Natural Luteolin from Methanolic Extract of Malaysian *Brucea javanica* Leaves Induces Apoptosis in HeLa Cell Lines

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ABSTRACT: There are a growing number of deaths of cancer patients due to toxicity of conventional chemotherapy. Therefore, continuous discovery of natural anticancer compounds with cytoselective actions would be an ideal strategy to overcome the problem. Thus, natural products from plant are still the alternative source in the search for anticancer drugs that can have a direct effect on both cancer cell removal and also minimize the side effects to the patients. Based on the traditional usage and pharmaceutical potential of *Brucea javanica* reported before, a study on the leaves of this plant was carried out. The aims of the study were to isolate the bioactive compound from *B. javanica* Leaves (BJL) extracts *via* bioassay-guided fractionation using several selected cancer cell lines, to determine the mode of cancer cell death induced by BJL's active compound and the molecular mechanism of apoptosis implicated in cancer cell lines by measuring the level of apoptotic protein expression such as bax, bcl-XL, caspase-3 and tumour suppressor p53. Among three crude extracts of BJL, methanol was the most potent against selected cancer cell lines which consist of cervical, breast, bone, ovarian and liver cancer cells. Cisplatin was used as positive control for the antiproliferative assay. Using a bioassay-guided fractionation, chromatography, NMR and mass spectrometry analysis we have isolated luteolin. It is a known compound from the flavonoid group which was found to be cytoselective. The IC_{50} value for HeLa is 8.02 μg/ml while for Vero is >99 μg/ml. Hoechst 33258 staining and flow cytometric analysis of Annexin V-FITC staining revealed that luteolin induces apoptotic cell death in cervical

cancer cell HeLa. Flow cytometric analysis also showed that luteolin induces apoptosis by increasing the p53, bax and caspase-3 protein expression.

Keywords: Anticancer activity, *Brucea javanica*, luteolin, methanolic extract, apoptosis

Introduction

Brucea javanica (L.) Merr. (BJ) is a shrub from family Simaroubaceae and it is known as 'lada pahit' among Malaysian due to its bitter taste. Its living locality is distributed from Southeast Asia to Northern Australia (Kamperdick *et al*., 1995; Kim *et al*., 2003). This plant has been used as traditional remedies for various types of ailments and a part of its therapeutic properties has been proven scientifically. Compounds from BJ have been shown to have various biological activities such as cytotoxic and antileukemic (Lee *et al*., 1979; Lee *et al*., 1984; Cassady and Suffness, 1980; Sakaki *et al*., 1986; Anderson *et al*., 1991; Fukamiya *et al*., 1992; Luyengi *et al*., 1996; Su *et al*., 2002; Kim *et al*., 2004), antimalarial (O'Neill *et al*., 1987), amoebicidal (Wright *et al*., 1988), anti-protozoan (Sawangjaroen and Sawangjaroen, 2005), anti-HIV (Okano *et al*., 1996), anti-inflammatory (Hall *et al*., 1983) and antibabesial (Subeki *et al*., 2007) effects. Chemical constituents reported from BJ included quassinoids (Polonsky *et al*., 1980; Kim *et al*., 2003; Kim *et al*., 2004), quassinoid glycosides (Lee *et al*., 1979; Sakaki *et al*., 1986; Fukamiya *et al*., 1992), apotirucallane-type triterpenoids, lignan (Luyengi *et al*., 1996), alkaloids (Liu *et al*., 1990) and alkaloid glycoside (Kitagawa *et al*., 1994).

As there are a growing number of deaths of cancer patients due to toxicity of conventional chemotherapy, therefore, the cytoselectivity actions of natural anticancer compounds would be an ideal strategy to combat the disease. Previous investigations on the chemical constituents of BJ have focused mainly on the fruits and seeds. Research on other part of this plant is limited, especially the leaves. Moreover, the type of active compounds from BJ leaves (BJL) that originated from Malaysia is still not known. This is because the constituents of compounds might vary according to different locality, due to different climate, latitude, longitude, soil pH and soil texture (Amaral *et al*., 2010; Oloumi and Hassibi, 2011).

Materials and Methods

Plant material

B. javanica leaves were collected from Kampung Nyok Kertau, Chenor, Maran, Pahang, Malaysia, specifically along the riverside of Sungai Pahang. BJL were authenticated by Mr Shunmugan, from the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia where voucher specimen (USM 11213) has been submitted for future references. The leaves were washed and dried in a drying oven overnight at $50^{O}C$, and were ground into powder.

Cell lines

The cell lines used for antiproliferative assay are U87 (human glioma cells), T24 (human bladder cancer cells), HeLa (human cervical cancer), SmGM2 (normal human bladder smooth muscle cells) and Vero (non-cancer of monkey kidney epithelium cell). All types of cells were obtained from American Type Culture Collection (ATCC), Virginia, USA.

Isolation of luteolin

Luteolin was isolated through bioassay-guided fractionation. Briefly, 800 grams of BJL powder was extracted successively using petroleum ether, methanol and water. The extracts from each solvent were tested for cytotoxicity towards several cancer cell lines (data not shown). The most active extract which is methanol, was subjected to vacuum liquid chromatography (VLC). The fractions from VLC were tested again on cervical cancer cell lines HeLa because this cell lines is the most sensitive towards methanol extract. The active VLC fractions were then pooled and subjected to silica gel column chromatography. The first pure compound managed to be isolated was a yellowish powder, and determined as luteolin through nuclear magnetic resonance (NMR) and mass spectrometric analysis; which were carried out using a Bruker (Avance) 600 MHz machine and Orbitrap HRESI mass spectrometer respectively.

Cell viability assay

Antiproliferative activity was measured by methylene blue assay which was carried out 3 days after the extract and cisplatin treatment. First, 22.5 µl of gluteraldehyde were added into each wells and was left on shaker for 15 min. Gluteraldehyde functions to fix the cell on the surface of the wells by acting on live cell membrane. Then gluteraldehyde and medium in the well were discarded. The microtiter plate was washed with 0.15 M NaCl solution 3 times to wash away dead cells, and then air-dried. Next 100 µl of 0.05% (w/v) methylene blue was added to each well and the plate was left on shaker for 15 min. The methylene blue was discarded and the plate was washed again with 0.15 M NaCl 3 times to wash away excess dye. After that, each well of the microtiter plate were added with 198 µl 0.33 M HCl and the microtiter plate was left on shaker for 15 min in order to get colour elution. Lastly, the plate optical density (OD) was read using ELISA reader at 655 nm to determine the amount of live cells remained in the wells. The color intensity of methylene blue is directly proportional to the number of living cells.

The relative viability of the treated cells as compared to the control cells was expressed as the percentage of live cells. IC_{50} value was determined from a graph of percentage of live cells versus log10 concentration (mg/ml) of extract. Percentage of control living cells was determined from the formula below:

$$\text{Percentage of living cells} = \frac{\text{OD treatment}}{\text{OD negative control}} \times 100\%$$

Apoptotic cell detection

Hoechst 33258 nuclear staining assay

The treated and untreated cells (0, 24, 48 and 72 hours, using the IC_{50} concentration of compound and cisplatin were trypsinized and centrifuged at 800 g to get the pellet which then washed with PBS. The cells were smeared onto microscope slides then fixed with

4% paraformaldehyde in PBS for 30 minutes at 4°C. After washing, the cells were incubated in Hoechst 33258 (Sigma Aldrich, St. Louis, USA) at a final concentration of 30µg/ml at room temperature (RT) for 30 minutes. Nuclear morphology was then examined under a fluorescent microscope (Hishikawa *et al*., 1999).

Apoptotic cells detection by flow cytometry

The number of apoptotic cell death induced by BJL's active compound was measured by flow cytometry using the BD Pharmingen™ Annexin V-FITC, BD 556420 kit. The treated and untreated cancer cells were harvested and washed with cold PBS. The cell pellet was re-suspended in 1x binding buffer at a concentration of 1 x 10^{6} cells/ml. 100 µl of cell suspension was transferred into a tube. 5 µl of Annexin V-FITC and 5µl of propidium iodide were added and incubated for 15 minutes at room temperature in the dark. An additional 400 µl of 1x binding buffer was added to each tube. Samples were analysed by Beckman-Coulter FACS Calibur-500 flow cytometry within 1 hour. 100,000 events was acquired using green channel FL1 for Annexin V-FITC and the red channel FL3 for PI.

Apoptotic protein detection

Cells were cultured in 25 cm^{2} culture flasks (5% CO2, 37°C) at seeding density of 5 x 10^{4} cells/ml. Plates were incubated overnight before the solutions of compound and cisplatin were added to each well. After 24, 48 and 72 hours of exposure, cells were harvested by trypsinization with trypsin/EDTA solution and centrifuged at 1500 rpm. Cells were washed twice with 1X PBS and fixed with ice-cold 70% ethanol for 1 hour at 4°C, then washed twice with 1X PBS and re- suspended in blocking buffer (2% BSA) for 10 minutes, followed by another washing step. Cell pellet was re-suspended in PBS to a concentration 1 x 10^{7} cells/ml and 100 µl of cells suspension (1 x 10^{6} cells/ml) were transferred into each sample tube and 20 µl of FITC/PE conjugated antibody were added into tubes followed by gently mixing. The antibodies used in this experiment are FITC-

conjugated Bcl-XL, PE-conjugated Bax and PE-conjugated p53; all were purchased from Santa Cruz Biotechnology, Inc. The tubes were incubated at RT for 20-30 minutes in the dark. The pellets were washed with 2 ml of 1X PBS and the supernatant were discarded. Cell pellets were re-suspended in 500 µl of 1X PBS and sent to flow cytometric analysis.

Results

Isolation of luteolin

The luteolin was obtained from column and thin layer chromatography techniques (Figure 1). The pure fractions were pooled and analyzed using nuclear magnetic resonance (NMR) (Figure 2).

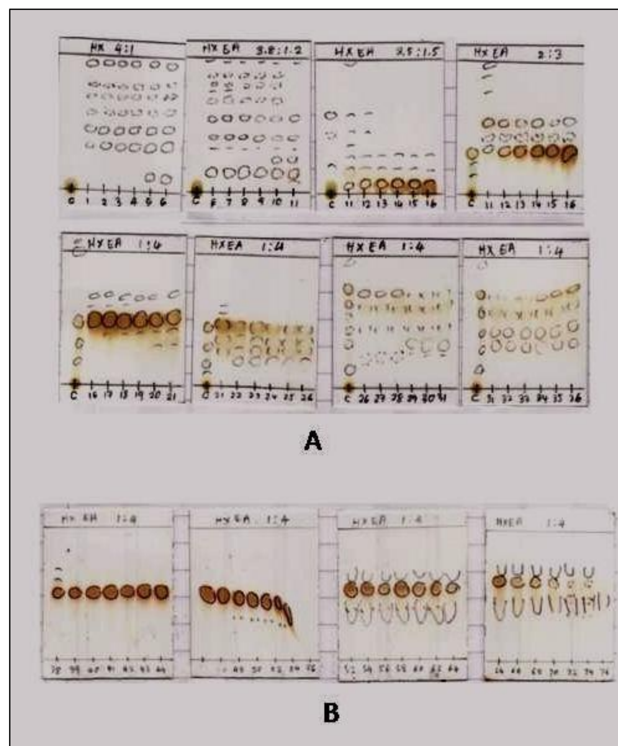

Figure 1: TLC profiles of fractions from column chromatography. (A) Fractions from first column; fraction with obvious yellow spot (13 to 21) were pooled and run to next column to get purer fractions. (B) Second silica gel column showed pure fractions; fraction 38 to 46.

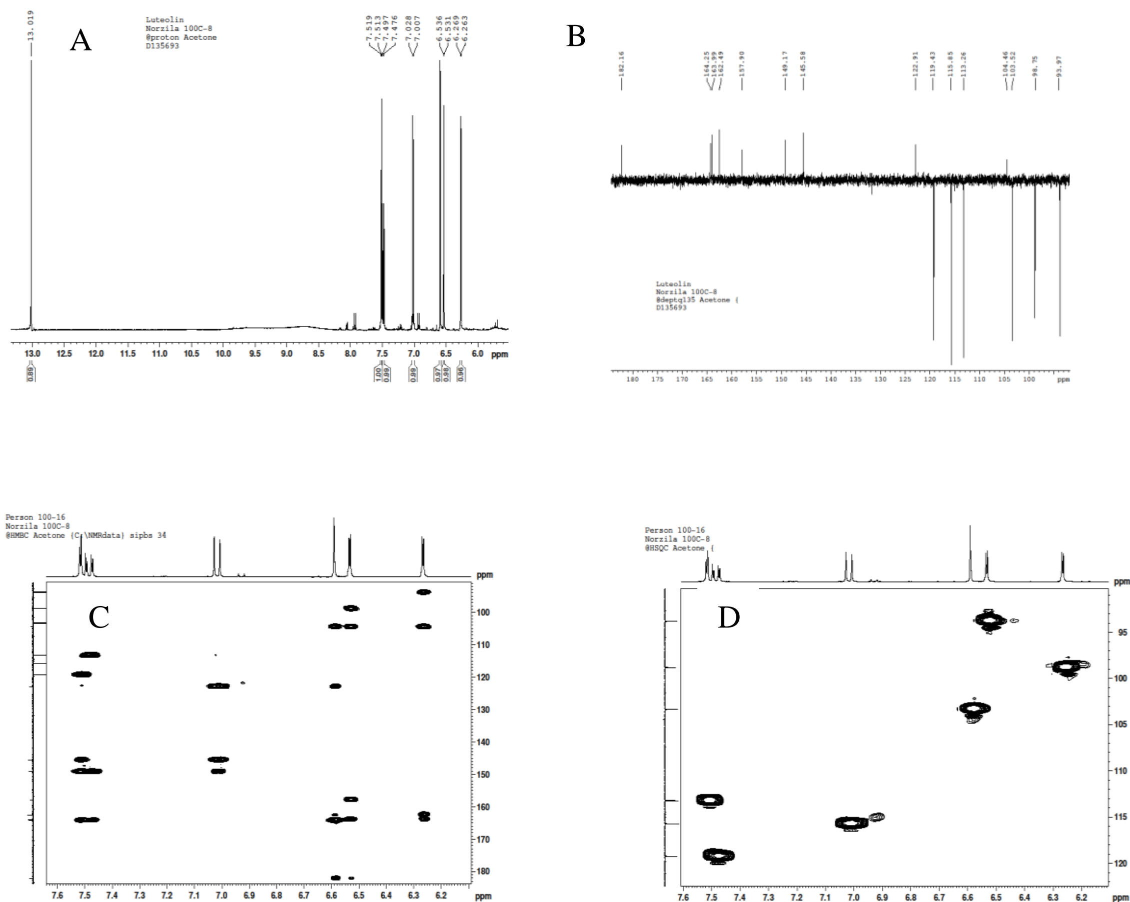

Figure 2: A: Spectrum of ^{1}H NMR (400 MHz, $(CD_3)_2CO$, JS-3). A highly de-shielded hydroxyl proton can be seen at δ13.02 ppm, indicating the typical 5-OH of a flavone. B: Spectrum of ^{13}C NMR (100 MHz, $(CD_3)_2CO$, JS-3). 15 carbon atoms can be seen in the spectrum, showing one carbonyl at 182.2 ppm, together with other 12 aromatic carbons, one methine at 103.4 ppm and one quaternary carbon at 164.25 ppm. C: Spectrum of HMBC NMR (400 MHz, $(CD_3)_2CO$, JS-3). This experiment is four times more sensitive that supports the ^{13}C NMR. It is for highly substitute compound which lack sufficient protons to 'track' the carbons. D: Spectrum of HSQC NMR (400 MHz, $(CD_3)_2CO$, JS-3). The cross-peaks show protons and carbons that are directly connected to each other.

Figure 3 showed the molecular structure of luteolin obtained after NMR analysis.

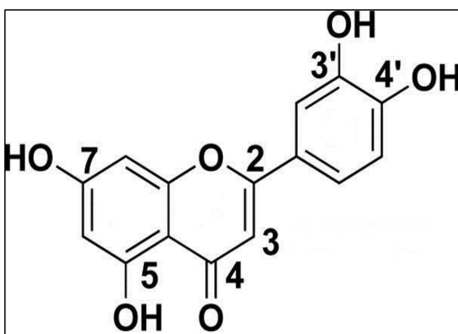

Figure 3: Molecular structure of luteolin

Antiproliferative activity of luteolin

The IC50 values for antiproliferative effect of luteolin towards selected cancer cell lines and normal cells are summarized in Table 1. The results showed that luteolin from BJ have cytoselective effect on cancer cells.

Table 1: The IC_{50} values of luteolin and cisplatin for selected cancer and non-cancer cell lines.

Cell types	IC_{50} values (µg/ml)	
	Luteolin	Cisplatin
HeLa	8.02 ± 0.91	73.95 ± 4.88
T24	8.33 ± 0.85	>99
U87	10.81 ± 1.01	2.20 ± 0.36
SmGM2	59.19 ± 5.97	>99
Vero	>99	24.64 ± 2.59

Nuclear morphology of luteolin-induced apoptotic cell death in HeLa cells

DNA fragmentation is the hallmark of apoptosis cell death. To asses this, the morphological changes in nuclear of HeLa cells treated with luteolin (8µg/ml) was further examined using Hoechst 33258. This fluorescent dye binds to AT-rich regions of DNA, allowing it to be used to detect DNA as well as a fluorescent stain for chromosomes (Latt and Stetten, 1976; Portugal and Waring, 1988). Figure 4 showed fragmentation and condensation of chromatin in HeLa cells treated with 8µg/ml luteolin for 24, 48 and 72 hours. The untreated HeLa cells showed no fluorescence in the nucleus, indicating the absence of DNA fragmentation. While in treated cells, the density of fluorescence was increased relatively according to the time of incubation. The fragmented of DNA can be seen as small fluorescent spots in the nucleus.

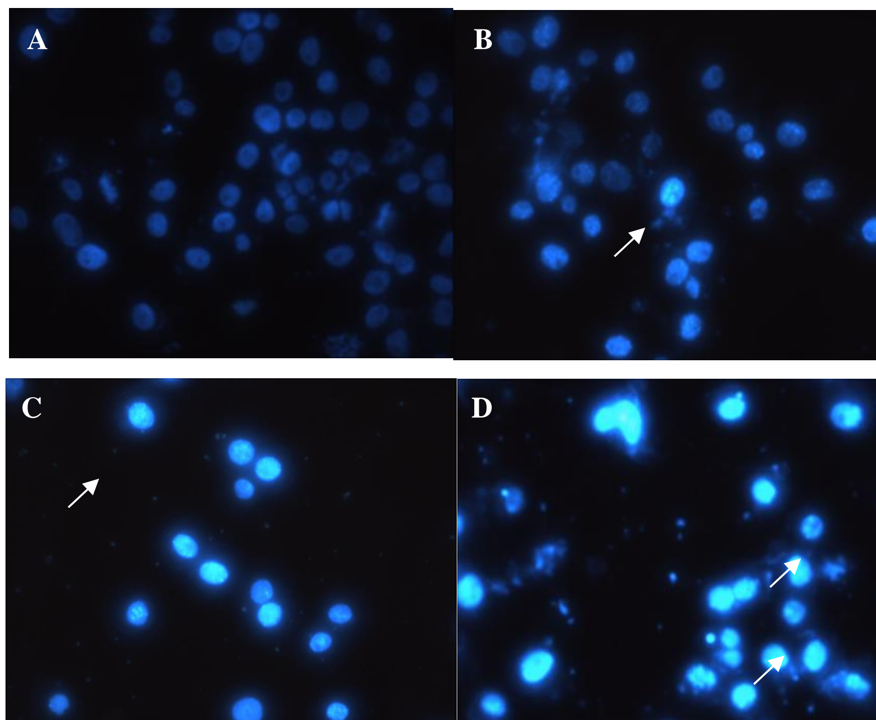

Figure 4: Representative picture of luteolin-induced apoptosis in human cervical cancer lines HeLa. Using Hoechst 33258 staining, the changes in nuclei of apoptotic cells can be observed under fluorescence microscope with magnification 40X. (A) HeLa cells treated with DMSO, (B) HeLa cells after treated with 8 µg/ml of luteolin for 24 hr, (C) 48 hr and (D) 72 hr.

A similar morphology was also observed in HeLa cells treated with 74µg/ml of cisplatin (Figure 5). Qualitative observation showed that the intensity of fluorescence in the nucleus was increased in time dependent manner.

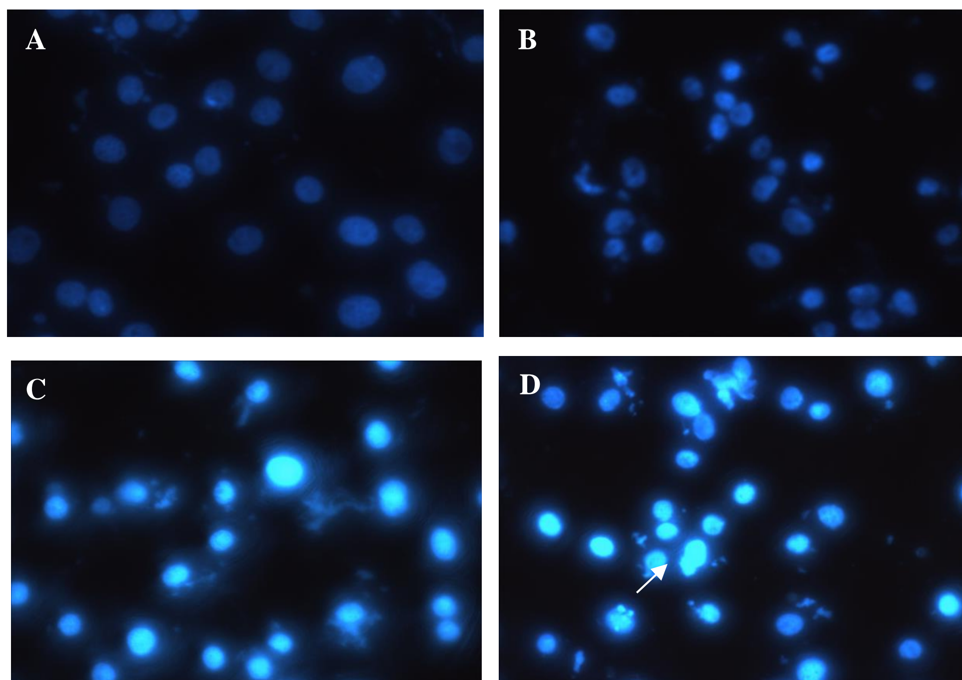

Figure 5: Representative picture of cisplatin-induced apoptosis in human cervical cancer lines HeLa. Using Hoechst 33258 staining, the changes in nuclei of apoptotic cells can be observed under fluorescence microscope with magnification 40X. (A) HeLa cells treated with DMSO, (B) HeLa cells after treated with 74µg/ml of cisplatin for 24 hr, (C) 48 hr and (D) 72 hr.

Flow cytometric analysis of luteolin-induced apoptotic cell death in HeLa cells

Early apoptotic cells were stained positively for Annexin V- FITC that bound to phosphatidylserine (PS), but negative for staining with propidium iodide (PI). Late apoptosis or dead cells are stained positive for Annexin V-FITC and PI, whereas viable cells are negative for both Annexin V-FITC and PI. During early up to late apoptosis, PS is redistributed from inner to the outer of cell membrane (Martin *et al.*, 1995).

Figure 6 showed the flowcytometric dot plot graphs of HeLa cells treated with luteolin for

24, 48 and 72 hours. The Q1 region represents the population of necrotic cells, Q2 is for late apoptosis or dead cells, Q3 is for healthy cells and Q4 is for early apoptotic cells. The result demonstrated that in non-treated cells, the percentage of apoptotic cell death was very low (Q2 and Q4), and became increase after treated with luteolin for 24, 48 and 72 hours. In contrast, the percentage of living cells (Q3) was gradually decreased with the increase of luteolin exposure time. The percentages of each cell population for 24, 48 and 72 hours were summarized in Figure 7.

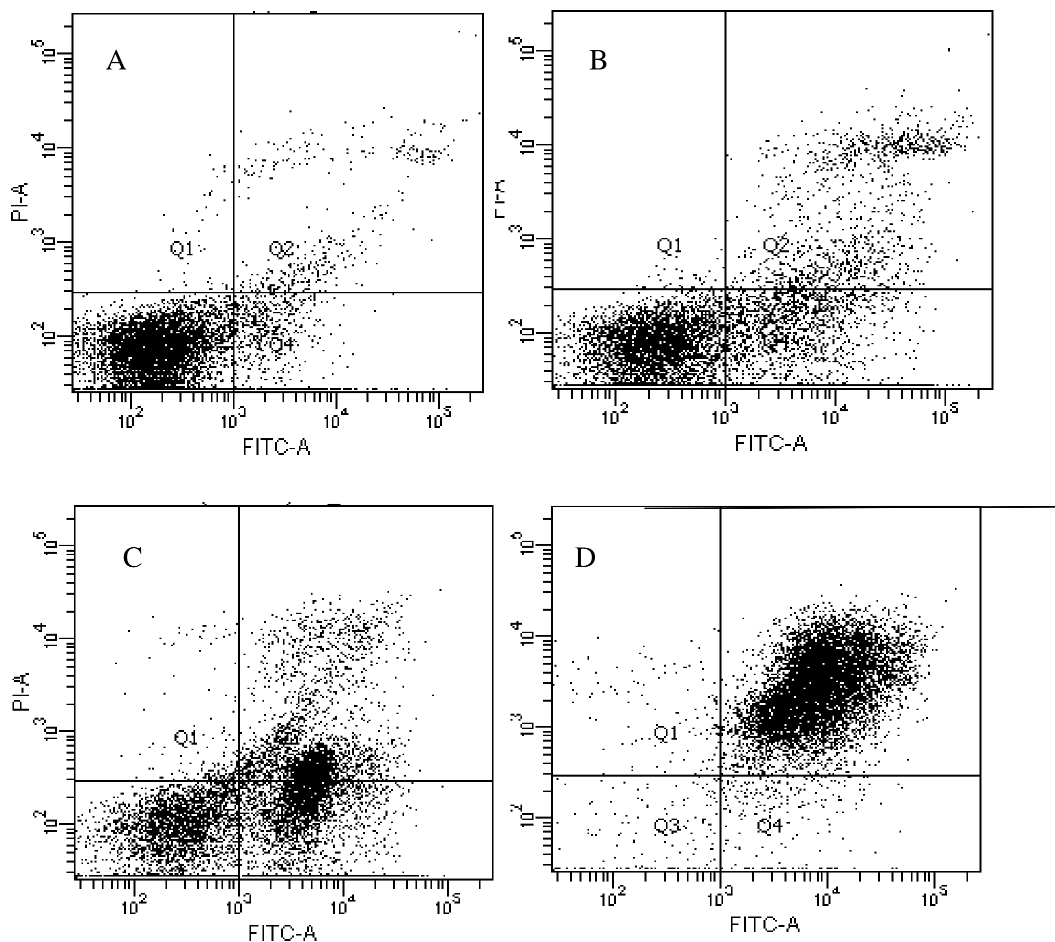

Figure 6: The Annexin V-FITC fluorescent staining of HeLa cells treated with 8 μg/ml of luteolin. Q1 represents necrotic cells, Q2 is late apoptosis or dead cell population, Q3 is living cell population and Q4 is early apoptotic cell population. (A) Cells without treatment showed the highest percentage of living cells. (B) Populations of cells after 24 hr treatment. (C) Populations of cells after 48h treatment. (D) Populations of cells after 72 hr treatment.

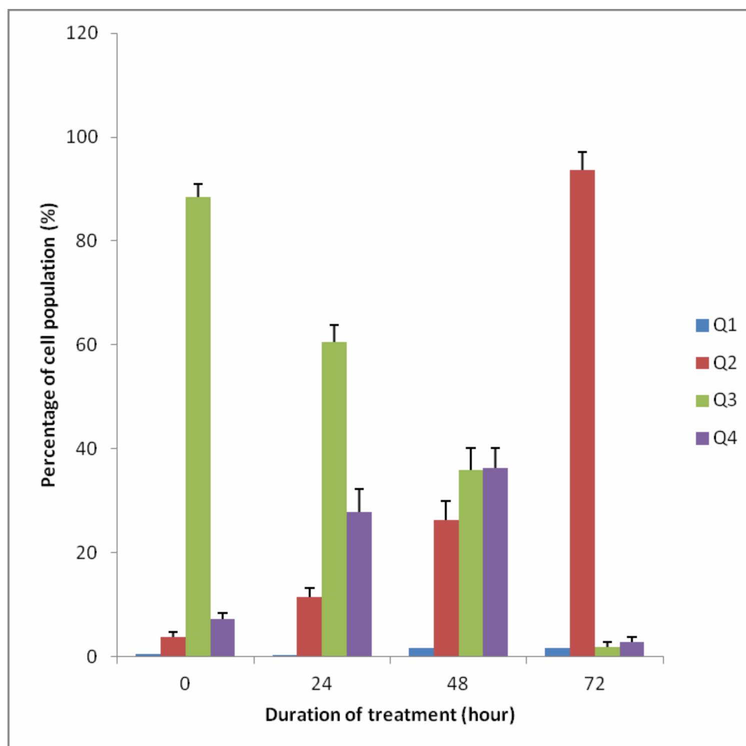

Figure 7: Percentages of cell populations for luteolin treated and non-treated cells. Q1 represents necrotic cells, Q2 is late apoptosis or dead cell population, Q3 is living cell population and Q4 is early apoptotic cell population. In time dependent manner, the living cells were decreased, while the apoptotic cells were increased. Percentage of cells were analysed using Facs Diva Software Analysis and expressed as mean ±S.E.M of three independent experiments (P<0.05).

Effects of luteolin on p53, Bax, Bcl-XL and Caspase-3 protein expression

Treatment of HeLa cells with luteolin revealed the apoptotic event throughout study periods. This event was further elucidated for regulation of tumor suppressor protein p53, Bcl-2 family proteins (proapoptotic Bax and anti-apoptotic Bcl-XL) as well as caspase-3 protein. These proteins were chosen because they determine the pathway of cell death event induced by luteolin. The protein expressions were analysed by flowcytometer summarized in Figure 8. The results also showed that the Bax: Bcl-XL ratio is increased, indicating apoptotic condition.

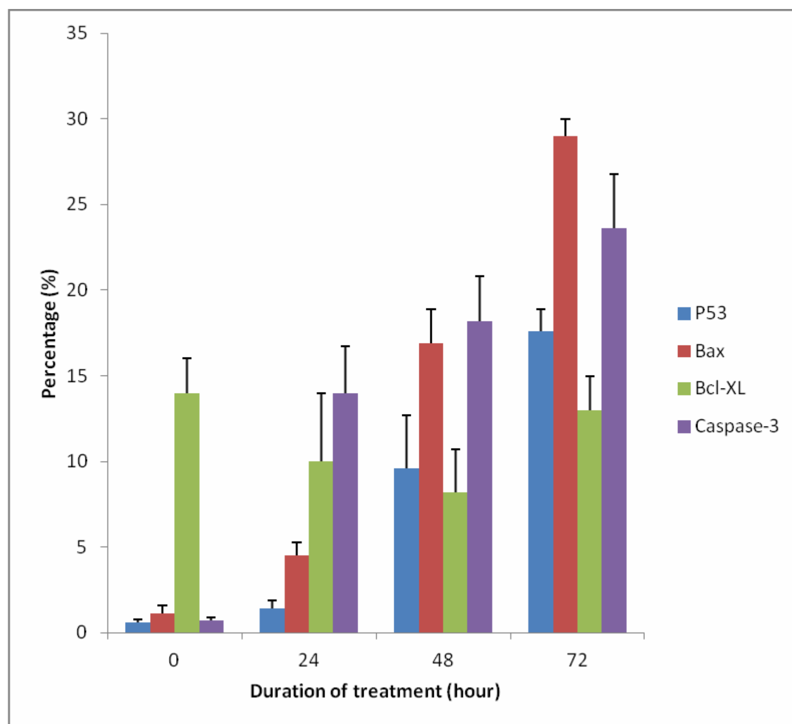

Figure 8: The bar chart indicates the percentages of p53, Bax, Bcl-XL and caspase-3 protein expression in HeLa cells after treatment of luteolin for 0, 24, 48 and 72 hours. p53, bax and caspase-3 protein expressions are increased in time-dependent manner, while Bcl-XL protein expression is not significantly changed. Results were expressed as mean S.E.M from three independent experiment ($P<0.05$).

Discussion

Luteolin belongs to flavones type (flavonoids) of polyphenolic group of compounds. Phenolics compounds contain one or more aromatic rings bearing one or more hydroxyl group with over 8000 structural variants and generally categorized as phenolic acids and analogs, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinines and many more based on the number of phenolic rings and the structural elements that link those rings (Fresco *et al*., 2006). Flavonoids are widely distributed and coloured phenolic derivatives.

In this study, the antiproliferative activity of luteolin from BJ was as effective as synthetic luteolin that is reported by other reseachers (Cherng *et al*., 2007). However, according to Lahlou (2014), natural compounds have more structural diversity and novelty compared to synthetic compounds. Furthermore, many natural chemicals are able to interact with proteins, and other biological molecules. On the other hand, synthetic drugs can cause adverse effects with less therapeutic activity. Besides, the luteolin was obtained from methanolic extract of BJ *via* simple column chromatography which is cheaper then to produce it synthetically.

The IC_{50} of HeLa in this study (28 µM) is consistent with previous research which reported that IC_{50} of luteolin for HeLa cells is 27 µM (Cherng *et al*., 2007). In this study, Cherng and colleagues have used synthetic luteolin that commercially available, which was also found effective in other cancer cell types, including carcinoma of the stomach, lung cancer and bladder cancer cells, with IC_{50} 25µM, 41µM and 68µM respectively (Cherng *et al*., 2007).

The cytotoxicity and antiproliferative activity of luteolin was reported to be associated with its molecular structure (Yanez *et al*., 2004). Yanez and colleages had conducted an *in vitro* study of cytotoxicity and antiproliferative activity of nine commercially available flavonoids including luteolin, quercetin, tangeretin and myricetin and they found that those flavonoids give different activities against melanocytes (Yanez *et al*., 2004). As flavonoids contain different hydroxyl and methoxyl group, the cancer cells react at different degrees of sensitivity (Yanez *et al*., 2004; Kuntz *et al*., 1999).

Cisplatin was used as positive control in this study because it is a highly reactive compound, capable of binding DNA, RNA, proteins and membrane phospholipids. The formation of DNA adducts is recognized as the primary cytotoxic mechanism of cisplatin (Pasetto *et al*., 2006; Wang and Lippard, 2005). However, the antiproliferative activity of cisplatin is very low in most of the tested cancer cells in this study, showing the emergence of resistance towards this drug (O'Grady *et al*., 2014; Pasetto *et al*., 2006).

The four types of proteins were chosen because their expressions are very important to determine the apoptotic mechanism in HeLa cell death. P53 function is crucial in the process of mitochondrial membrane permeability which will further unleash the caspase cascade activation and chromatin degradation (Vaseva and Moll, 2009). Apart from that, we also studied the pro- apoptotic bax and anti-apoptotic bcl-XL protein expression because the bax: bcl-XL ratio is important to determine an apoptotic condition in cancer cells. The imbalance of this ratio contributes to apoptosis impairment and carcinogenesis. The increasing of bax:bcl-XL ratio in compound or drug-treated cancer cells reflects a good clinical values (Kang and Reynolds, 2009). Finally, the expression of caspase-3 protein was also studied because caspase-3 mediates both intrinsic and extrinsic pathway of apoptosis and the increase of caspase-3 protein will favour apoptosis. Caspase-3 activates the downstream caspases such as caspase-6 and -7 which will induce the cleavage of target proteins that will contribute to apoptotic features in cells (Ghobrial *et al.*, 2005).

In this study, it has clearly shown that luteolin from *Brucea javanica* leaves induces apoptosis in cervical cancer cell lines HeLa, as the morphological changes of the cells could be seen after treated with luteolin. The cells that undergo apoptosis would show morphological changes such as condensation of cytoplasm, condensation of chromatin at the nuclear membrane to delineated masses and cell fragmentation into apoptotic bodies (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). This apoptotic morphology was supported with the flowcytometric analysis, showing the increase of cell the undergone apoptosis due to treatment of luteolin. Based on previous studies, luteolin also induces apoptosis by showing such morphological changes using different type of staining on various types of cancer cells such as liver cancer cells HepG2 and leukemia cells HL-60 (Lee *et al.*, 2005; Cheng *et al.*, 2005).

Apart from apoptotic morphology, the expression of p53, Bax, Caspase-3 and Bcl-XL proteins were also evaluated in HeLa cells treated with luteolin. Abundants of literatures have reported that luteolin induce apoptosis whether *via* mitochondria or death receptor pathways in various cancer cell lines (reviewed by Seelinger *et al.*, 2008).

P53 was the first tumor suppressor gene associated with apoptosis. Its mutation occur in the majority of human tumours and often related to advanced tumour stage and poor patient prognosis (Wallace-Brodeur and Lowe, 1999). Our result showed that p53 protein expression in HeLa cells was increased after treated with 28µM luteolin in time dependent manner. The present finding seems to support the previous research by Shi *et al*., (2007); which demonstrated that luteolin-induced p53 up-regulation in HepG2 and HCT116 is achieved at post- transcriptional level through enhanced p53 protein stabilization.

Conclusion

The present findings demonstrated that luteolin from BJ was obtained *via* simple column chromatography displayed capability to kill cervical cancer cells through apoptosis, without affecting the normal cells. It is therefore suggested that luteolin from BJ leaves possess similar anticancer activity as reported on synthetic luteolin and luteolin from other sources. Hence, luteolin might be responsible for anticancer properties exhibited by BJ methanolic leaves extract.

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