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High Performance Liquid Chromatography (HPLC) Profiling Analysis and Bioactivity of *Baeckea frutescens* L. (Myrtaceae)

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Abstract

Leaves of *Baeckea frutescens* were extracted by methanol, and then were subjected to chemical compound analysis through qualitative High Performance Liquid Chromatography (HPLC) profiling, bioactivity property. The total weight of *B. frutescens* leaves crude extract obtained was 2.532 g from 10 g of initial weight. The HPLC profiling used three solvents that are, Methanol, Acetonitrile and water. The profile spectrums from HPLC showed a few peaks that represent the chemicals that lie in the methanolic extract especially in methanol. The results from HPLC crude extract profile for leaves extract of *B. frutescens* showed many peaks at the retention time between 0 to 60 minutes. This showed that a lot of compounds have already been flushed out based on time and polarity range of the solvents. The two types of bioactivity study are antibacterial and cytotoxicity assay. In antibacterial assay, six pathogenic bacteria, were selected and used by using agar well diffusion method. All methanolic extract did not show any antibacterial activity or was not effective at all concentrations of 12.5-100 mg/µL. As a result, methanolic extract was not subjected to broth dilution method for the quantitative measurement of the microbiostatic (inhibitory). In cytotoxic assay the cell line used was HL- 60 that were treated with *B. frutescens* methanolic extract to evaluate the IC_{50} value, that is, which concentration of test compounds that cause 50 % inhibition or cell death. It is to compare the cytotoxic effect with 3T3 which acted as a control. *B. frutescens* methanolic extract is not effective for normal cell line. The methanolic extract of *B. frutescens* was cytotoxic to HL-60.Their IC_{50} of the crude extracts was moderate 21µg/ml.

Keywords: *Baeckea frutescens* L. (Myrtaceae), high performance liquid chromatography, antibacterial, Cytotoxicity activity, methanolic extract

1. Introduction

Traditional *Baeckea frutescens* L. (Myrtaceae) commonly known as 'cucur atap', is an important medicinal plant in the East Coast of Peninsular Malaysia and is used as antimicrobial and anticancer. This species which is from the family Myrtaceae (Bean, 1997a) was investigated for their chemical components (Tsui et al., 1996) and its biological activities (Fujimoto et al., 1996).In China, the leaves are used as a medicinal tea which is drunk for recovery from fever. Mardisiswojo (1985) mentioned that in Indonesia, *B. frutescens* is one of the raw materials of traditional folk medicine. Research on *B. frutescens* by Ibrahim et al. (2004) has reported that the chemical variation in this leaf oils analyzed by capillary GC and GC-MS which possess a few compositions depend on different areas. In related study by Tsui et al. (1996) the chemical elements of leaves of *B. frutescens* are pinenes, g-terpinenes, 1, 8-cineole, p-cymene, limonene, linalool, a-terpineol, b–caryophyllene, a-humelene and various type of sesquiterpenes such as humulene epoxide, eryophyllene epoxide and clovane-2, 9-diol. It also yielded many series of chromones. Another study by Makino et al. (1996) has found that the constituents from the leaves of *B. frutescens* have led to the isolation of three new flavanones such as BF-4, BF-5 and BF-6. The result were tested and showed BF-4 and BF-5 have a strong cytotoxic activity (IC_{50}=0.2-0.5 µg/ml) against leukemia cells (L1210) in tissue culture.

According to Fujimoto et al. (1999) from analysis of methanolic extract from the dried leaves of *B. frutescens* which produced isolates BF-1, BF-2 and BF-3, only BF-2 showed strong cytotoxic activity (IC_{50}=5.0 µg/ml) against leukemia cells (L1210) in tissue culture. The determination supported the essential oil can be used in the treatment of rheumatism. Satake et al. (1999) had found five chromones C-glycosides from the leaves.

According to Nor Azah et al. (2000) the methanolic extract of *B. frutescens* showed the antifungal activity against dermatophytic fungi such as *Trichophyton mentagrophytes* and *Microsporum canis*. Methanolic extract of *B. frutescens* also showed the antioxidant property in auto oxidation of linoleic acid in water-alcohol system assays. The study also found that methanolic extract of *B. frutescens* contains about 2.0% sweet odor of essential oil which have potential as constituent of skin care product. Hwang et al. (2004) reported that the methanol extracts of *B. frutescens* has anticariogenic activity against *Streptococcus mutans* which can cause dental caries. The methanolic extracts of *B. frutescens* at all concentrations tested exhibited strong bacterial activity against *S. mutans*.

The present study of this species is chosen to determine its antibacterial and cytotoxic properties according to its use in Malaysian traditional medicine. The potent crude extracts are analyzed by High Performance Liquid Chromatography (HPLC) to examine chemical variability (Tsui et al., 1996) to select member of the alcohol group which is good for functional antimicrobial (Hwang et al., 2004) and cytotoxicity (Fujimoto et al., 1996) to improve the traditional medicine.

2. Materials and Methods

2.1 Plant Materials

The fresh samples of *B. frutescens* leaves (Fujimoto et al., 1999; Tsui et al., 1996) were collected from Batu Rakit, Kuala Terengganu, Malaysia. Leaves were first washed and dried in ventilated oven at 40^{o}C for three days. The dried samples then were grounded using the grinder until fine powder was obtained. Ten grams of dried fine powder mixture of leaves and small branches was weighed and soaked in 99% methanol to be submerged with solvent (in ratio 1:10) in a 100 ml Erlenmeyer flask and then was placed in dark room for three days. The mixture was filtered using filter paper (Whatman paper 1mm). In order to obtain the crude extract of *B. frutescens*, the aqueous solution was evaporated by using a rotavapor machine (BUCHI R-210) utilizing a water bath (BUCHI B-491) at 40^{o}C. The methanolic extract were weighed, labeled, and stored in chillier at 4^{o}C.

2.2 Qualitative Analysis of High Performance Liquid Chromatography (HPLC) Profiling

The analysis also used to look at the chemical profiling of the crude extract of *B. frutescens* by using High Performance Liquid Chromatography (HPLC) at Chemistry Laboratory, Kampus Kota, Universiti Sultan Zainal Abidin (UniSZA). Methanolic extract was first flushed with the n-hexane to make sure the lipid and the fatty acid compound have been flushed away to avoid column in the HPLC to clog or stuck. The 10 grams of methanolic extract was mixed with 10 grams of silica and forward to the column chromatography. 0.1 g of sample was diluted with 10 ml of methanol to get 0.01 g/ml concentration. Next, 10µl of diluted sample was injected to the HPLC and the separation was realized into a C18 reverse phase HPLC column (250 x 4.6 mm) (stationary phase) with a low pressure gradient. The flow rate of the solvent used in HPLC was 1.0 ml/min, starting at 10% Methanol, 15% Acetonitrile, 75% deionised water and continue with increasing polarity of the solvent.

2.3 Bioactivity

Two bioactivities involved are screening of antibacterial and cytotoxicity. Antibacterial activity was evaluated at the Microbiology Laboratory, while cytotoxicity activity was evaluated at the Animal Cell and Tissue Culture Laboratory, Department of Agriculture and Biotechnology, UniSZA.

2.3.1 Antibacterial Assay

The antibacterial investigation was performed in three replicates using the agar wells diffusion technique against six species of human pathogenic bacteria to determine the presence of antibacterial properties of *B. frutescens*. The six selected bacteria were *Escherichia coli* (ATCC 35218)*, Morganella morganii* (IMR MM99), *Serratia marcescens* (ATCC 8100), *Salmonella typhii* (ATCC 19430), *Listeria monocytogenes* (IMR L10), and *Klebsiella pneumonia* (ATCC 10273). Antibacterial assay was carried out to determine the potential of the crude extracts of *B. frutescens* to inhibit microbial growth.

For the qualitative determination, the bacteria were first grown in Mueller Hinton Broth (MHB (Difco)) overnight at 37^{o}C.The bacterial suspensions were then standardized to an OD_{600} of 0.5 using spectrometer machine (SHIMAZU/UV- Mini 1240).These bacteria were first cultured onto nutrient agar, (Difco) to obtain the pure single colony by appropriate four quadrants streaking followed by incubating the agar overnight at 37^{o}C. A clear zone around the well indicates inhibition of growth. The diameter of clear zone was measured to differentiate the inhibition of bacterial growth whether weak, moderate or strong.

The wells were prepared on the Mueller Hinton Agar (MHA (Difco) using Graham tube which has a standard

diameter of six millimeters. The lawn of bacteria was prepared by pipetting and spreading the bacteria evenly using sterile cotton buds onto the agar in petri dishes. The crude extract were diluted with diluents (20% DMSO).Twenty µl of the crude extract was pipetted into all wells and allowed to dry before leaving overnight in a incubator at 37^{o}C. Compounds that show positive antibacterial activities with the wells diffusion assay were subjected to the broth dilution method for the quantitative measurement of the microbiostatic (inhibitory) activity in 96 well plates. The lowest concentration which completely inhibits the visible bacterial growth was recorded as the minimum inhibitory concentration (MIC, µg cm^{-3}).

2.3.2 Cytotoxicity Assay

Human Promyelotic Leukemia (HL-60) and Mouse Embryonic Fibroblast (3T3) cell lines were purchased from American Tissue Culture Collection (ATCC, USA) were maintained grown in 25cm^2 tissue culture flask (Nunclon^{tw}, Germany) in 5% CO_2 incubator (Heracell 150 Thermo electron Corporation) at 37^{o}C with 90% humidity. Then 10% fetal bovine serum (Culture laboratory), 1% of penicillin (100IU/mL) and Streptomycin (100 µg/mL) were added to the culture medium prior to use. The cell counts were determined by using haemocytometer prior to treatment which the consistent numbers of cells were resulted from maintaining optimum growth and standardize technique using cell cultures.

The assay was carried out in a flat bottom 96 well plate (Nunclon^{tw}, Germany). The treated cells were incubated for 24 to 72 hrs or three days in a humidified 5% CO_2 incubator at 37^{o}C. Microtitration cytotoxic assay was carried out the cell viability in four hrs in humidified 5% CO_2 incubator at 37^{o}C based on the 20 microliter yellow tetrazolium salt MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide). A volume of 150 µl medium was removed from the wells and replaced with 100 µl of DMSO. Then the plate was placed in the sonicator for a few minutes and allowed to stand at room temperature for five to ten minutes. Then, the solubilised formazan product was quantified spectro photometrically. The absorbance at 570nm with 630nm as a reference was recorded using microplate reader (Tecan 200). Finally, the concentrations of dose extract that can inhibit concentration of 50% of cell viability (IC_{50}) were determined from plotting of graph cell viability percentage versus concentration of crude extract.

3. Result and Discussion

The total weight of *B. frutescens* crude extract obtained was 2.532 grams from 10 grams of initial weight of leaves.

3.1 Qualitative Analysis of High Performance Liquid Chromatography (HPLC) Profiling

The results from HPLC crude extract profile for leaves extract of *B. frutescens* showed a lot of peaks at the retention time between 0 to 60 minutes. After 60 minutes, compound within this range of polarity have already been flushed out which indicates the polarity of the solvents have increased by increasing the percentage of the MeOH used to 20, 30, 40 % at the 45, 50 and 55 retention time respectively. After 40% MeOH were used, there were no peaks appeared anymore to indicate that all compounds were being flushed out from the column. It could be seen that the peak spectrum of leaves extract in different solvents showed quite the same trend at the beginning, such as at 1 to 8 minutes and only differ in the heights of peak. The peak spectrum of leaf in 10% of MeOH showed the highest peak spectrum at 2 to 3, 4 to 5 and 5.5 to 6 minutes. While, at 6.5, 10 to 12, 15.5 to 16, 16.5, 17, 18.5, and 19 to 20.5 minutes showed the moderate peak spectrum. The pattern of peak spectrum which showed the similar pattern is between the highest peak spectrum at 2 to 6 minutes and the moderate peak spectrum at 15.5 to 20.5 minutes.

However, water almost showed the linear peak spectrum at 0 to 60 minutes. It indicates that water did not show any kind of peak spectrum pattern as compared to other two solvents. The result showed that the crude extract of *B. frutescens* may have some compounds which was not flushed out by the Acetonitrile after 40 minutes and above. It has proved that in using MeOH, some compounds showed the low peak spectrum at 40 to 60 minutes and after 60 minutes there is no peak shown and it indicates that all compounds were being flushed out from the column. The results are shown in figure 1 to figure 3.The results from HPLC crude extract profile for leaves of *B. frutescens* showed a lot of peaks at the retention time between 0 to 60 minutes using Methanol, Acetonitrile as compared to water as solvent. The atomized wavelength for this analysis uses the 254 nm absorbance. This is because the wavelength of 254 nm is the best as this wavelength needs for detection of organic compound. By choosing the leaves of the plants in this study, investigation has found that it contained diverse groups of chemical compound as reported by Ibrahim et al. (2004). In related study by Tsui et al. (1996), Makino et al. (1996), Satake et al. (1999), Nor Azah et al. (2010), it were reported that there were varieties of chemical elements of leaves of *B. frutescens.*

Table 1. Percentage (%) of solvent combination and the retention time (min) that is used as the mobile phase in the HPLC profiling of crude extract of *B. frutescens*

Retention Time (min)	Solvent (%)		
	Methanol	Acetonitrile	Water
5	10	15	75
10	10	20	70
15	10	30	60
20	10	40	50
25	10	50	40
30	10	60	30
35	10	70	20
40	10	80	10
45	20	0	80
50	30	0	70
55	40	0	60

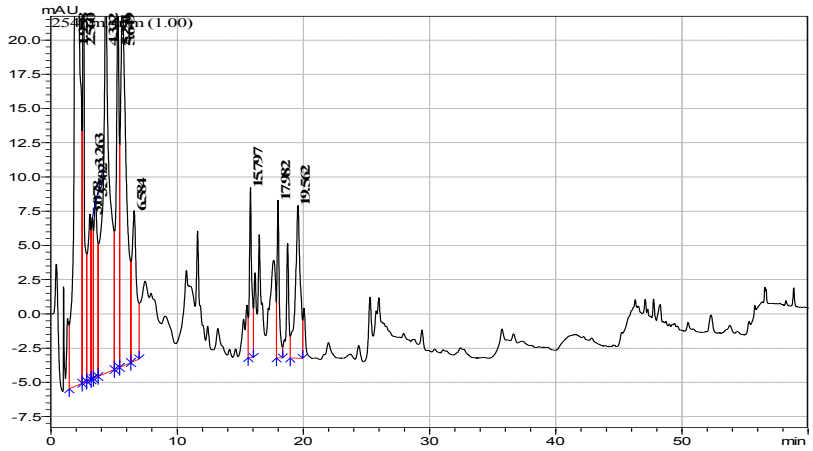

Figure 1. HPLC spectrum of the leaves crude extract of *B. frutescens* at 254 nm absorbance using Methanol as solvent

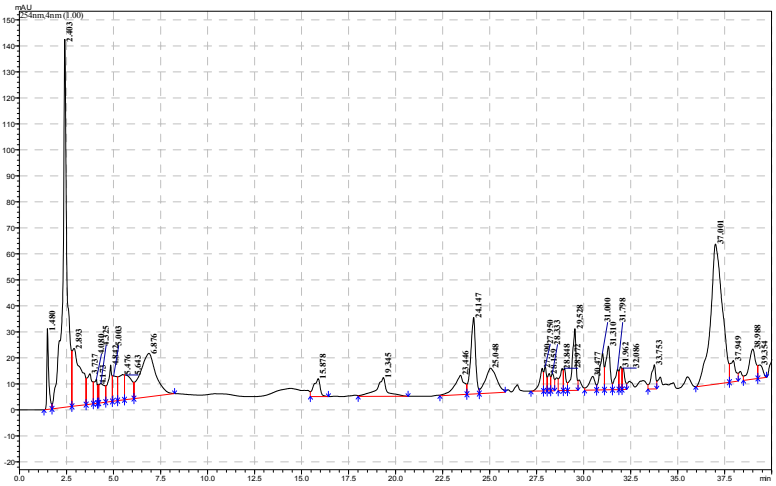

Figure 2. HPLC spectrum of the leaves crude extract of *B. frutescens* at 254 nm absorbance using Acetonitrile as solvent

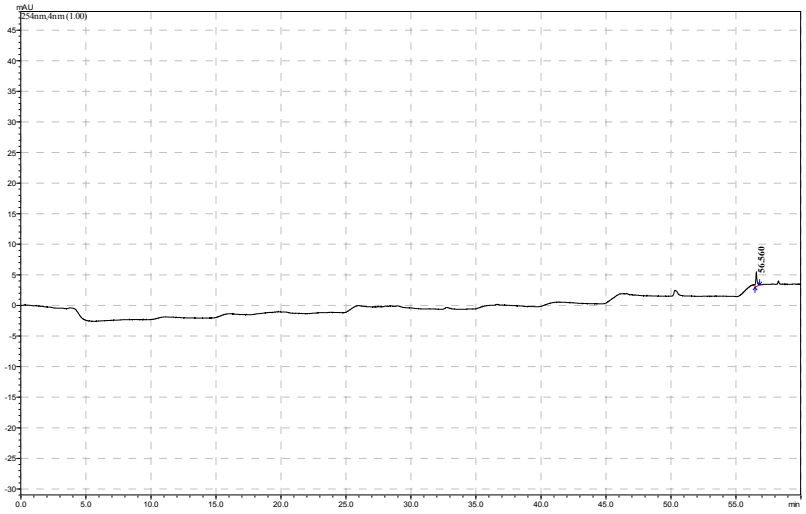

Figure 3. HPLC spectrum of the leaves crude extract of *B. frutescens* at 254 nm absorbance using water as solvent

3.2 Antibacterial Assay

All methanolic extract did not show any antibacterial activity or was not effective at all concentrations treated by 12.5, 25, 50 and 100 mg/µl. This was proved when none of inhibition zone was observed around the well to indicate inhibition of growth which is similar to that shown in the control (DMSO). However, Erythromycin as a standard in this study showed the active antibacterial activity as shown by 21 to 22 mm zone of inhibition for all treatments through all concentrations. As a result without a clear zone, the different measurement for inhibition of bacterial growth whether weak, moderate or strong failed to be determined. Only the compounds that show the positive result in qualitative antibacterial activities will be subjected to the quantitative antibacterial activity assay.

Interestingly, although showing a negative result, six of pathogenic bacteria have showed a tremendous growth for all different concentrations. After three days of treatments, the extract of *B.frutescens* promoted their growth with the good nutrients and the growth of *Serratia marcescens* (ATCC 8100) and *Klebsiella pneumonia* (ATCC 10273) as compared to other four species of bacteria. Through some reports, the antibacterial activity could be contributed by p – cymene, a bactericidal compound which can be found in essential oil of fennel and several species (Burt, 2004). The negative result in this study showed that the compound was not found in leaves of *B. frutescens* methanolic extract.

However, there are few reports on crude extracts and their biological activities of *B. frutescens* in the literature mostly against species of *Staphylococcus*. Previous studies on the antibacterial activity of *B.frutescens*, Habsah et al. (2008) reported that essential oil of *B. frutescens* showed high antibacterial property against *Staphylococcus aureus* with inhibition zone ranging from 10.57 to 17 mm. Hwang et al. (2004), reported that the methanolic extract of the leaf of *B. frutescens* at all concentrations tested exhibited antibacterial (anticariogenic) activity against *Streptococcus mutans* which can cause dental caries.

Table 2. *In vitro* antibacterial activity of the methanol extract of *Baeckea frutescens* against six human pathogenic bacterial strains based on agar well diffusion method

Zones of inhibition (mm)						
Bacterial strains	*Ec*	*Mm*	*Sm*	*St*	*Lm*	*Kp*
Concentration of extract (mg/ml)						
100	–	–	–	–	–	–
50	–	–	–	–	–	–
25	–	–	–	–	–	–
12.5	–	–	–	–	–	–
Control	–	–	–	–	–	–
Standard	23	22.4	21	23.4	21	23.2

Ec= Escherichia coli (ATCC 35218)*; Mm =Morganella morganii* (IMR MM59)*; Sm = Serratia marcescens* (ATCC8100); St =*Salmonella typhii* (ATCC 19430); *Lm =Listeria monocytogene* (IMR L10) and *Kp = Klebsiella pneumonia* (ATCC 10273); Control = DMSO; Standard = Erythromycin and - = No zone of inhibit

3.3 Cytotoxicity Assay

The percentage of viability of 3T3 cell line shows almost linear pattern at 10 to 30 µg/ml concentration of this crude extract. The positive result showed was expected of *B. frutescens* crude extract against normal cell because the best potential of medicinal plant should have no effect to the normal cell. The 40% increase in growth of 3T3 proved that *B. frutescens* extract promoted the growth of 3T3 cell line and it can be used as an internal drug. This is because the negative result for cytotoxicity of *B. frutescens* crude extract found in this study suggests that *B. frutescens* crude extract should be consumed orally. The growth of 3T3 was increased at 3.75 µg/ml concentration of *B. frutescens* as shown in Figure 4.

The cytotoxicity effects of *B. frutescens* methanolic extract against HL-60 cell line were determined by measuring the cytotoxic dose that kill 50% of cell population as compared to the untreated control for various periods using colorimetric cytotoxicity assay (MTT). Figure 4 shows the effectiveness of 0 to 30.0 µg/ml concentrations of *B. frutescens* crude extract towards cell viability of HL-60 to 50% of control cells responses after 72 hours exposure. This graph showed the drastically declined in percentage of cell viability from 100% of HL-60 to 20% after exposed to 30µg/ml of *B. frutescens* extract. From 100% to 80%, the graph showed a small curve after exposed to 4.5 µg/ml of *B. frutescens* methanolic extract. Meanwhile, in 50% of control cells showed that 21 µg/ml concentration of *B. frutescens* extract inhibit HL-60 was successful with the long curve of graph. The results obtained indicated that *B. frutescens* inhibited the growth and proliferation of HL-60 in a dose. As a result, *B. frutescens* crude extract has potential as anticancer against HL-60 cell line when it showed the moderate effectiveness in cytotoxicity activity.

Previous study by Fujimoto et al. (1999), reported that the leaves of *B. frutescens* methanolic extract which produced new phloroglucinols named BF-2 showed strong cytotoxity activity (IC_{50}=5.0 µg/ml) against Mouse Lymphotic leukemia cells line (L1210) in tissue culture. The result from this study was further investigated if effect of phloroglucinols (BF-2) of *B. frutescens* methanolic extract also has strong cytotoxic against human leukemia cell line similar to Mouse leukemia cell. However, the effectiveness is not the same but *B. frutescens* methanolic extract can still be commercialized as anti-leukemia.

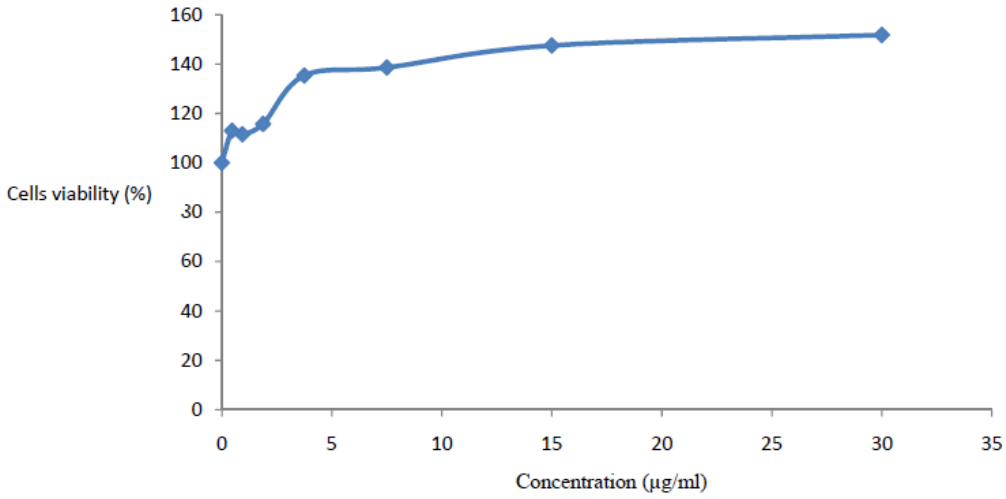

Figure 4. Cytotoxicity of *B. frutescens* crude extract against the Human Normal cell (3T3) cell line

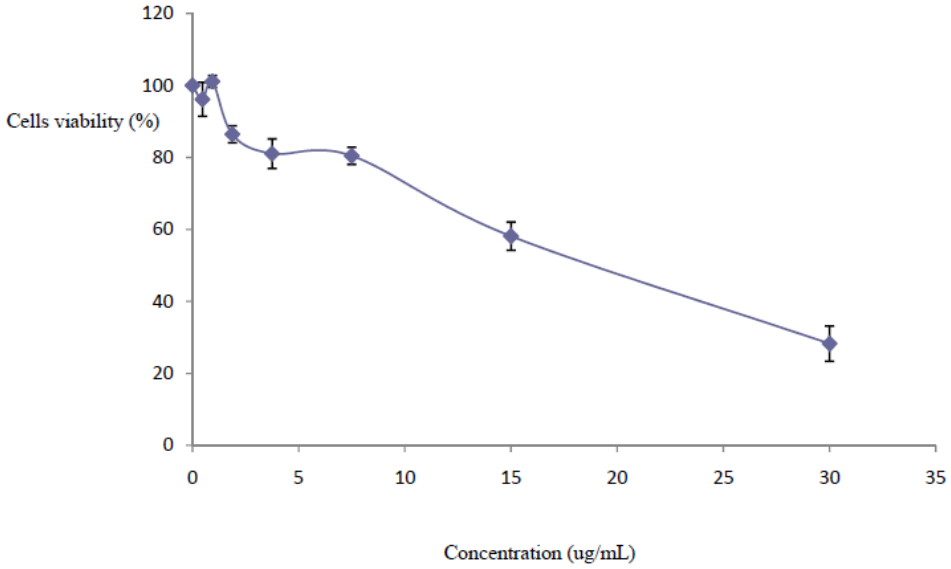

Figure 5. Cytotoxicity of *B. frutescens* crude extract against the Human Promyelotic Leukemia (HL-60)

4. **Conclusion**

The result of this study demonstrated that leaves of *B. frutescens* extract does contain bioactive compounds according to a few peaks pattern that were shown in HPLC profiling analysis result by using Methanol and Acetonitrile as solvents. These peaks confirmed the presence of the potential chemical content of *B. frutescens* extract and possibly their potential in the pharmaceutical industry. However, *B. frutescens* methanolic extract did not show any antibacterial activity against six human pathogenic bacteria. The results concluded that *B. frutescens* were successfully extracted by using methanol extraction method when it showed the moderate range of cytotoxicity activity against HL-60, an indication of the possession of a wide range of pharmacological activities especially as anti-leukemia.

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