancer is estimated in 2020 with 604,127 cases and the mortality associated with this cancer type was 341,831 cases [1]. In the year 2020, cervical cancer was classified as the fourth most diagnosed malignancies among Malaysian women [2]. It begins in the cervix and spreads to other tissues and organ such as lungs and liver. Cervical cancer symptoms include irregular menstruation, weight loss, heavy menstruation, abnormal menstruation, vaginal discomfort, and pelvic pain. [3].

Surgery, radiation, and chemotherapy, as well as conventional therapy, are now used to treat cervical cancer. The goal of cancer treatment is to trigger apoptosis, or cell death, without being harmful to healthy cells [4]. The advancement of cancer research and the discovery of anticancer agents with cyto-selective, anti-proliferative, apoptosis induction, and differentiation events have brought new insights into cervical cancer

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treatment [5]. Even though these treatments have prolonged survival rates, the substantial and long-term adverse effects such secondary cancer development and infertility remain as obstacles for the success of the treatment [6]. Furthermore, rising resistance to cancer treatment has prompted researchers to search for new cancer chemoprevention strategies based on herbal resources, which might be utilized to treat cancer effectively [7].

The oak tree, *Quercus infectoria* Olivier. belongs to the Fagaceae family which can be found in Greece, Asia Minor, Syria, and Iran. The use of *Q. infectoria* galls by Malay women primarily as post-partum remedies was thought to improve blood flow, speed up uterine contractions and tighten vagina, and stomach [8], as well as being used a as health supplements, known as "Jamu". [9,10]. Also, the anti-inflammatory, antiviral, antidiabetic, aantibacterial, antiulcerogenic, and astringent activities of the galls are highlighted as the most significant medicinal value potentials of the galls [9]. Previously, ethanol, methanol [11], and ethyl acetate extracts [12] of *Q. infectoria* were reported to suppress the proliferation of a few cancer cell lines, including human cervical cancer cell lines, HeLa [11,12]. The ethyl acetate extract of *Q. infectoria* exerted more potent cytotoxicity effect towards HeLa cells through induction of apoptosis [13]. Moreover, this extract revealed cyto-selective effect towards only cancer cells [12]. In the present study, we examined cell death mechanisms of aqueous and supercritical fluid extracts of *Q. infectoria* on HeLa cells.

Materials and Method

Ethical considerations

All ethics involved in the entire research have been considered.

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, streptomycin and penicillin (Gibco Thermo scientific, USA), gallic acid and cisplatin (Sigma Chemical Co., USA) DMSO (Sigma, USA) Annexin V-Fluorescein isothiocyanate (V-FITC) apoptosis kit (BD Biosciences, USA) were used. Acridine orange, Propidium iodide (Nacalai Tesque, Japan), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Invitrogen, USA), CycleTEST^{TM} PLUS DNA Reagent Kit (Beckton Dickinson, USA). anti-p53, anti-Bax, anti-Bcl-2 antibody (Santa Cruz Biotechnology, USA), Cell Fixation & Permeabilization Kit (Flow Cytometry) (ab185917) (Abcam, USA), FAM-FLICA® Caspase-8 Assay Kit, FAM-FLICA® Caspase-9 Assay Kit (Biorad, USA)

Plant material

The galls of *Q. infectoria* (Figure 1) were purchased in 2013 from local Chinese herbal market in the city of Kota Bharu, Kelantan, Malaysia. The galls were identified based on morphology parameters such as external colour [14]. The galls were authenticated in the year 2020 by Assoc. Prof. Dr. Khamsah Suryati Mohd and a sample was deposited at the Herbarium of Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin (UniSZA) with voucher specimen number: UniSZA 00423.

Figure 1. The physical appearance of *Quercus infectoria* galls

One kg of the galls was ground into powder. The aqueous extract was prepared by 50 g of homogenised *Q. infectoria* powder in 1 L beaker containing 200 mL distilled water in a water bath for 24 hours at 50 °C. After that, the solution was filtered and frozen [15]. The aqueous extract was further freeze-dried to a obtain powder form of the extract. The extract was weighed, and percentage of yields were calculated based on equation below and stored in -20˚C until used [16].

$$\text{Extraction yield (\%)} = \frac{\text{Final weight (g)}}{\text{Initial weight (g)}} \times 100$$

Supercritical fluid extraction (SFE) was obtained

by using specific apparatus with oven extraction system, model: OV-SCF-1000 (Supercritical Technology Co., Ltd, Taiwan). We have outsourced the extraction process to the company which provided the services. Briefly, one kg of the galls was ground to a coarse powder prior to extraction. The extraction was resumed at temperature and pressure of 50.4 °C and 2508 PSI, respectively. Supercritical carbon dioxide extracts both waxes and essential oils that make up the concrete. Waxes were separated from the essential oils through subsequent process with liquid carbon dioxide at lower extraction temperature achieved in the same extractor. Lower temperature process prevents the decomposition and denaturing of compounds. The pressure is reduced to ambient temperature and the carbon dioxide reverts to a gas, leaving no residue indicating complete extraction process.

Cell culture

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

In vitro cytotoxicity assay

Cytotoxicity assay of both extracts was carried out by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction [17]. The extract was diluted in DMSO to obtain a stock solution of 10.00 mg/mL. The cells (3×10^4/mL) were treated with various concentration of extract (0-100 µg/mL) in 96-well culture plates for 72 hours. Prior to the experiment, we optimised the treatment hours of 24 and 48 hours according to usual doubling time for HeLa cells. However, no activities were recorded at 24 and 48 hours (not published). Then the treatment duration was extended to 72 hours and found the half minimal concentration of 12.33±0.35 µg/mL and 14.33±0.67µg/mL, respectively for aqueous and supercritical fluid extract (SFE).

After 72 hours of incubation, 10 µL of MTT solution (final concentration; 0.5 mg/mL) was added to each well and the cells were further incubated for another 4 hours at 37 °C. Then, 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. The IC_{50} value of each extract was calculated using non liner regression analysis (percent inhibition versus concentration) and used in subsequent experiments. A platinum based anticancer drug, cisplatin, was used as the positive control.

Cell morphology analysis

Acridine orange and propidium iodide (AO/PI) staining was used to determine the apoptosis features by morphological changes of nucleus. [18]. HeLa cells at a density of 5×10^4 cells/mL were seeded into 6 well plates and then treated with aqueous and SFE extracts for 24, 48 and 72 hours using IC_{50} concentrations in triplicates. The treated cells were trypsinized and harvested with 1 mL cold phosphate buffered saline (PBS), followed by centrifuged at 300 g 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 placed on 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 the negative control and cisplatin was used as the positive control.

Determination of phosphatidylserine externalization

Annexin V-FITC Apoptosis Detection Kit 1 (Beckton Dickinson, USA) was used in determining the phosphatidylserine externalization. The kit contains Annexin V conjugated with fluorochrome FITC, propidium iodide and binding buffer. Briefly, HeLa cells (5×10^4 cells/mL) were treated with IC_{50} concentration of aqueous and SFE extracts and cisplatin for 3, 6 and 9 hours. After completing the treatment hours, the cells were harvested, washed trice with cold PBS, followed by addition of 100 µL of binding buffer to the tubes. Then, 3 µL of FITC-conjugated Annexin V (Annexin V-FITC) and 3 µL of propidium iodide (PI) were added. The mixtures were then incubated at room temperature in the dark for 15 min. The stained

cells were diluted using binding buffer (400 µL) and were 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 representing the viable cells (Annexin V-FITC and PI negative), early apoptotic cells (Annexin V-FITC positive and PI negative), late apoptotic cells (Annexin V-FITC and PI positive) and necrotic cells (Annexin V-FITC negative and PI positive).

Cell cycle analysis

CycleTEST^{TM} PLUS DNA Reagent Kit was used to analyse the cell cycle (Beckton Dickinson, USA). The analysis was carried out according to the manufacturer's instructions. HeLa cells ($5x10^4$ cells/mL) were cultured overnight in a 6-well plate and treated with aqueous and SFE extracts and CIS for 24, 48 and 72 hours at the IC_{50} concentrations in triplicates. The cells were trypsinized and harvested in a similar manner with AO/PI staining protocol. The cells pellet was resuspended in 250 µL of buffer solution A (trypsin buffer in a spermine tetrahydrochloride). The cells were then incubated for 10 min at room temperature. A 200 µL of solution B (trypsin inhibitor and ribonuclease A) was added and further incubated for 10 min at room temperature. Then, 200 µL of solution C containing propidium iodide was added and incubated in a dark place at 4 °C for 10 min. The cellular DNA content was measured by using CytoFlex flowcytometer (Beckman Coulter, USA). Untreated cells served as the negative control and cells treated with cisplatin served as the positive control.

Expression of p53, Bax and Bcl-2 in the treated cells

The detection of protein expression p53, Bax and Bcl-2 in the treated cells were modified according to Zakaria et al. [19]. HeLa cells were seeded into a 6-well plate and incubated for 24 hours at 37 ºC with 5% CO_2 and 90% humidity prior to treatment with aqueous and SFE extracts and cisplatin for 3 hours. After that, the cells were harvested and washed trice with cold PBS. Anti-Bax, anti-Bcl-2 and anti-p53 antibody FITC and Cell Fixation & Permeabilization Kit (Flow Cytometry) were used to determine the expression of Bax, Bcl-2 and p53 proteins in treated HeLa cells. Staining, fixation and permeabilization were done following the manufacturer's protocol. Briefly, the cells were resuspended thoroughly and 100 µL of fixative solution was added into each tube and incubated for 15 min at 4 ºC. After 5 min, the cells were washed twice with PBS 0.2% and Tween 20. A 100 µL of permeabilization solution was added into each tube and incubated for 15 min at 4ºC. After 15 min, the cells were washed twice with PBS 0.2% and Tween 20. Next, the cells were blocked using 5% PBS for 15 min and washed again twice with PBS 0.2% and Tween 20. For staining, 10 µL of fluorochrome-conjugated primary antibodies was added and incubated for 20 min at 4 ºC in the dark followed by washing twice with PBS 0.2% and Tween 20. Then, cells were centrifuged at 400 g for 5 min and resuspended in 500 µL of ice-cold PBS, 10% fetal calf serum (FCS) and 1% sodium azide. Finally, the cells were analysed using flow cytometer.

Caspases analysis

Using the FAM FLICA ^{TM} Caspases Kit, the activities of caspase-8 and caspase-9, were evaluated. The analysis was carried out in accordance with the manufacturer's instructions. Initially, the FLICA ^{TM} was prepared by adding DMSO to solubilize it, diluted with PBS prior to adding into the sample solution. After 6 hours of treatment, disassociated cells from the treated and untreated cells groups of HeLa cells were harvested. The cells were centrifuged at 300 g for 10 min and then the supernatant was discarded. After that, 290 µL of fresh media was added. Next, 10 µL of FAM-FLICA working solution was added to the samples at a v/v ratio of 1:30 and mixed well in the cell suspension to disperse the FAM-FLICA reagent, then, it was incubated in CO_2 incubator at 37 ºC for 40 min. After incubation, 2 mL of 1x Apoptosis Wash Buffer was added and gently mixed, then the cells were centrifuged at 300 g for 10 min. These steps were repeated twice. Then, 300 µL of Apoptosis Wash Buffer was added and placed on ice before proceeding to the flow cytometer for analysis. The assay was carried out in three independent replicates for each sample. The data were quantitatively represented as percentage activity of caspase-8 and caspase-9 in comparison to the untreated cells group.

Statistical analysis

The results were obtained from three independent experiments. Data were expressed as the mean ± standard error of the mean (SEM) and analysed by 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 the control (untreated cells).

Results and Discussion

The yield aqueous extract was 35.5%, while the yield obtained by supercritical fluid extraction was 0.09%.

The organoleptic properties of *Quercus infectoria* galls revealed obvious characteristics for galls identification (Table 1).

The cytotoxic effect of the extracts and cisplatin was determined at the concentration that induced 50% inhibition of the HeLa cells growth in triplicates. Both extracts reduced HeLa cells growth (Figure 2) at IC_{50} of 12.33±0.35µg/mL and 14.33±0.67µg/mL for the aqueous and SFE extracts, respectively (Table 2). However, both extracts did not exert any cytotoxic effect on Vero cells, proving its cyto-selective properties.

Morphological changes determine mode of cell death of treated Hela cells. Staining with AO/PI (Figure 3) revealed the state of cell death morphology with difference color appearance. Viable cells (V) stained green with intact nucleus structure, early apoptotic cells (E) stained green with condensed nuclear structure, cell shrinkage and formation of apoptotic bodies while late apoptotic cells (L) stained bright orange with areas of condensed chromatin in the nucleus. Conversely, necrotic cells (N) stained with uniform red color.

Apoptotic cells were detected using flow cytometry and annexin V/PI analyses in a time-dependent manner. Figure 4 depicts the distribution of HeLa cells over four quadrants. Q1 (An-, PI+) represents necrosis, Q2 (An+, PI+) represents late apoptosis, Q3 (An-, PI-) represents viable cells, and Q4 (An+, PI-) represents early apoptosis. Most of the cells are viable in the untreated groups, with intact nucleus in which all the plots seen are in Q3. 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 the HeLa cells were treated with AQ, SFE and CIS, the plots are dispersed into quadrant 1, 2, and 4. In all the three quadrants (Q1, Q2, and Q4), the percentage of cells in early apoptosis, late apoptosis, and necrosis increased significantly ($p<0.05$) with longer treatment durations of 3, 6, and 12 hours compared to UT cells. These time points (3, 6 and 12 hours) were chosen because the externalization of phosphatidylserine from the inner layer of the plasma membrane to the outer surface occur at early event of apoptosis. Apoptosis can be initiated and completed in as quickly as 2–3 hours. Thus, if the assay is performed too soon or too late, a false negative result can be observed [20]. In this study, all treatment groups indicated the initiation of cell death as early as 3 hours of after treatments.

Table 1. Organoleptic properties of *Quercus infectoria* galls

No	Characterics	Result
1	External colour	Dark yellowish brownish
2	Odour	Astringent
3	Size	Small (diameter 1-1.5 cm)
4	Surface	Rough and horny
5	Texture	Hard and woody

Table 2. IC_{50} of extracts and cisplatin on HeLa and Vero cells

	IC_{50} (µg/mL)	
Treatments	**HeLa**	**Vero**
Aqueous extract	12.33 ± 0.35	-
SFE extract	14.33 ± 0.67	-
Cisplatin	1.85 ± 0.15	14.33 ± 0.88

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

After 3, 6, and 12 hours of aqueous extract treatment, the population of early apoptotic cells (Q4) was 1.26±0.02%, 3.58±0.07%, and 2.4±0.06%, respectively. Similarly, the percentage of late apoptotic cells gradually increased with longer treatment period but then slightly decreased at 12 hours with 1.9±0.03%, 9.38±0.25% and 5.51±0.2%, respectively. The trend of apoptotic cells in HeLa cells treated with SFE, were similar to aqueous extract treated cells for longer treatment period (Figure 5). Meanwhile, the percentage of viable population (Q3) for both aqueous and SFE extract treated cells decreased significantly ($p<0.05$) after 3, 6, and 12 hours of treatment. Hence, the overall changes in the population of cells revealed the capability of extracts to cause cell death in HeLa cells at early stage of apoptosis.

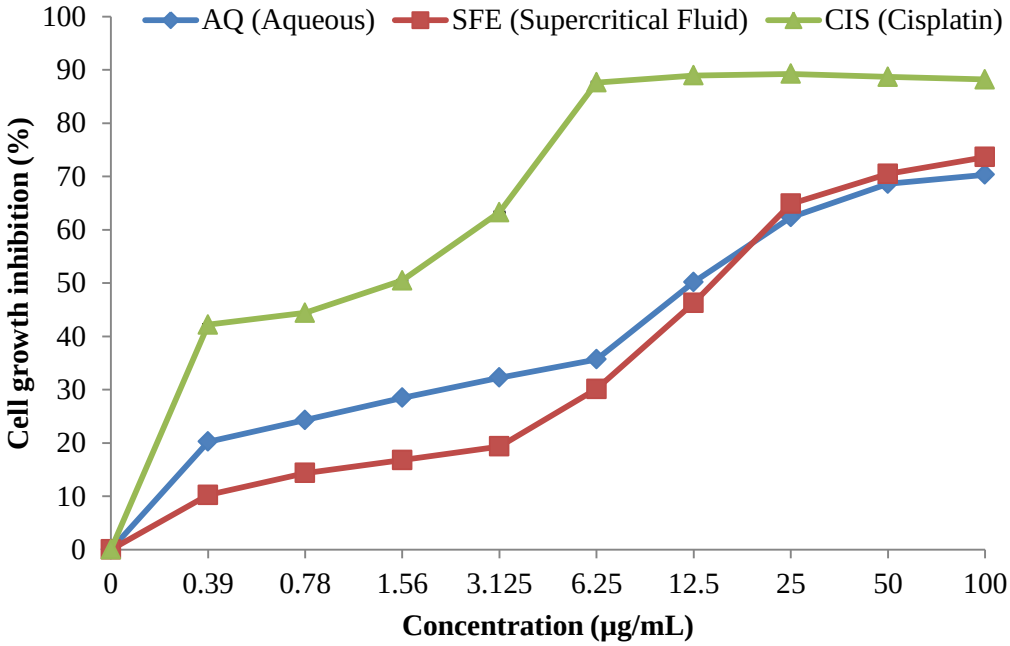

Figure 2. Cytotoxic effect of SFE, AQ extracts of *Quercus infectoria* and CIS on HeLa cell line; the cells were treated with various concentrations of extracts and cisplatin (0-100 µg/mL) in 96-well culture plates for 72 hours. The results were expressed as mean of IC_{50} concentration of three independent experiments.

Figure 6 shows cell cycle distribution of HeLa treated with IC_{50} concentration of aqueous and SFE extract and cisplatinat 24, 48, and 72 hours. The sub G0 population for aqueous and SFE extract and cisplatin increased significantly ($p<0.05$) throughout treatment periods compared to untreated cells in a time dependent manner. At 24 hours post aqueous extract treatment, the sub G0 population was 5.76±0.24%. After 48 and 72 hours, the population increased to 11.47±0.3% and 29.11±0.41%, respectively (Figure 7). Meanwhile, the sub G0 population for SFE increased further from 24 to 48 and 72 hours with the mean percentage of 6.13±0.05% to 15.3±0.16% and 35.85±.0.16%. A similar increment of sub G0 cell population was also observed in cisplatin treated cell for longer treatments duration. The increase in sub G0 population reveals the induction of apoptosis, as sub G0 peak is reported to be a quantitative apoptosis marker. Meanwhile, the stage of G0/G1 and G2/M phase decreased for longer treatment duration suggesting that the cell population entered the cell cycle phase. The expression of p53, Bax and Bcl-2 in HeLa cells treated with aqueous and SFE extracts and cisplatin were analysed by flow cytometry analysis. The current study analysed individual expression of p53, Bax and Bcl-2 protein in the treated HeLa cells, without taking into account Bax/Bcl-2 expression ratio. On the other hands, the experiment protocol used is only applicable for qualitative evaluation [21].

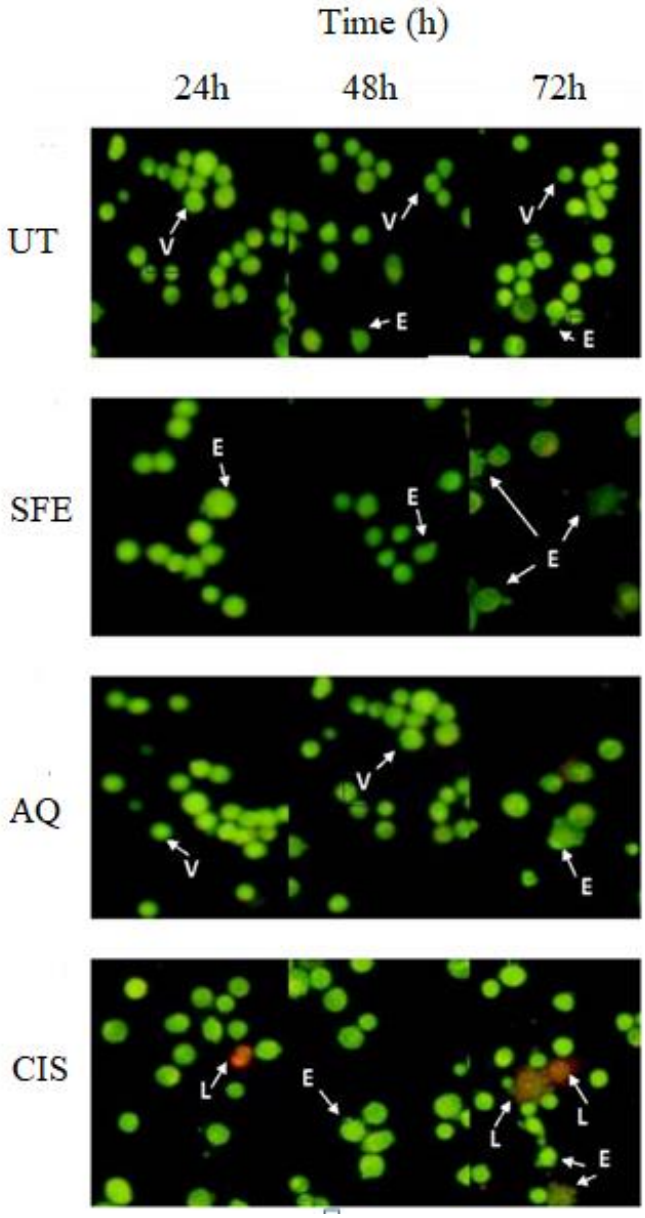

Figure 3. AO/PI staining of Hela cells under a fluorescence microscope at 24, 48 and 72 hours. V: viable cell; E: early apoptotic cells; L: late apoptotic cells; N: necrotic cells. The cells were treated with IC_{50} concentration of aqueous extract (AQ) 12.33 µg/mL, supercritical fluid (SFE) extract, 14.33 µg/mL and cisplatin (CIS), 1.85 µg/mL. The untreated cells (UT) served as negative control.

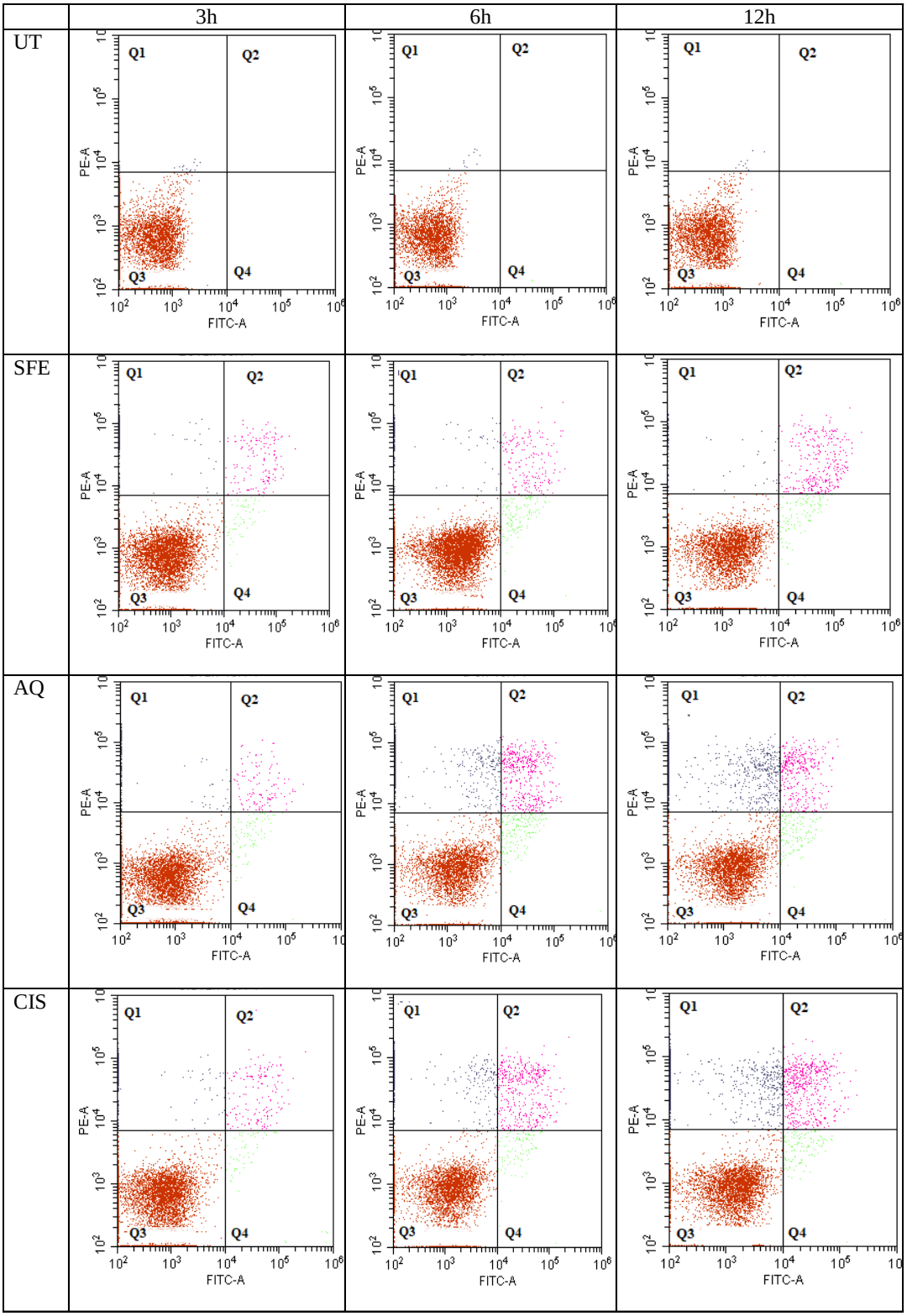

Figure 4. Annexin V(AV) / propidium iodide (PI) staining of HeLa cells represent untreated (UT), treated with aqueous extract (AQ), supercritical fluid extract (SFE), and cisplatin (CIS) at 24, 48 and 72 hours. IC_{50} concentration of AQ (12.33 µg/mL), SFE (14.33 µg/mL) and CIS (1.85 µg/mL) were used to execute the treatments. The Y-axis indicates the PI-labeled population, whereas the X-axis indicates the FITC-labeled 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).

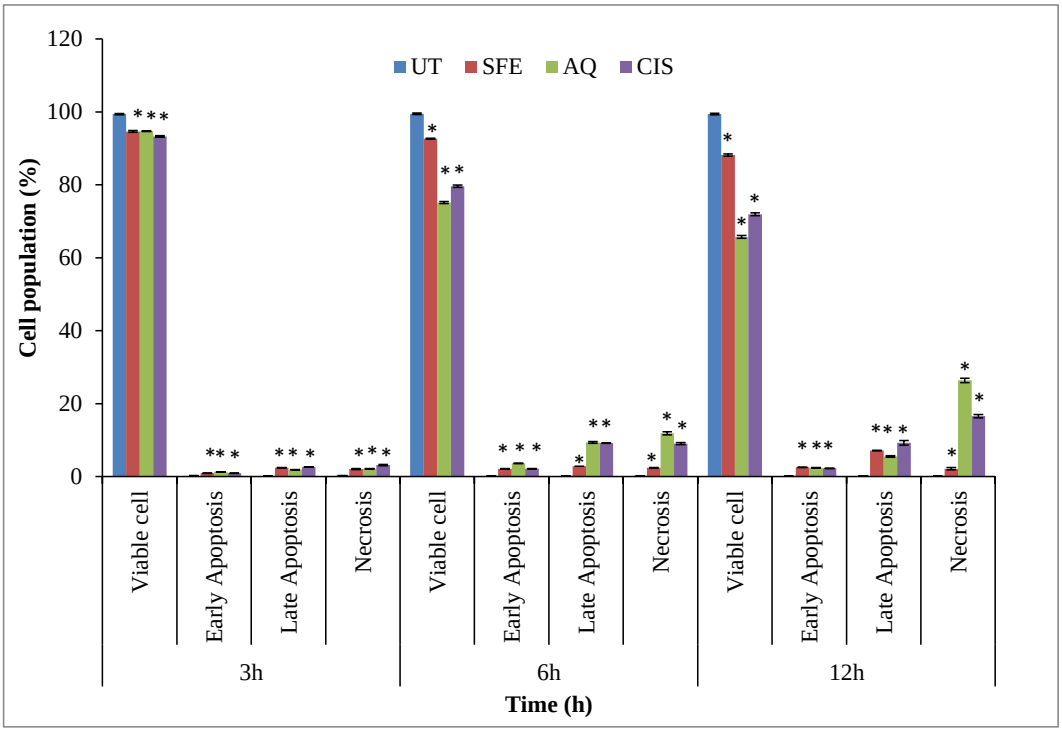

Figure 5. Percentages of HeLa cell population after treatment with aqueous extract (AQ), supercritical fluid extract (SFE) and cisplatin (CIS) at 3, 6, and 12 hours detected by apoptosis assay Annexin V-FITC; values are mean±SEM (n=3); * indicates significant value ($p<0.05$) as compared with untreated group (UT). The cells were treated with AQ, SFE and CIS at IC_{50} concentration of 12.33 µg/mL, 14.33µg/mL and 1.85 µg/mL respectively.

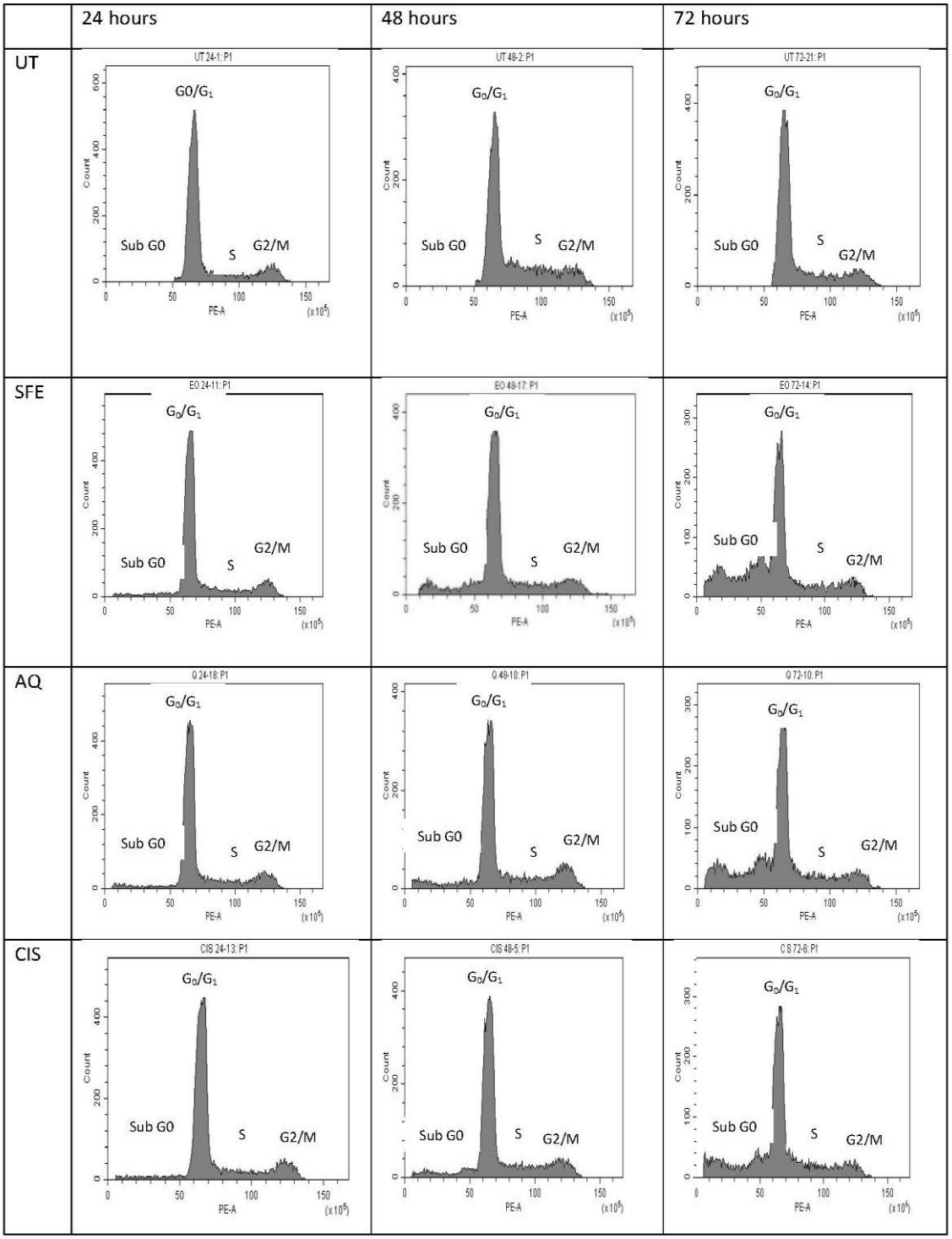

Figure 6. Cell cycle distribution of HeLa cells treated with aaqueous extract (AQ) and supercritical fluid extract (SFE) and cisplatin (CIS) as the positive and untreated cells (UT) as the negative controls at 24, 48 and 72 hours of treatment. The cells were treated with AQ, SFE extracts and CIS at IC_{50} concentration of 12.33 µg/mL, 14.33 µg/mL and 1.85 µg/mL respectively.

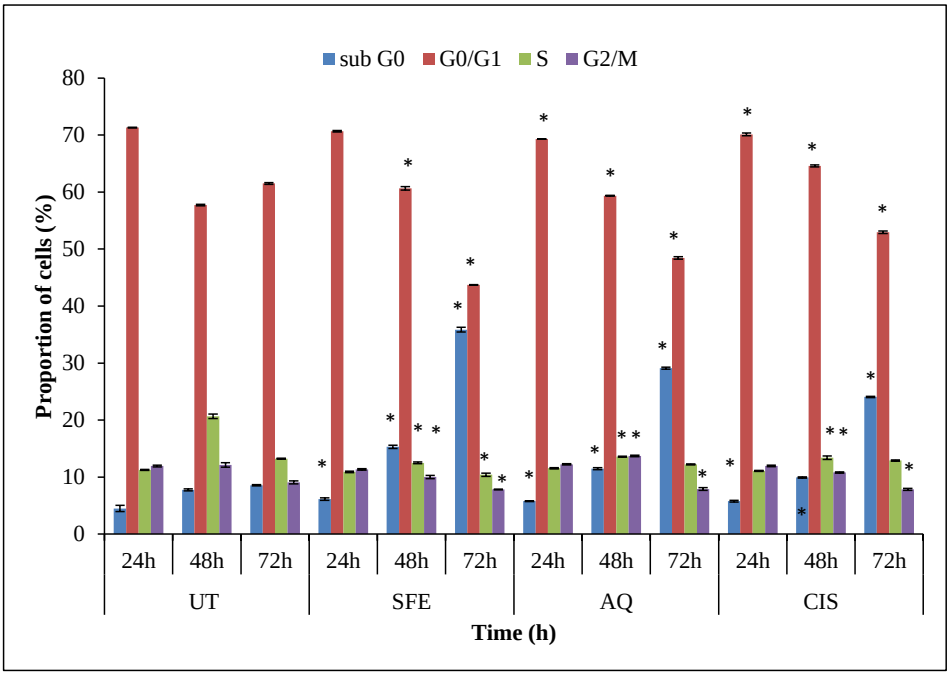

Figure 7. Percentage of cell cycle distribution of HeLa cells treated with IC_{50} concentration of aqueous extract (AQ) (12.33 µg/mL), supercritical fluid extract (SFE) (14.33 µg/mL) and cisplatin (CIS) (1.85 µg/mL) at 24, 48 and 72 hours. Cisplatin served as positive control and untreated (UT) cells served as negative control. Values are expressed as mean ± S.E.M of triplicate. * indicates a significant value ($p<0.05$) as compared with untreated group (UT).

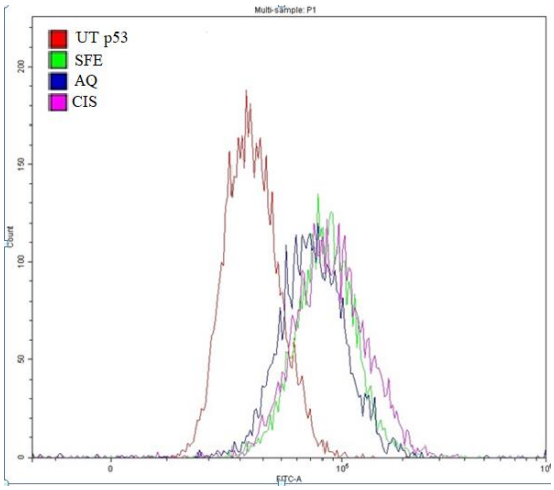

Figure 8. The expression of tumor suppressor protein p53 in HeLa cells after treatment with IC_{50} concentration of aqueous extract (AQ) (12.33µg/mL), supercritical fluid extract (SFE) (14.33 µg/mL) and cisplatin (CIS) (1.85 µg/mL) at 24, 48 and 72 hours.

Based on Figures 8 and 9, all treated groups showed expression of p53 and Bax proteins as the pattern of expression slightly shifted the cells population to the right compared to untreated cells. The trend of Bax expression in untreated cells, aqueous and SFE extracts and cisplatin are quite similar. On the other hand, the Bcl-2 proteins were not expressed in all treated groups (Figure 10).

These finding suggested that aqueous and SFE extracts and cisplatin induced apoptosis by enhancing the pro-apoptotic expressions of p53 and Bax while inhibiting the anti-apoptotic protein expression of Bcl-2.

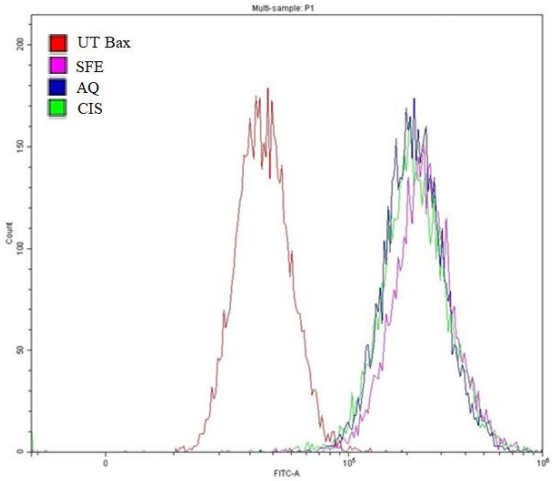

Figure 9. The expression of Bax in HeLa cells after treatment with IC_{50} concentration of aqueous extract (AQ) (12.33 µg/mL), supercritical fluid extract (SFE) (14.33 µg/mL) and cisplatin (CIS) (1.85 µg/mL) at 24, 48 and 72 hours

For each sample, three independent replicates of the caspases analysis were performed. No expression was observed in the untreated cells (Figure 11).

After 6 h of treatment with aqueous and SFE extracts and cisplatin, the expression of caspase-8 and caspase-9 increased from 0.3% to 26.54%o, 56.58%, and 54.18% for caspase-8, respectively, and 39.02% 45.00% and 52.30% for caspase-9, respectively. From the results, it shows that aqueous extract was capable to induce expression of both caspases compared to SFE.

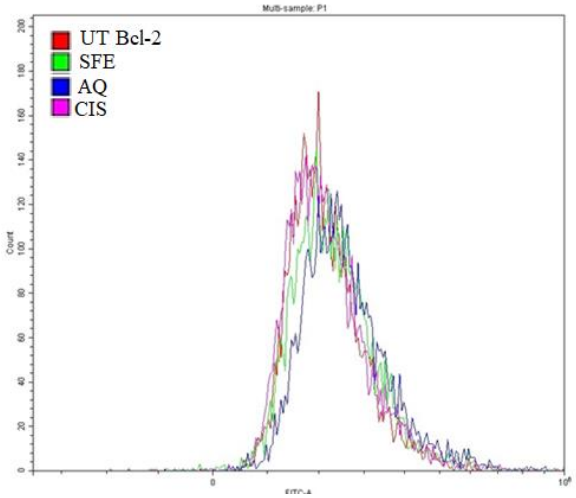

Figure 10. The expression of Bcl-2 in HeLa cells after treatment with IC_{50} concentration of aqueous extract (AQ) (12.33 µg/mL), supercritical fluid extract (SFE) (14.33 µg/mL) and cisplatin (CIS) (1.85 µg/mL) at 24, 48 and 72 hours.

The IC_{50} values of aqueous and SFE extracts on HeLa cells were less than 20 µg/mL indicating its potential as anticancer agent as previously reported [12,19]. The results showed an increasing trend in percentage of HeLa cell growth inhibition following higher concentration of aqueous and SFE extracts treatment on HeLa cells. The aqueous extract showed greater anti-proliferative effect compared to SFE extracts. Both extracts were cytoselective towards HeLa cells without affecting the growth of normal cells, Vero.

AO/PI staining indicated the morphological changes of HeLa cells as hallmark of apoptosis in the cells treated with extracts and cisplatin. According to Kerr et al., the morphological changes of apoptotic cells include chromatin aggregation, nuclear and cytoplasmic condensation, and partition of cytoplasmic and nucleus into membrane-bound vesicles [22]. The cells treated with aqueous and SFE extracts, at different time intervals exhibited viable cells with intact nucleus structure and apoptotic morphological changes like early apoptotic cells with condensed nuclear structure, cell shrinkage and formation of apoptotic bodies while late apoptotic cells showed areas of condensed chromatin in the nucleus as reported previously [23]. Phosphatidylserine externalization is a typical characteristic of early phase apoptosis [21]. During apoptosis, phosphatidylserine is translocated and externalized on the outer surface of cell membrane that enables binding to annexin V. The propidium iodide stains late-apoptotic or necrotic cells with impaired cell membrane integrity. In this study, the results of AO/PI staining confirmed phosphatidylserine externalization in HeLa cells treated with both aqueous and SFE extracts, showing cell death through apoptosis. However, after treatment with aqueous extract during 3-6 hours, the percentage of cell necrosis increased suggesting that aqueous extract displayed similar pathway with cisplatin which induced cell death through both apoptosis and necrosis as reported by previous research [24,25].

The activation of cell cycle arrest can control cell cycle progression which can be an appropriate approach for cancer therapy as cell cycle progression governs cell proliferation [26]. In this study, there was significant accumulation of cells in the sub G0 phase, along with the decrease in the percentage of G0/G1, S, and G2/M phases compared to untreated cells. The cell arrest occurs at the checkpoint of sub G0 phase to prevent the entry of damaged cells into the G0 phase and allowing for cell repair which then inhibits the damaged cell cycle progression [26]. Both extracts exerted potential apoptosis inducing effects.

Wild-type p53 is another important feature involved in cell arrest regulation, and p53 mutations, deletions, or lack of normal function may be the underlying cause of numerous types of cancers [26]. In this study, p53 expression was observed for aqueous and SFE extracts, which is likely due to their effects in restoring p53 expression in the HeLa cell line. Bcl-2 also plays significant role as an apoptosis cell death regulator while pro-apoptotic Bax promote cell death [27].

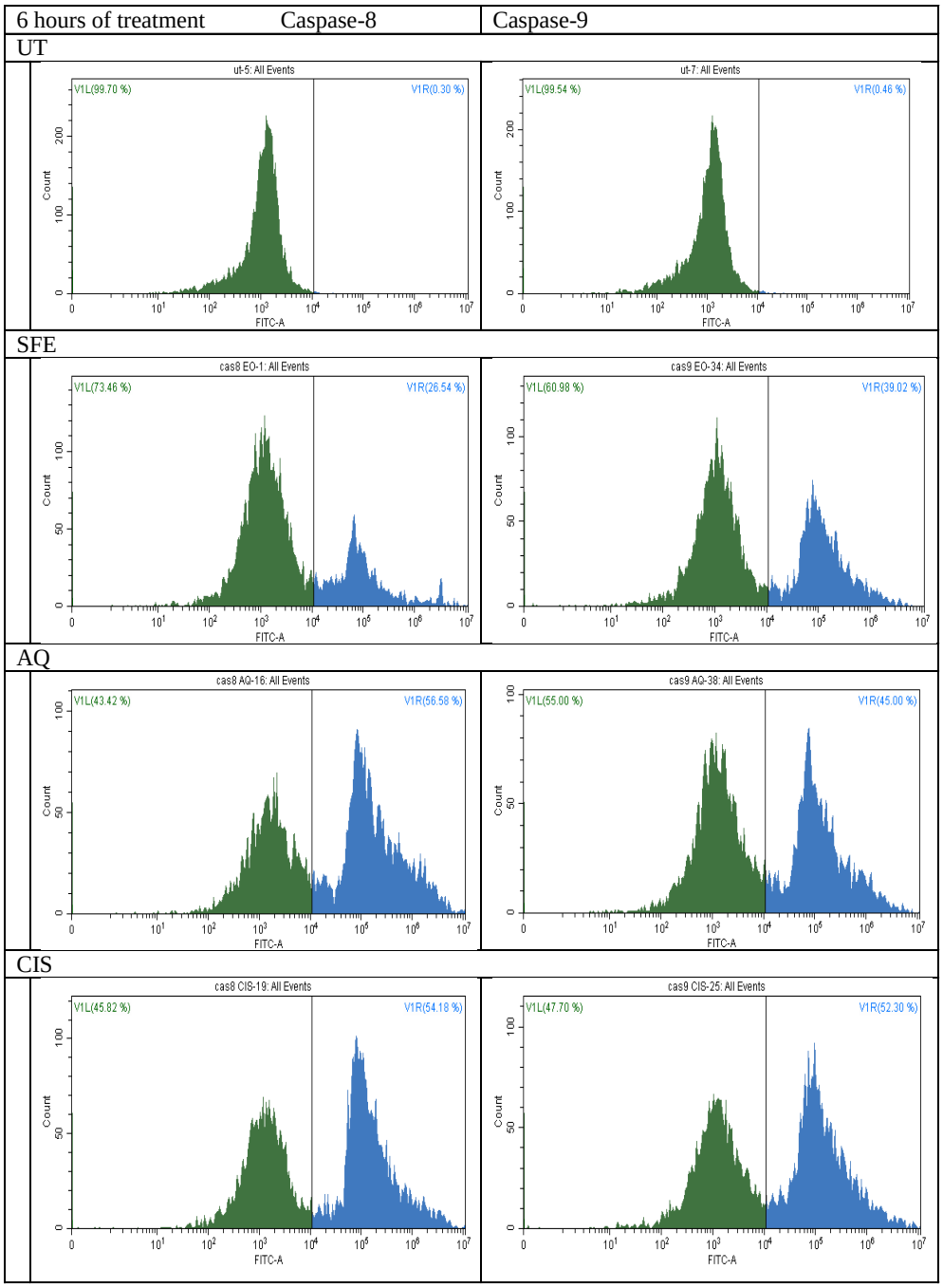

Figure 11. The expression of caspase-8 and caspase-9 in HeLa cells after treatment with IC_{50} concentration of aqueous extract (AQ) (12.33 µg/mL), supercritical fluid extract (SFE) (14.33 µg/mL) and cisplatin (CIS) (1.85 µg/mL) at 24, 48 and 72 hours.

In this study, p53 expression was observed for aqueous and SFE extracts, which is likely due to their effects in restoring p53 expression in the HeLa cell line. Bcl-2 also plays significant role as an apoptosis cell death regulator while pro-apoptotic Bax promote cell death [27]. Obviously, the treatments of aqueous and SFE extracts on HeLa cells utilise the apoptotic cell death mechanism pathway. Apart from that, caspases are produced as inactive zymogens in most cells and are closely related to apoptosis. As reported, caspase-8 and caspase-9 are active initiator caspases and can often activate other downstream caspases termed executioner caspases (caspase-3, -6, -7), thus causing caspase cascade, which strengthens the apoptotic signaling pathway

resulting in cell death [28]. Activity of caspase-8 and caspase-9 involved in the extrinsic and intrinsic apoptotic stimuli were expected in the treated HeLa cell line as previously reported [23]. The aqueous extract exhibited higher elevated levels of caspase-8 and caspase-9 compared to SFE. However, both extracts could induce expression of caspase-8 and caspase-9 to promote cell apoptosis and have potential for effective treatment of tumors.

Conclusion

In conclusion, aqueous and SFE extracts from *Quercus infectoria* showed cytotoxic effects towards cervical cancer cell line. Morphological changes revealed the apoptotic hallmarks of the treated cells at first 48 hours. The phosphatidylserine externalization indicated the apoptosis pathway in the cells treated with aqueous and SFE extracts. In addition, SFE halted the cell cycle progression better than aqueous extract through the activation of cell cycle arrest at sub G0 phase. The expression of pro-apoptotic protein p53, Bax and the activation of caspase-8 and caspase-9 indicated that the extracts could induce apoptosis via both extrinsic and intrinsic pathway. The bioactive markers of aqueous and SFE extracts need to be explored to rule out the phytochemical constituents responsible for cytotoxic activity and cell death of HeLa cells.

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Author contributions

Hasmah Abdullah was involved in idea, concept of the research design and manuscript writing; Illyana Ismail conducted the experiments and data analysis; Rapeah Suppian gave advise on the study design and data analysis of flowcytometry experiments.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

HeLa: human cervical cancer cells; Vero: African Green Monkey kidney cells; DMEM: Dulbecco's modified Eagle's medium; Annexin V-FITC: Annexin V-Fluorescein isothiocyanate; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ATCC: American type culture collection: DMSO: dimethylsulfoxide; AO/PI: acridine orange and propidium iodide; PBS: phosphate buffer saline; FBS/FCS: fetal bovine /calf serum; SEM: standard error mean; ANOVA: one-way analysis of variance; SPSS: statistical package for the social sciences; An: Annexin; h: hour; UniSZA; Universiti Sultan Zainal Abidin.