

Full Length Research Paper

Antiproliferative activity of *Erycibe elliptilimba* against human leukemic cancer cell lines

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Erycibe elliptilimba Merr and Chun., family Convolvulaceae, is a Thai traditional medicine that has long been used as a remedy for various infectious and malignant diseases. Biological examination of extracts from *E. elliptilimba* showed that the methanol fraction (F3) had significant antiproliferative effects on four human leukemic cell lines (NB4, HT93A, Kasumi and K562) with IC₅₀ values of 57.74, 63.76, 69.55 and 47.58 µg/ml, respectively. It induced apoptosis without significant change in the cell cycle process of those cell lines, especially NB4 and HT93A. The significant levels of apoptosis on NB4 and HT93A were 89.13 and 61.21%. The methanol fraction was further chromatographed to produce special extracts F3.1-3.7. The antiproliferative test showed growth inhibition of human leukemic cell lines produced by F3.6 with IC₅₀ values of 22.68, 31.54, 28.88 and 47.72 µg/ml, respectively. Isolation of the major component in F3.6 was performed and resulted in compound E, which was identified as a mixture of β-sitosterol and stigmasteryl-O-β-glucopyranosides. In conclusion, this active fraction (F3.6) from *E. elliptilimba* Merr and Chun. contained β-sitosterol and stigmasteryl glucosides (compound E) as a major compound. It will be used as a marker compound in the standardization of the active fraction (F3.6), which will be further studied for the antiproliferative effect.

Key words: *Erycibe elliptilimba*, antiproliferative effect, NB4, K562.

INTRODUCTION

Leukemia is one of the most common hematologic malignancies in Thailand with an estimated ASR of 4.1 per 100,000 in men and 3.5 per 100,000 in women, according to a report by Thai National Statistics (Jootar, 2003). Acute promyelocytic leukemia (APL) is characterized by increased promyelocytes in the marrow harboring specific chromosomal translocations that generate fusion proteins such as PML-RAR α and PLZF-RAR α (X-RAR α) (Melnick and Licht, 1999). This oncogenic gene product is believed to act through disruption of the transcription-modulating function of RAR α and consequently induces leukemogenesis. Acute myeloblastic leukemia with maturation (AML-M2) is one type of acute leukemia. It is found in 10 to 15% of AML

patients, whereas more than 90% of t(8;21)-positive leukemias have FAB AML-M2 morphology (Hagemeyer et al., 1992). The t(8;21) disrupts the AML1 gene (also referred to as CBFA2 or PEBP2 α B) at 21q22 and the ETO gene (also CDR or MTG8) on chromosome 8q22, generating a fusion of AML/ETO1 gene rearrangement (Miyoshi et al., 1993; Erickson et al., 1992; Nisson et al., 1992; Maruyama et al., 1994). This translocation results in the production of a fusion protein known as ETO/AML1, of which the ETO portion from chromosome 8 is involved in histone deacetylation. This, in turn, serves to block activation of AML1 gene expression, resulting in a lack of IL-3 and GM-CSF receptor activation as well as loss of cellular differentiation/maturation. Chronic myeloid leukemia (CML) is a myeloproliferative disorder usually involving a chromosome abnormality called "Philadelphia chromosome" that harbors a specific gene named bcr-abl. This fusion gene product increases the activity of tyrosine kinase which signals the proliferation of stem cells

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(Deininger et al., 2000). As standard treatment, chemotherapy in acute or chronic myeloid leukemia diminishes symptoms and improves the quality of life. However, many patients who suffer from leukemia still employ alternative or traditional medicine, including herbal therapies, for treating and minimizing their symptoms.

E. elliptilimba is a Thai medicinal plant in the Convolvulaceae family known as “pra-kan-chai-sri”. It is distributed throughout several regions of Thailand. For centuries, its stem has been widely used in Thai traditional medicine for the treatment of various diseases including malignancies (Sintusarn, 2002). Based on a recent study using bioassay-guided screening, the methanolic fraction (F3) of *E. elliptilimba* exhibited growth inhibition against several cancer cell lines, especially MDA-MB435, the human breast cancer cell line, by regulation of the cell cycle at G2/M phase (Kummalue et al., 2005, 2007). Interestingly, this special fraction also induces apoptosis in the breast cancer cell line in a dose and time dependent manner. However, analysis of the antiproliferative effect of *E. elliptilimba* in human leukemic cell lines has not yet been performed.

In this study, the antiproliferative effect of the special extracts from *E. elliptilimba* on human leukemic cell lines was investigated using bioassay guided fractionation with the MTT method in combination with apoptotic study. Isolation and identification of the major compound in the active special extract were performed.

MATERIALS AND METHODS

Plant identification

E. elliptilimba was collected from Ratchaburi Province in the central region of Thailand in 1994. It was identified by Miss Leena Phuphathanapong, a taxonomist at the Forest Herbarium (BKF), Royal Forest Department, Ministry of Natural Resources and Environment, Bangkok, Thailand, where voucher specimen #015461 was deposited.

Plant extraction procedure

Extraction of the plant material was performed according to a previous study (Kummalue et al., 2007). In brief, the dried stems and leaves were cut into small pieces and ground into powder. The powdered drug was macerated with three liters of 95% ethyl alcohol. The extract was concentrated under reduced pressure to yield crude ethanolic dry extract. The dry extract was dissolved in water. The water soluble part was then chromatographed on a Diaion® HP20 column (Mitsubishi Chemical Corp., Japan) and eluted with water.

The water insoluble part was dissolved in water-methanol (1:1), which was further chromatographed on the same column and eluted with water-methanol. The water-methanol insoluble part was dissolved in methanol and worked up in the same manner as above. The methanol insoluble part was dissolved in ethyl acetate. The fractionation of the crude extract from the column provided four fractions which were water (F1), water-methanol (F2), methanol (F3) and ethyl acetate (F4) fractions. Further chromatographic fractionation of F3 was performed using silica gel column

chromatography. Seven fractions were obtained: F3.1, F3.2, F3.3, F3.4, F3.5, F3.6, and F3.7. Thin-layer chromatograms of all fractions were performed to isolate the major compound. Identification of the major compound in F3.6, which was the active fraction, used the spectroscopic technique as follows: melting points were performed on an Electrothermak 9100, (Eng. Ltd); UV and IR-spectra using a UV spectrophotometer (Hitachi, U-320 spectrophotometer); the IR and NMR were measured on a Vector 22 (Switzerland, Bruker 500 Hz, Switzerland); and mass spectra using a Micromass (LCT, England).

Preparation of the plant extract

All fractions in dry extract form were dissolved in DMSO (Sigma, USA), except the water dry extract which was dissolved in sterilized water. For all experiments, the extracts were diluted with culture medium to obtain final concentrations varying from 1 to 500 µg/ml.

Cell lines and culture

Four leukemic cell lines namely NB4 and HT93A (acute promyelocytic cell lines), Kasumi (acute myeloblastic leukemia cell lines, AML-M2) and K562 (chronic myelocytic leukemia cell line) were used (Lanotte et al., 1991; Kishi et al., 1998; Asou et al., 1991; Lozzio and Lozzio, 1975). HT93A was kindly provided by Dr. Kenji Kishi, Tokai University, Japan. The other cell lines were kindly provided by Ms. Setsuko Miyanishi, Tenri Institute of Medical Research, Japan. All of these cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (Stem Cell Technology, Canada) and 1% penicillin plus streptomycin at 37°C in a 95% humidified atmosphere containing 5% CO₂.

Cell proliferation assay

The viability of cells was assessed by MTT [(3, 4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which is based on the cleavage of yellow tetrazolium salt MTT to purple formazan crystal by metabolically active cells (Skehan et al., 1990). Briefly, 1x10⁵ cells/ml of NB4 and K562, 1x10⁶ cells/ml of HT93A and Kasumi (the number of the cells depended on the growth rate) were seeded in 96-well plates and treated with all extracts, fractions 1 through 4, including crude extract, at final concentrations of 1, 10, 100 and 500 µg/ml. In the same condition, these cell lines were treated with F3, F3.1, F3.2, F3.3, F3.4, F3.5, F3.6, or F3.7 at final concentrations of 10, 20, 40, 60 and 80 µg/ml, respectively. A final concentration of 0.1% DMSO was used as the negative control group. Vinblastin is classified as a plant alkaloid which is an anti-cancer chemotherapy drug was used as the positive control group at a final concentration 0.1 µg/ml. After 48 h of exposure, 50 µl of 1 mg/ml of MTT (Sigma, USA) in phosphate buffer saline (Sigma, USA) was added to each well and incubated for 4 h. 100 µl of 10% SDS in 0.01 M HCl was then added to each well and the plates were incubated overnight in the incubator at 37°C with 5% CO₂. The optical density of the dissolved solutions was measured at 595 nm by a microplate ELISA reader (Bio-Rad, USA). Data was calculated as the percentage of cell viability using the following formula:

$$\text{Cell viability (\%)} = (\text{sample O.D.} / \text{control O.D.}) \times 100$$

Cell cycle analysis

To determine cell cycle distribution, 1x10⁵ cells/ml of NB4 and K562, 1x10⁶ cells/ml of HT93A and Kasumi were cultured in each well of the 12-well plates. For NB4 and K562 cell lines, cells were treated

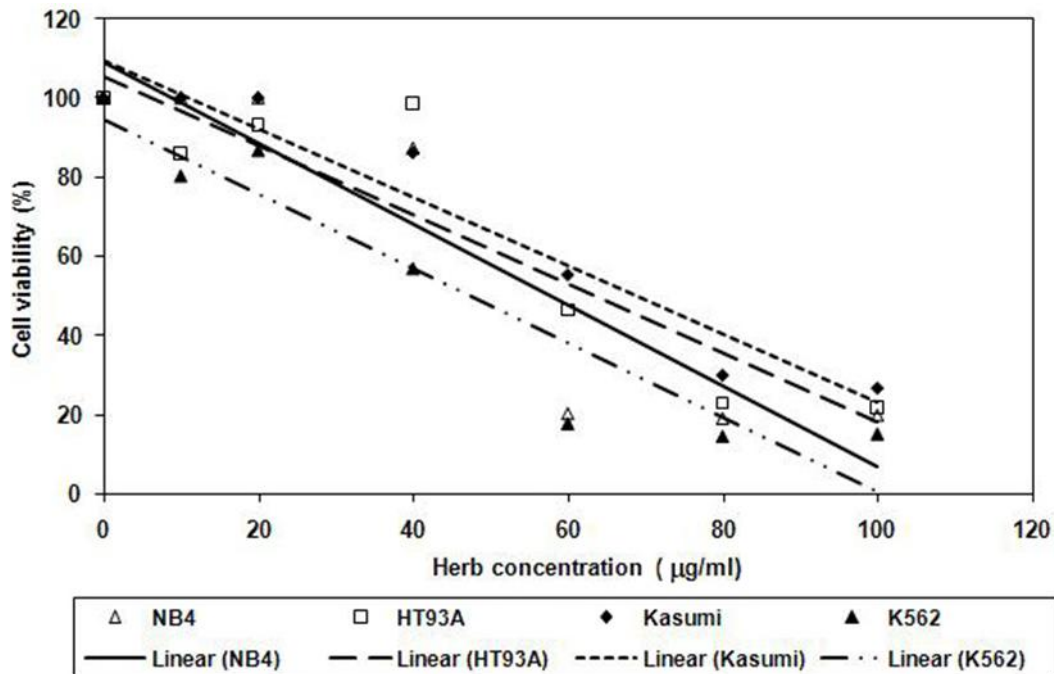


Figure 1. Effect of *Erycibe elliptilimba* methanol fraction, F3, on NB4, HT93A, Kasumi and K562 cell viability. Four experiments for each cell line were performed. Cells were plated in 96 well plates with different concentrations of the herb extract for 48 hours. The cell viability was then determined by MTT assay. Results expressed as mean cell viability as a percentage of untreated control samples ($n=4$). The IC_{50} of the herb on NB4, HT93A, Kasumi and K562 cell line were 57.74 ± 0.01 , 63.76 ± 0.07 , 69.55 ± 0.13 and 47.85 ± 0.05 $\mu\text{g/ml}$, respectively.

with herb extract fraction 3 at final concentrations of 0, 50 and 100 $\mu\text{g/ml}$, whereas HT93A and Kasumi cells were treated with the herb extract at final concentrations of 0, 70 and 140 $\mu\text{g/ml}$. The cell cycle analyses were conducted after 24 and 48 h of incubation using the CycleTEST™ PLUS DNA Reagent Kit (BD, Biosciences, USA). Briefly, cells were collected, washed and incubated with the A, B, and C solutions, respectively, as recommended by the manufacturer. All samples were analyzed within 3 h by FACScalibur using CellQuest software.

Statistical analysis

All experiments were performed with three independent experiments (except four independent experiments for IC_{50} of the herb extract from methanol fraction; F3.1, F3.2, F3.3, F3.4, F3.5, F3.6, and F3.7). Each independent experiment was performed in triplicate. Data were expressed as mean \pm standard deviation. The R-square equation was used to calculate the IC_{50} value. A P-value less than 0.05 were considered statistically significant using regression coefficient analysis.

RESULTS

Effect of *Erycibe elliptilimba* on NB4, HT93A, Kasumi and K562 cell lines

The results of the growth inhibition of *E. elliptilimba* on

NB4, HT93A, Kasumi and K562 were demonstrated. The cell viability of all cell lines was significantly decreased when treated with the *E. elliptilimba* methanol fraction (F3). The IC_{50} value of NB4, HT93A, Kasumi and K562 were 57.74 ± 0.01 , 63.76 ± 0.07 , 69.55 ± 0.13 and 47.85 ± 0.05 $\mu\text{g/ml}$, respectively, as shown in Figure 1. This inhibitory effect was observed to be dose dependent. However, crude extract and other fractions did not exhibit any inhibitory effect on these cell lines under the same conditions. In the positive control groups, the viability of all cell lines was lower than 40% after being treated with 0.1 $\mu\text{g/ml}$ vinblastin.

The IC_{50} value of fractions extracted from methanol fractions (F3.1, F3.2, F3.3, F3.4, F3.5, F3.6, and F3.7) on four cell lines are shown in Table 1. Only fraction 3.6 of methanol fraction inhibited all cell types at concentrations lower than 50 $\mu\text{g/ml}$. The IC_{50} value of NB4, HT93A, Kasumi and K562 cell lines treated with F3.6 were 22.68 ± 0.10 , 31.54 ± 0.15 , 28.88 ± 0.30 and 47.72 ± 0.06 $\mu\text{g/ml}$, respectively.

Erycibe elliptilimba induced apoptosis in NB4, HT93A, Kasumi and K562 cell lines

The cell cycle analysis of four leukemic cell lines NB4,

Table 1. IC₅₀ of fraction extract from methanol fraction, F3, on four cell lines detected by MTT assayed.

fraction extract from methanol fraction (fraction 3)	IC ₅₀ of herb on cell lines (mean±SD) (µg/ml)			
	NB4	HT93A	Kasumi	K562
Fraction 3.1	36.08±0.23	65.50±0.45	99.86±0.72	58.54±0.48
Fraction 3.2	43.11±0.22	51.37±0.19	29.55±0.09	44.22±0.34
Fraction 3.3	>100	>100	52.75±0.02	65.69±0.36
Fraction 3.4	>100	60.32±0.27	28.10±0.31	76.18±0.06
Fraction 3.5	53.35±0.14	32.58±0.03	25.28±0.05	46.49±0.14
Fraction 3.6	22.68±0.10	31.54±0.15	28.88±0.30	47.72±0.06
Fraction 3.7	24.96±0.11	59.28±0.13	48.70±0.24	42.50±0.21

HT93A, Kasumi and K562 cells treated with *E. elliptilimba* fraction were analyzed using flow cytometry. Based on our results, only the herb fraction 3 treated cells were investigated because this fraction was detected to have inhibitory effects on all cell lines. The histogram of cell cycle analysis of HT93A treated with *E. elliptilimba* fraction 3 (F3) at concentrations of 0, 70 and 140 µg/ml after 24 h is shown in Figure 2. The herb extract at 70 and 140 µg/ml induced the apoptosis of HT93A cells from 2.60 to 9.48 and 40.19% when compared with the control as shown in Table 2. Moreover, the number of HT93A cells in the G2/M phase significantly decreased from 17.01 to 6.74%, but no other significant changes were observed in either the G0/1 or the S phase cell cycle progression. The other cells were affected in the same manner. The same patterns of cell cycle were observed after 48 h. Methanol fraction significantly induced apoptosis in NB4 and K562 at levels of 89.13 and 61.21% compared to the control cells, as shown in Table 2. The inhibitory effect was shown to operate in a dose dependent manner.

Identification of active compound

Thin-layer chromatograms of F3.6 are shown in Figure 3. Compound E was isolated and identified. Compound E, which appeared as a white powder, had a melting point of 230 to 236°C (with decomposition). The structure of compound E was identified by spectroscopic methods as a mixture of 3-O-β-D-glucopyranosyl-24ζ-ethylcholesta-5-ene or β-sitosterol glucoside (C₃₅H₆₀O₆), and 3-O-β-D-glucopyranosyl-24ζ-ethylcholesta-5, 22-diene or stigmasteryl glucoside (C₃₅H₅₈O₆), as shown in Figure 4.

DISCUSSION

This study showed that the methanolic fraction (F3) of *E. elliptilimba* inhibited growth and induced apoptosis of four human leukemic cell lines: NB4, HT93A, Kasumi and K562. After 48 h incubation, the IC₅₀ values of F3 on NB4,

HT93A, Kasumi and K562 were lower than 70 µg/ml. In addition, it significantly induced apoptosis in NB4 and K562 by more than 60% compared to control cells. The result suggested that the herb extract fraction could suppress all four cell lines via the apoptotic process. Therefore, it was further fractionated into 7 fractions: F3.1, F3.2, F3.3, F3.4, F3.5, F3.6 and F3.7 which were examined for antiproliferative activities. F3.6 was shown to have the highest potential anticancer properties with IC₅₀ values on NB4, HT93A, Kasumi and K 562 ranging from 22.68±0.10 to 47.72±0.06 µg/ml. The exhibition of the growth inhibitory effect demonstrated a modest pattern.

From the thin-layer chromatogram of F3, compound E was the most prominent band. It was isolated and identified as the sterols, a mixture of β-sitosterol and stigmasteryl glucosides. Compound E had been reported as plant sterols acting as selective DNA polymerase β lyase inhibitors and also potentiators of bleomycin cytotoxicity in the A549 human lung cancer cell line (Li et al., 2004).

In conclusion, *E. elliptilimba*, a Thai herbal plant commonly used for treating malignancies in traditional medicine had an antiproliferative effect. The plant active fraction (F3.6) contained β-sitosterol and stigmasteryl glucosides (compound E) as major compounds. It will be used as a marker compound in the standardization of the active fraction (F3.6), which will be further studied for antiproliferative effects.

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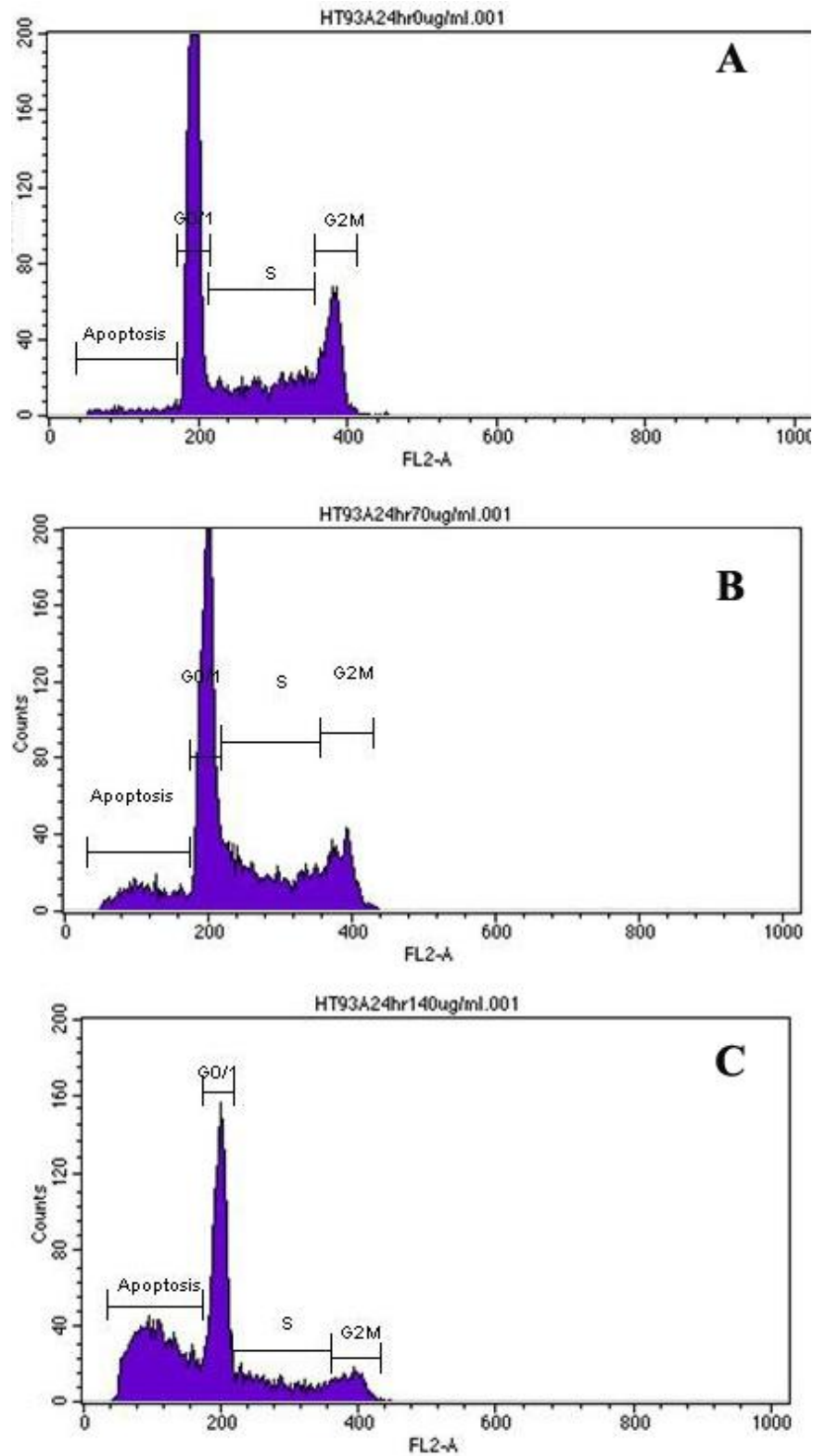
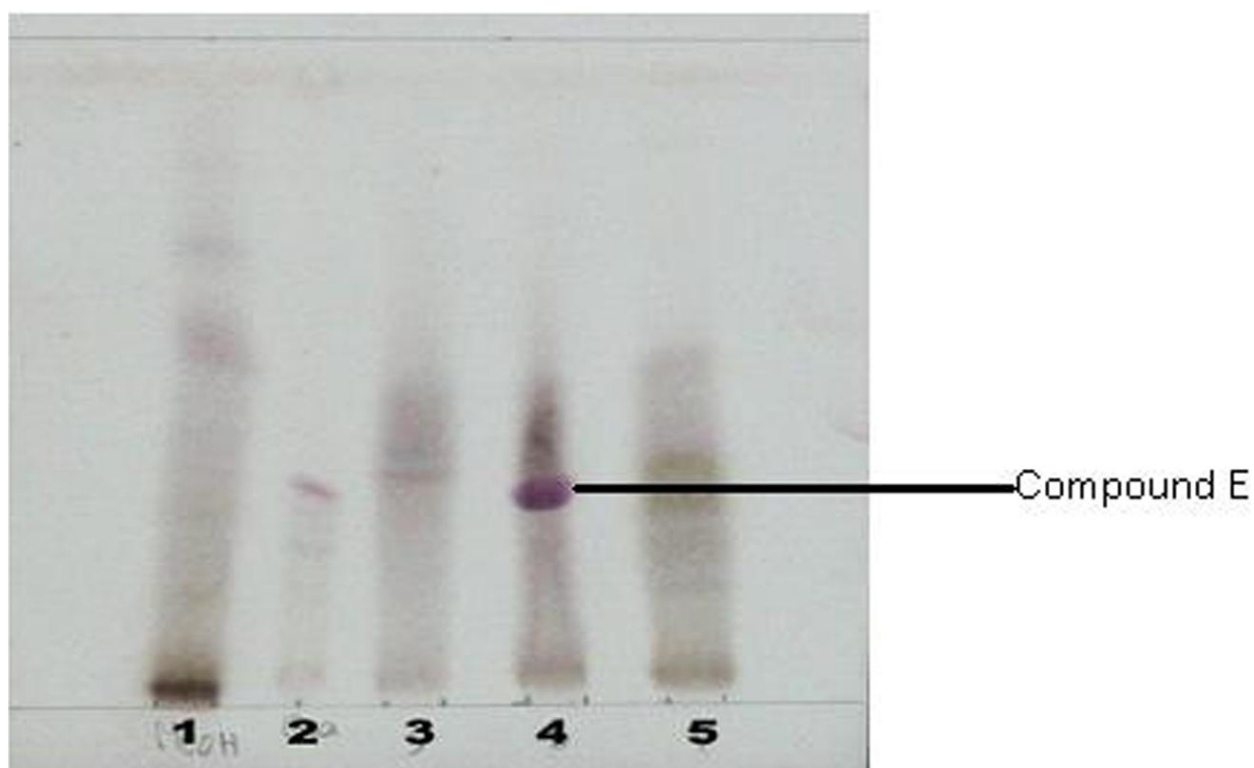


Figure 2. Cell cycle analysis of HT93A promyelocytic cell line by flow cytometry was analysed by using CycleTEST™ DNA Reagent Kit. Cells were treated with *Erycibe elliptilimba* methanol fraction at concentrations of 0 (A), 70 (B) and 140 (C) µg/ml for 24 hours. The percentage of apoptotic cells treated with 140 µg/ml of the herb methanol fraction increased significantly from 2.60 to 40.19 when compared with the control untreated cells.

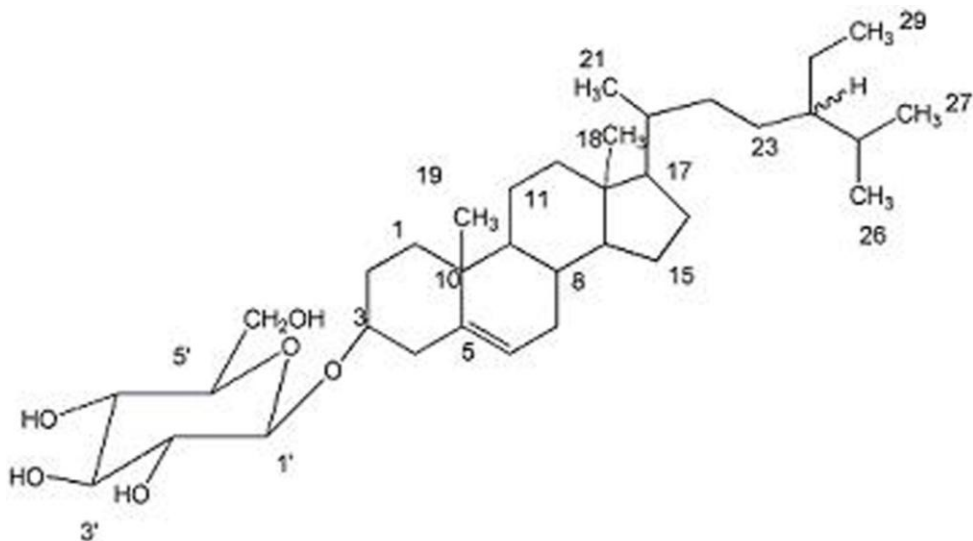
Table 2. Cell cycle analysis by flow cytometry of NB4, K562, HT93A and Kasumi cells treated with herb extract methanol fraction, F3, for 24 h.

Type of cells	Herb ($\mu\text{g/ml}$)	% DNA in cell cycle phase			
		G0/1	S	G2M	Apoptosis
NB4	0	49.03	32.46	17.72	1.71
	50	43.38	16.50	25.95	13.35
	100	3.52	7.66	0.15	89.13
K562	0	39.64	35.39	21.70	5.23
	50	18.29	23.47	12.87	46.11
	100	8.86	23.64	7.29	61.21
HT93A	0	60.58	20.38	17.01	2.60
	70	51.38	26.22	13.50	9.48
	140	40.76	13.48	6.74	40.19
Kasumi	0	74.20	14.55	8.82	2.59
	70	69.09	18.91	4.51	7.95
	140	57.18	13.63	1.89	27.81

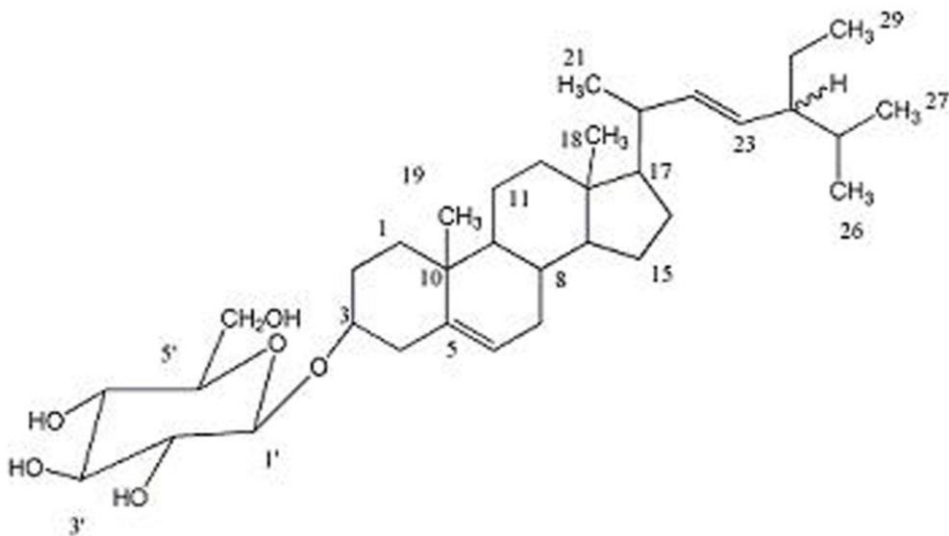
**Figure 3.** Thin-layer chromatogram of the fractions included methanol fraction, F3, (1), phytosteryl glucoside, G1a, (2), F3.5 (3), F3.6 (4) and F3.7 (5) were performed and detected with 10% H_2SO_4 in methanol. Compound E was isolated from F3.6.

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Sitosteryl glucoside, ($C_{35}H_{60}O_6$), $M_R = 576$
(3-O- β -D-glucopyranosyl-24 ζ -ethylcholesta-5-ene, E 1)



Stigmasteryl glucoside, ($C_{35}H_{58}O_6$), $M_R = 574$
(3-O- β -D-glucopyranosyl-24 ζ -ethylcholesta-5, 22-diene, E 2)

Figure 4. Structure of compound E, a mixture of sitosteryl- and stigmasteryl-O- β -D glucopyranoside.

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