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Antiproliferative and Apoptotic Studies of the Standardised Extracts of *Etlingera elatior* on Human Colorectal Carcinoma Cells

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Abstract

Etlingera elatior (Zingiberaceae), locally known as kantan, has been used in Traditional Malay Medicine for various ailments. Plants belong to *Zingiberaceae* family are well known for antiproliferative effects on various cancer cells. Only two preliminary scientific reports were found in the literature on the antiproliferative activity of *E. elatior* on human breast (MCF-7), T-lymphoblastoid (CEM-SS) and glandular cervix (HeLa) cancer cells. Low incidence of colorectal cancer in malay community may be attributed to their dietary habits, especially ulams. Therefore, we investigated the antiproliferative activities of the standardised ethanol (95%) and acetone extracts from leaves, rhizomes, stems and flowers of *E. elatior* on human colorectal cancer (HT-29) and non-cancerous Chinese Hamster Ovary (CHO) cells using MTT Assay. The extracts were standardised by determining their total phenolic content, total flavonoid content and total anthocyanin content. The acetone extract from leaves of *E. elatior* was the most potent with an IC_{50} value of 170±0.05µg/mL on HT-29. It did not inhibit the proliferation of CHO cells at concentrations up to 250 µg/mL. The cell death induced was through apoptosis as detected by phosphatidylserine translocation and caspase-3 activation. The extent of necrosis was negligible compared to the extent of apoptosis.

Introduction

Cancer has been a threat to the human population over several decades. In 2003, colon cancer ranked third in the overall cancer incidence in Malaysia. The Malay population has the lowest colon cancer incidence followed by Indians and Chinese. Lower incidence in Malays may be due to their genetic, culture and daily dietary habits, especially ulams [1].

Etlingera elatior (*E. elatior*) (Zingiberaceae) is most popular and widely used in ulam preparations. It is also known as kantan. The Malay community uses the young inflorescence as "ulam" or as a flavouring agent in laksa and curry with vegetables. The processed flowers and leaves can eliminate body odour [2, 3, 4]. Malays believe that taking the raw inflorescence regularly can reduce the risk of diabetes and hypertension. They also believe that the shredded inflorescence with betel leaf can relieve flatulence, especially after giving birth [5]. Ethanol extract of flower shoots of *E. elatior* have shown cytotoxic activities on the glandular cervix cancer cells (HeLa cells) [6]. Ethyl acetate extract of rhizomes showed cytotoxic effect on human breast (MCF-7) and human T-lymphoblastoid (CEM-SS) cancer cells [4].

Since there was no detailed studies on antiproliferative activity of *E. elatior* and it is known to have beneficial actions on gastrointestinal tract, we investigated the antiproliferative activity of standardised extracts from leaves, rhizomes, stem and flowers of *E. elatior* on human colorectal cancer (HT-29) cells. Its mode of action was also investigated. The extracts were standardised by determining the total phenolic content (TPC), total flavonoid content (TFC) and total anthocyanin content (TAC).

Experimental

Materials

Authenticated fresh plant materials (rhizomes, leaves, flowers, stems) of *E. elatior* were collected from the Floranika Nursery (Sungai Buloh, Malaysia). Human colorectal cancer (HT-29) and Chinese hamster ovary (CHO) cells were purchased from the American Type Cell Culture (ATCC) (Rockville, MD, USA). The following reagents and chemicals were obtained from respective suppliers: 5-fluorouracil (Calbiochem, China); acetone (Merck, Germany); dimethylsulfoxide, 95% ethanol, sodium carbonate anhydrous, gallic acid monohydrate, quercetin dihydrate, 2M Folin-Ciocalteu Reagent (Sigma, USA); potassium chloride (Darmstadt, Germany); aluminium chloride, sodium acetate anhydrous (Systerm, Malaysia); Dulbecco's Modified Eagle Medium (DMEM), phosphate-buffered saline (PBS) (Mediatech Inc., USA); fetal bovine serum (FBS), penicillin-streptomycin, sodium pyruvate (PAA Laboratories, Austria); accutase™ (Innovative Cell Technologies, USA); trypan blue (Gibco, USA); MTT Assay Kit and Dual Apoptosis Kit (Biotium, USA); Cell Death Detection ELISA^{PLUS} Kit (Roche, Germany)

Preparation of E. elatior Extracts

The *E. elatior* plant parts (rhizomes, leaves, flowers, stems) were collected and authenticated. Fresh plant materials were washed to remove dirt, cut into small pierces and were dried in a hot air oven (at temperature not more than 50^{o}C) until complete dryness. The dried plant materials were powdered using an electric

blender. Extraction of powdered dried plant materials was carried out with ethanol (95%) and acetone separately in a soxhlet extractor until the solution becomes colourless in the siphon of the extractor. The extracts were filtered under vacuum, concentrated in a rotary evaporator at temperature not more than 50ºC and lyophilised using a freeze dryer until constant weight was obtained. Solution of dimethyl sulfoxide in water, DMSO (50%) was used as a vehicle to dissolve the extracts. The extracts were filtered through a 0.22µm nylon filter and were kept under sterile condition until further experimentation.

Standardisation of Extracts

Total phenolic content (TPC) of extracts was determined using the Folin-Ciocalteu assay reported by Singleton and Rossi [7]. TPC of the extracts were determined by measuring the absorbance of the solutions at 765nm. TPC was expressed as gallic acid equivalents (GAE) in milligram per 100g of *E. elatior*. The calibration equation for gallic acid was

$$y = 0.7075x - 0.0595\ (R^2 = 0.9922)$$

Total flavonoid content (TFC) was determined according to the procedure reported by Woisky and Salatino [8]. TFC of the extracts were determined by recording the absorbance of the extract solutions at 415nm. TFC was expressed as quercetin equivalent (QE) in milligram per 100g of *E. elatior*. The calibration equation for quercetin was

$$y = 0.033x - 0.021\ (R^2 = 0.9922)$$

Total anthocyanin content (TAC) of extracts was determined using the pH differentiate method as reported by Fuleki and Francis [9]. The total anthocyanin content was expressed as cyanidin-3-glucoside equivalents (CGE) in milligram per 100g of fresh plant.

Cell Culture

Human colorectal cancer (HT-29) and Chinese Harmster Ovary (CHO) cells, were cultured in DMEM complete medium, with 10% FBS, 1% penicillin-streptomycin and 1% sodium pyruvate in a humidified 5% carbon dioxide incubator. HT-29 and CHO cells were cultured using the standard culture procedure. Accutase™ was used to detach the cells from culture flasks.

Cell Proliferation Assay

It was carried out using MTT assay kit according to the manufacturer's protocol. Briefly, 99µL of cell suspension containing 10,000 cells were seeded into each well of a sterile 96-well plate and incubated for 24 hours at 37°C in a humidified incubator. The ethanol and acetone extracts of rhizomes, leaves, flowers and stems from *E. elatior* were tested at concentrations ranging from 50 to 250µg/mL. The final volume in each well was 100µL and final concentration of DMSO in each well was 0.5%. The plates were further incubated for 48 hours. Five microliters MTT reagent was added to each well and incubated for 4 hours. The plates were centrifuged at 1000rpm for 5 min at 4°C. MTT solution and medium were aspirated from the wells without disturbing the formazan crystals at the bottom of the well. Buffered DMSO (200µL) was added into each well to dissolve the formazan crystals. The plates were shaken for five minutes and the absorbance was recorded on a microplate reader at the wavelength of 570nm and a reference wavelength of 630nm. The percentage of cell viability of treated cells was calculated using the following formula:

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of the treated cells - Absorbance of the blank}}{\text{Absorbance of the negative control - Absorbance of the blank}} \times 100\%$$

5-Fluorouracil was used as a positive control while 0.5% DMSO was used as a negative control. Negative control did not affect the proliferation of cells. Cell proliferation at each concentration of the extract was tested in triplicate in each 96-well plate and whole experiment was repeated three times. The IC_{50} value for each extract was determined from dose-response curve.

Determination of Apoptotic Activity of Extracts

In order to determine the mode of cancer cell death, apoptosis induced by the extracts was determined using dual apoptosis assay kit according to the manufacturer's protocol and details were published in our previous report [10]. The advantage of using this kit is that it can detect two important events of apoptosis in a single experiment. It can detect the presence of phosphatidylserine (PS) on the outer leaflet of cell membrane, which occurs at the early stage of apoptosis and activation caspase-3 which occurs at the terminal stage of apoptosis. If both events are present, it is confirmed that the apoptosis is taking place. PS translocation and caspases-3 activation were identified by observing the stained cells using Texas-Red filter (510 nm-560 nm) and fluorescein isothiocyanate (FITC) filter (450-490 nm) respectively under fluorescent microscope (Eclipse 80i, Nikon, Japan).

The extent of apoptosis and necrosis induced by extracts was quantified using Cell Death Detection ELISA^{PLUS} assay as recommended by the manufacturer and experimental details were published in our previous report [10]. It was expressed as enrichment factor which was calculated using the following formula.

$$\text{Enrichment Factor} = \frac{\text{Absorbance of the sample - Absorbance of blank}}{\text{Absorbance of the negative control - Absorbance of blank}}$$

Statistical Analysis

All data were reported as means ± standard deviation (S.D.) from three individual experiments. Statistical difference between treated and negative control was analysed using one-way analysis of variance (ANOVA) followed by *post hoc* Dunnet's *t*-test. If *p* value less than 0.05 ($p<0.05$), the difference was considered as statistical significant. The SPSS (version 15.0) for Windows was used to analyse the results.

Results

Standardisation of Extracts

TPC, TFC and TAC of extracts were shown in the Table 1. In general, TPC and TFC content of ethanol extracts were higher than those of acetone extract for all parts. TAC was detected only in flowers.

Table 1: Total Phenolic Content, Total Flavonoid Content and Total Anthocyanin Content of *Etlingera elatior* Extracts

Etlingera elatior's Part	Extraction Solvent	TPC (mg GAE per 100g fresh plant)	TFC (mg QE per 100g fresh plant)	TAC (mg of CGE per 100g fresh plant)
Leaves	Ethanol	3064 ± 52	320.4 ± 14.2	-
	Acetone	902 ± 21	13.9 ± 2.3	-
Flowers	Ethanol	1928 ± 116	324.8 ± 3.7	-
	Acetone	244 ± 3	17.6 ± 0.3	1.05 ± 0.13
Rhizomes	Ethanol	642 ± 11	-	-
	Acetone	393 ± 5	23.7 ± 1.7	-
Stems	Ethanol	556 ± 14	37.9 ± 1.5	-
	Acetone	228 ± 22	16.0 ± 1.3	-

The values are mean± SD. The symbol "-" indicates the extract does not contain any flavonoid or anthocyanin. GAE, QE and CGE are gallic acid equivalent, quercetin equivalent and cyaniding-3-glucoside equivalents respectively.

Antiproliferative Activities of Extracts on HT-29 and CHO Cells

Ethanol extracts from all parts of *E. elatior* did not inhibit the proliferation of HT-29 cells at all concentrations. Among the acetone extracts, only leaves extract showed an IC_{50} value less than 250µg/mL, which was 170±0.05µg/mL (Figure 1).

Since acetone extract from leaves of *E. elatior* was the most potent, it was further tested for its antiproliferative activity on CHO cells to determine the selectivity of extract towards cancerous cells (figure 2). The acetone extract of leaves did not inhibit the proliferation of CHO cells at all concentrations.

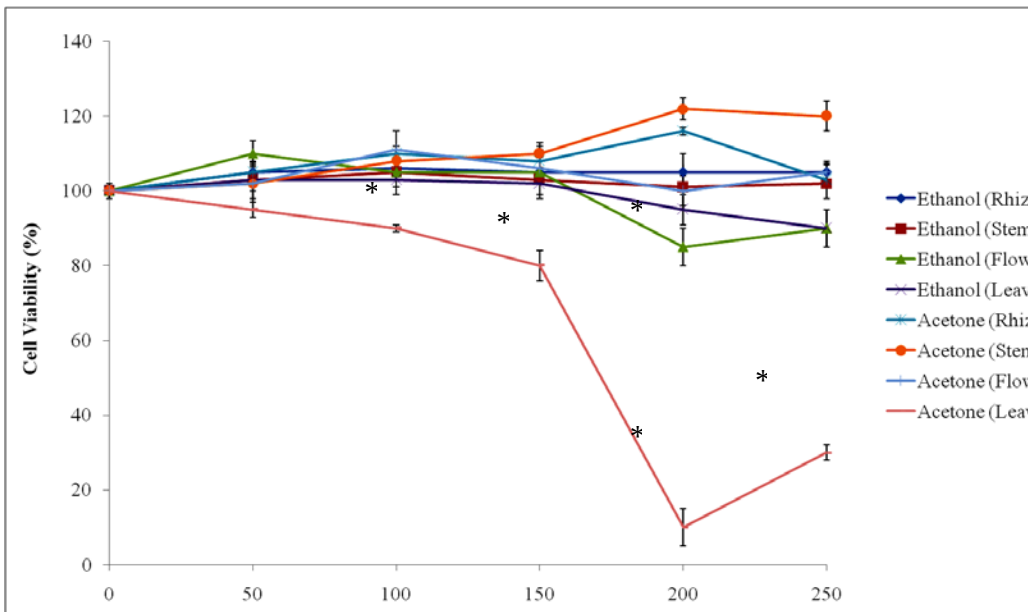

Figure 1. Antiproliferative activities of *E. elatior* ethanol (95%) and acetone extracts on HT-29 cells. Results are expressed as percentage of cell viability (mean ± S.D. of triplicate). Statistical significant different from the negative control are indicated as * $p<0.05$ by one-way ANOVA *post hoc* Dunnet's *t*-test.

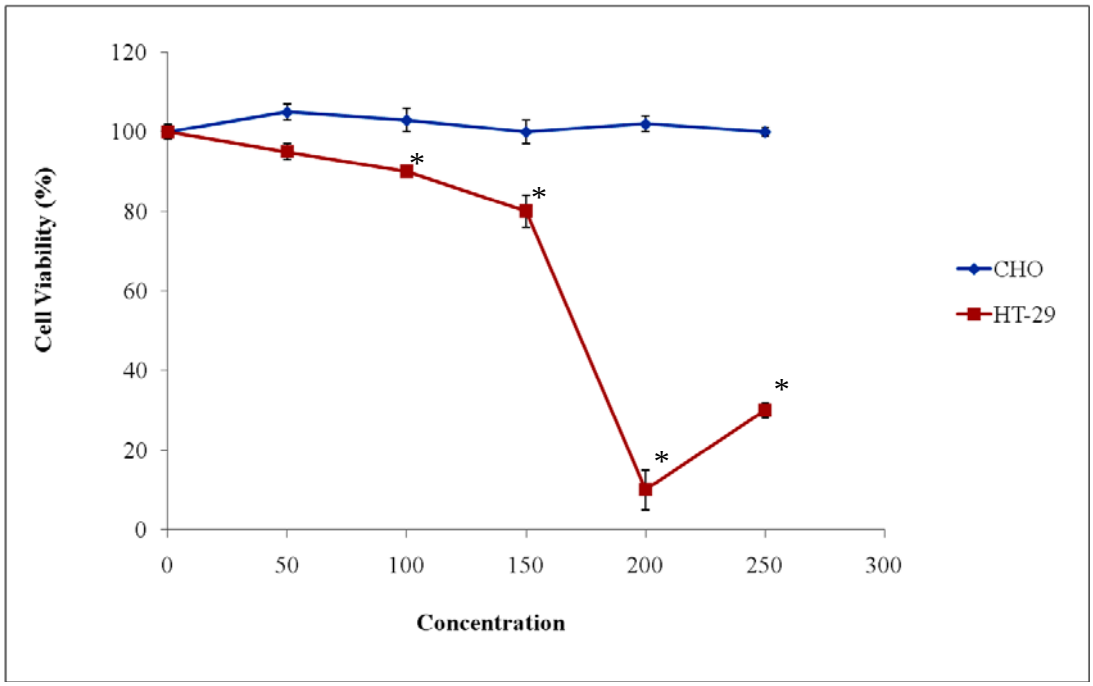

Figure 2. Antiproliferative activities of acetone extract of *E. elatior* **leaves on CHO and HT-29 cells.** Results are expressed as percentage of cell viability (mean ± S.D. of triplicate). Statistical significant different from the control are indicated as **p*<0.05 by one-way ANOVA *post hoc* Dunnet's *t*-test.

Apoptotic Activity of Acetone Extracts from E. elatior Leaves

Cell death induced by acetone extract from leaves of *E. elatior* was investigated for apoptotic activity by monitoring the PS translocation and caspases-3 activation. The morphological changes were observed under microscope. As showed in figure 3, at the 6 hours, the outer surface of most of the treated HT-29 cells was stained red ring, indicated PS translocation. While at 12 hours, most of the nuclei of cells were stained green and membrane was stained with a red ring, which indicated both PS translocation and caspase-3 activation. Apoptosis was confirmed on HT-29 cells which stained with both indicators.

To quantitate and further support the apoptotic activity of acetone extract from *E. elatior* leaves, we investigated the extent of apoptosis and necrosis induced using the Cell Death Detection ELISA^{PLUS} kit. Compared to negative control, the leaves extract induced 2.353±0.009-fold and 0.229±0.003-fold increases in apoptosis and necrosis respectively (Figure 4).

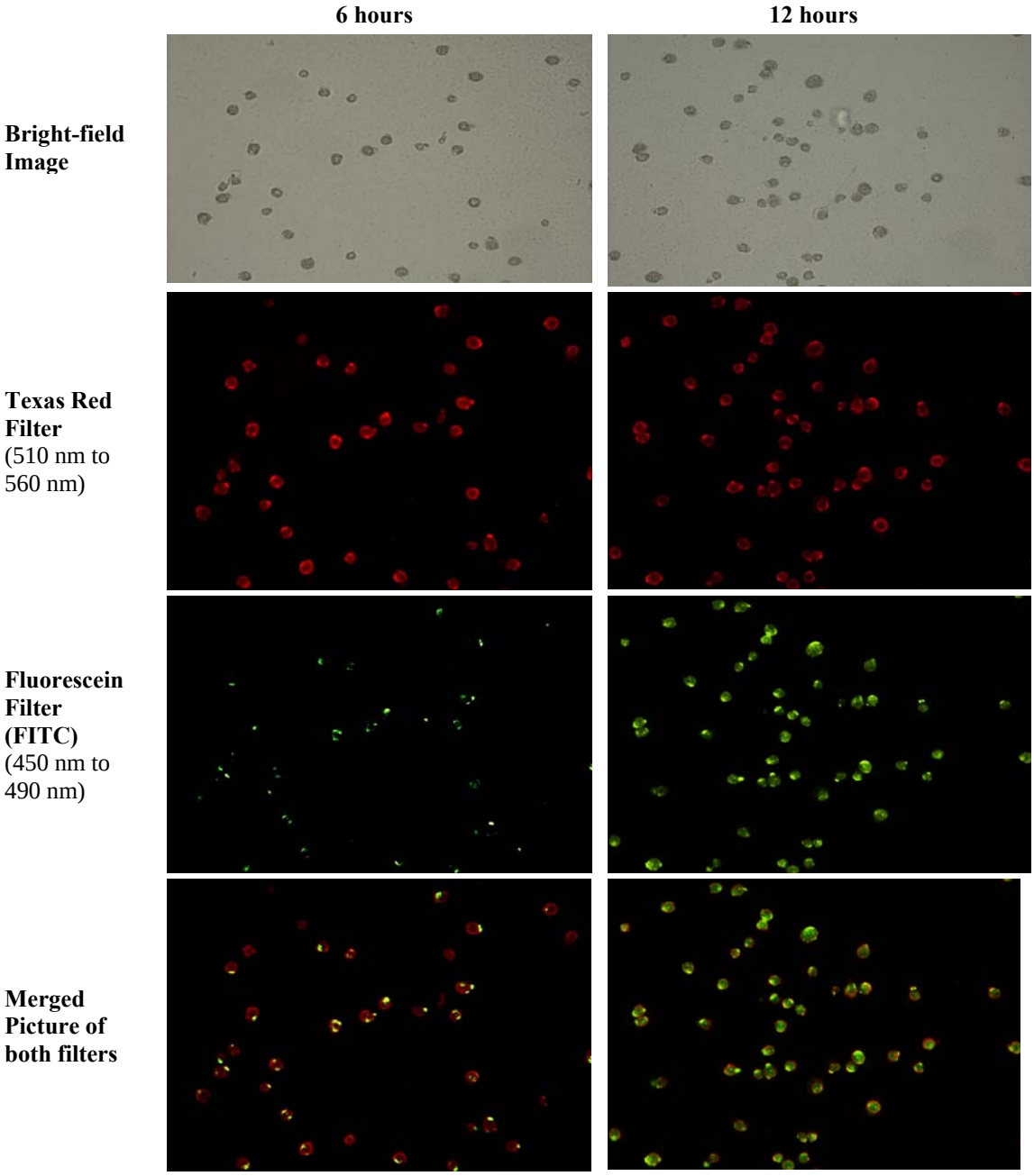

Figure 3. Treated HT-29 cells with 200µg/mL of acetone extract of leaves stained with sulforhodamine-101 Annexin V and NucView™ 488 Caspase-3 substrate. The images were taken using Nikon Eclipse 80i fluorescent microscope at 200× magnification.

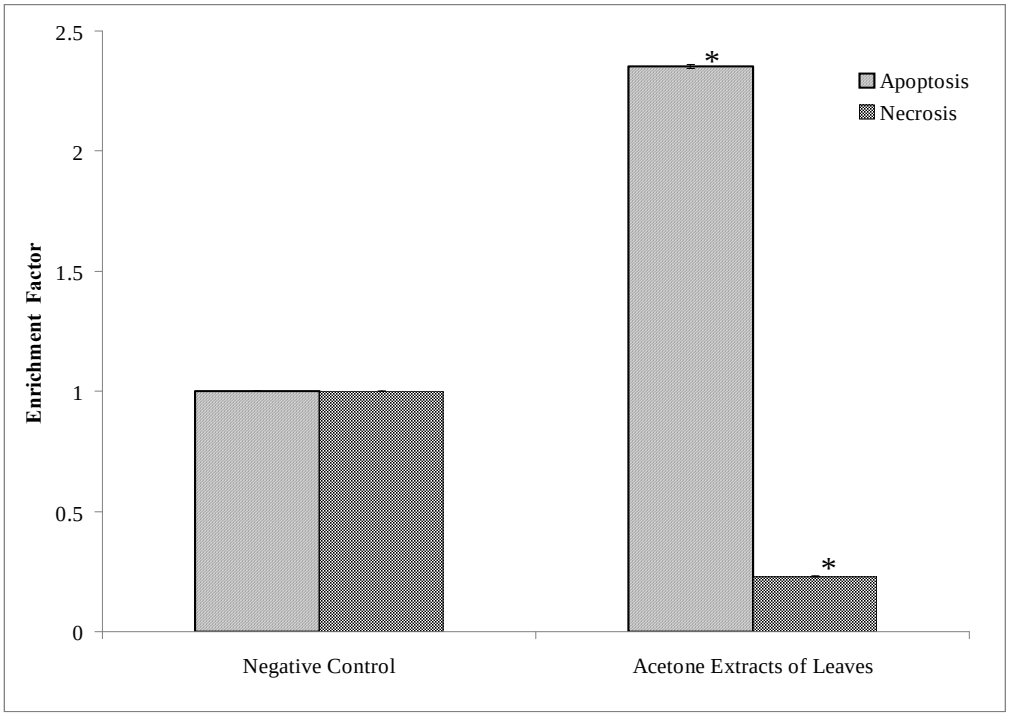

Figure 4. Effect of acetone extract of leaves on apoptosis induction on HT-29 cells. Enrichment factor (mean ± S.D) was calculated as the ratio between the absorbance of treated and negative control. Statistical significant different from the control are indicated as **p*<0.05 by one-way ANOVA *post hoc* Dunnet's *t*-test.

Discussion

Past research studies on *E. elatior* showed beneficial effects on MCF-7, CEM-SS and HeLa cells [4,6]. However these studies were confined only to the roots and flowers and mode of cell death induced by it was not reported. In our study, we investigated the antiproliferative activities of standardised extracts from all parts of *E. elatior* on HT-29 cells using MTT assay. The mode of cell death induced by it was also investigated by monitoring the two important apoptotic events and quantifying the extent of apoptosis and necrosis.

Even though TPC and TFC are higher in ethanol extracts compared to acetone extracts, ethanol extracts did not inhibit the proliferation of cells. These results suggest that only few of the phenolic and flavonoid compounds present in extracts are inhibiting the proliferation of cells. The other possible reason for the ethanol extract not showing activity could be the presence of inorganic elements, sugars and amino acids [2]. Since ethanol is more polar than acetone, ethanol can extract all these compounds where as acetone cannot. Therefore, the effective concentration of the active compounds in ethanol extract would be less than that in acetone extract at concentrations used in this study.

Antiproliferative activity of acetone extracts are in good correlation with their TPC. Rhizomes contain highest TFC followed by flowers, stems and leaves. The antiproliferative activity of different parts of *E. elatior* was not in good agreement with their TFC. These results suggest that the phenolic compounds which are not flavonoid in nature may be responsible for its antiproliferative activity.

The acetone extract of leaves had shown good selectivity towards human colorectal cancerous cells compared to normal Chinese hamster ovary cells. Its IC_{50} value on HT-29 was 170±0.05 µg/mL whereas it did not inhibit the proliferation of CHO cells at all concentrations.

Cell growth reduction can be attributed to a decreased proliferation rate or an enhanced cell death by apoptosis or necrosis. Apoptosis is a programmed cell death, which eliminates redundant or damaged cells. Cancer cells have deregulated proliferation and they are not able to undergo apoptosis naturally. Therefore, this study also investigated whether the extracts can induce apoptosis by monitoring both apoptotic markers, phosphatidylserine (PS) translocation and caspase-3 activation. The acetone extract of leaves induced apoptosis changes on HT-29 cells. At the initial stage of apoptosis, PS in apoptotic cells of HT-29 cells was translocated from inner to outer leaflet of the plasma membrane. Sulforhodamine 101-annexin V, which has high affinity to PS, stained the surface of the apoptotic cells. At the later stage of apoptosis, cells undergone caspase-3 activation and PS translocation [10].

To quantify and further support the finding that acetone extract of leaves causes apoptosis on HT-29 cells, this study used the Cell Death Detection ELISA^{PLUS} kit. In apoptotic cells, the DNA fragments remain intact within the cell membrane or apoptotic body. Conversely, lysosomal enzymes in necrotic cells digest the cell membrane and cause the release of DNA

fragments from the cells. By quantifying the DNA fragments, the level of apoptosis and necrosis induction on HT-29 cells treated with the acetone extract of leaves were studied [10]. At the concentration close to $IC_{50,}$ acetone extract of leaves induced 2.353±0.009-fold increases in apoptosis and 0.229±0.003-fold increases in necrosis compared to negative control on HT-29 cells. These findings correspond with the results from the proliferation assays and suggest that antiproliferative activity of acetone extract of leaves appears to be explained in part by only the induction of apoptosis.

Conclusion

E. elatior extracts (ethanol (95%) and acetone) showed antiproliferative activities on HT-29 cells. Interestingly, acetone extract of leaves, the most potent extract, exhibited less inhibition of non-cancerous cells, CHO cells. Its apoptosis induction was confirmed with the detection of PS translocation and caspase-3 activation. At the concentration close to $IC_{50,}$ acetone extract of leaves induced 2.353±0.003-fold increase in apoptosis and negligible increase in necrosis compared to negative control on HT-29 cells. Thus, the mechanism of cell death was suggested to be through apoptosis. Further identification and isolation of the compounds from the plant is important in investigating the compounds that are responsible for the activity.

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