widely grows and is cultivated in Southeast Asia for medicinal purposes. Conventionally, it is used to treat stomach aches and wounds, relieve headaches, and increase appetite.[6] The major active compound present in *Z. zerumbet* is zerumbone.[7] Kirana *et al.* discovered that zerumbone is effective as an anticancer agent through the stimulation of apoptosis and antiproliferation effects.[8] Studies have shown that zerumbone can also act as antileukemic drugs by inhibiting the growth of leukemia cells through cytotoxic effects and continuous induction of apoptosis toward leukemic cells.[9] Previous studies also found that zerumbone showed cytotoxic effects on leukemia cell line K-562.[10] Kaempferol, one of the active compounds isolated from this plant, has been found to increase the effect of cisplatin by promoting the formation of apoptosis in ovarian cancer cells.[11] Kaempferol and quercetin are believed to provide synergistic effect in reducing breast cancer cell division.[12] Therefore, a combination of these active compounds that might be present in the ethyl acetate extract of the rhizome has to be studied to identify its effectiveness as cytotoxic inducing agent through the changes in morphology of the cell. Therefore, the objectives of this study were to determine the cytotoxic effect and morphological changes of *Z. zerumbet* ethyl acetate crude extract on leukemia cell line K-562 using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and phase-contrast microscopy.

MATERIALS AND METHODS

Extraction of *Zingiber zerumbet*

Rhizomes of *Z. zerumbet* (voucher specimen UKMB-29952 deposited in the herbarium of the Faculty of Science and Technology, UKM) were obtained from a herbal farm in Temerloh, Pahang. The rhizomes were extracted using ethyl acetate extraction. Stocks from extract solution were prepared by dissolving the 2 mg of extract *Z. zerumbet* with 1 ml dimethylsulfoxide (DMSO) to produce 2 mg/ml of stock concentration.

Media, reagents, and chemicals

Eagle's minimum essential medium, Dulbecco's modified Eagle's medium, Iscove's modified Dulbecco's medium, solution of MTT, phosphate buffer saline, trypsin/ethylenediaminetetraacetic acid 0.25%, and doxorubicin hydrochloride were used in this study.

Cell Line

Cell line used is K-562 leukemia cells (erythroleukemia) obtained from the American Type Culture Collection. Cells were cultured in Biocompatibility and Toxicology Laboratory, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur Campus.

Ethyl acetate extraction of *Zingiber zerumbet* rhizomes

Z. zerumbet rhizomes that had been dried were immersed in hexane for 3 days at room temperature. The extract obtained was then filtered to separate the extractant from the rhizomes. Filtrate used was hexane and the immersion process was performed three times for the same solvent. This extraction process was repeated with ethyl acetate to yield ethyl acetate extract. Rotary evaporator was used to separate the solvent from the extract samples, thus producing the concentrated extract.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Cell viability for K-562 leukemia cells was determined using MTT assay by Mosmann's method.[13] All cells were treated with the extract of *Z. zerumbet* and doxorubicin (positive control), based on their exposure to time point, respectively. After the incubation period, 20 µl of MTT solution was added into each well and incubated for 4 h at 37°C. After that, 200 µl of the solution was removed from each well without disturbing the formazan crystals formed in the bottom of the 96-well plate. Then, 180 µl DMSO was added to each well to dissolve the formazan crystals and incubated again for 15 min. The absorbance was determined by ELISA plate reader at 570 nm. The IC_{50} was obtained from the viability curves and assessed as the concentration that reduces the percentage of viability of cell to 50% as compared to controls. The level of toxicity of the extract can be classified into four categories based on IC_{50} toxicity cell viability value according to How *et al.*[14]

Observations of cell morphology changes

K-562 cell was cultured in 6-well plates treated with IC_{50} concentrations of extracts and doxorubicin for 24 and 48 h. Then, the observation of morphology was done using phase-contrast microscopy.

Statistical analysis

The results were analyzed statistically and are expressed by means ± standard error of mean using SPSS version 17 (IBM Corporation, Chicago, United States), while the morphological changes were analyzed descriptively.

RESULTS AND DISCUSSION

Cytotoxicity Effects of *Zingiber Zerumbet* extract on K-652 Cell Line

For cells treated with the extract of *Z. zerumbet* at 48 and 72 h, the graph showed that the IC_{50} values obtained are 13.83 ± 1.82 and 10.13 ± 1.67 µg/ml, respectively [Figure 1 and Table 1]. The cytotoxic effects of this extract on K-562 cells therefore fall into less

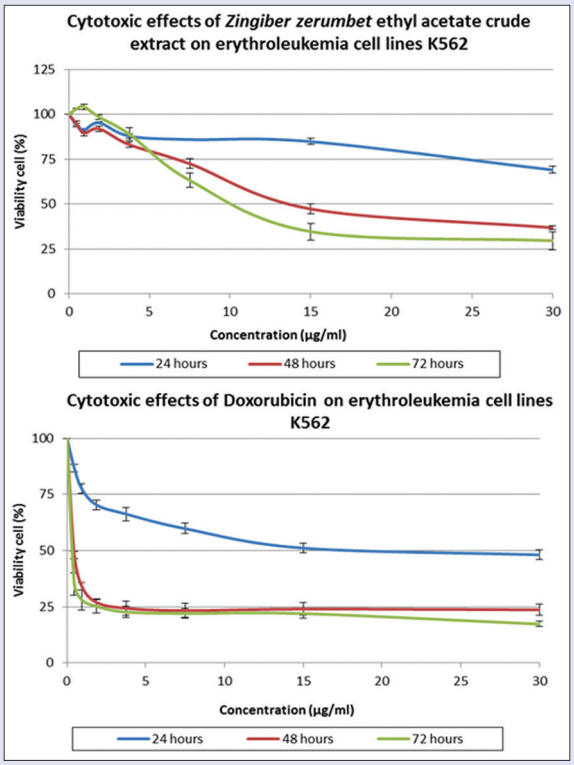

Figure 1: Cytotoxic effects of *Zingiber zerumbet* ethyl acetate extract and doxorubicin on K-562 erythroleukemia cell line at 24, 48, and 72 h of exposure time

toxic categories as described in How *et al.* [Table 2]. This result is also supported by the plant screening program conducted by the National Cancer Institute (USA) that reported a crude extract is considered as having cytotoxic activity to the cancer cell if it gives the IC_{50} value <20 µg/ml. A previous study that tested the main active ingredient of this plant, which is zerumbone (found in this extract), reported that it possessed cytotoxic effects and inhibited the cell growth of erythroleukemia cell line K-562.[10] The active ingredient also showed cytotoxic and antiproliferative effects on cancer cells and was postulated as a potential chemoprevention agent.[15,16] There was also a significant decrease in cell viability occurred with the increasing of the extract concentration and time. This suggests that the ethyl acetate crude extract of *Z. zerumbet* on erythroleukemia cell line K-562 showed cytotoxic effects in a dose- and time-dependent manner. This study is supported by previous studies which prove that the active ingredient found in *Z. zerumbet,* zerumbone, can inhibit the growth of leukemia cell, HL-60, in a time- and dose-dependent manner.[17,18]

When comparing the potential of the combination of various active compounds in this extract on K-562 erythroleukemia cell line with pure compound zerumbone as shown by Asmah *et al.*,[10] the IC_{50} value of K-562 cells treated with crude extract of *Z. zerumbet* is 10.133 ± 1.665 µg/ml, while the IC_{50} for cells treated with zerumbone is 9.72 ± 0.076 µg/ml. Both IC_{50} values for these two studies showed an almost similar value. Therefore, it can be concluded that the crude extract of *Z. zerumbet* was able to give almost the same cytotoxic effect as the main active compound found in this plant, zerumbone, at almost the same concentration. This is because the rhizomes of *Z. zerumbet* contained high concentration of zerumbone that can inhibit the growth of leukemia cells by cytotoxic effects and followed by apoptosis induction.[19]

Morphological changes

For the K-562 cells treated with *Z. zerumbet* extract, the morphological changes can clearly be seen such as cell shrinkage (yellow arrows), membrane blebbing (red arrows), and formation of apoptotic bodies (blue arrows) which are the characteristics of apoptosis [Figure 2]. While cells treated with doxorubicin showed cell shrinkage and some cells showed swelling (green arrows), which is an early sign of necrosis. Meanwhile, in the case of the cells treated with doxorubicin, the number of cells observed decreased rapidly and morphological characteristics such as cell shrinkage and membrane blebbing were also observed.

Morphological characteristics for K-562 cells were similar as shown in the studies done by Asmah *et al.*[10] which revealed features of apoptosis such as cell shrinkage, membrane blebbing, and formation of apoptotic bodies. These features of apoptosis are in line with the description by Kerr *et al.* in 1972. According to Sakinah *et al.*, zerumbone can stimulate the HepG2 cell apoptosis through regulation of Bax/Bcl-protein through functional p53 activity.[20] This findings is also in agreement by Murakami et al. (2002) and Murakami et al. (2004) which revealed an apoptotic effects of Zerumbone on colon cancer cell lines and skin tumour respectively.[21,22]

CONCLUSIONS

This study reveals that *Z. zerumbet* ethyl acetate extract exhibited cytotoxic effect, which is in a less toxic category on erythroleukemia cell line K-562. Based on the morphological changes, *Z. zerumbet* ethyl acetate extract induced apoptosis-related morphological changes on apoptosis-resistant K-562 cells as an event of cytotoxicity. Therefore, further studies are needed to ascertain its mechanism of action and its potential as antileukemia agent.

Table 1: IC_{50} values for erythroleukemia K-562 treated with *Zingiber zerumbet* ethyl acetate crude extract and doxorubicin as positive control

K-562 cell treatment	IC_{50} (µg/ml±SEM)		
	24 h	48 h	72 h
Zingiber zerumbet ethyl acetate crude extract	ND	13.83±1.82	10.13±1.67
Doxorubicin	17.88±3.36	0.44±0.03	0.33±0.06

ND: Not detected; SEM: Standard error of mean

Table 2: Category of the toxicity based on IC_{50} value

Category	IC_{50} value (µg/ml)
Very toxic	<5.0
Medium toxic	5.0≤10
Less toxic	10.0-25.0
Not toxic	≥25

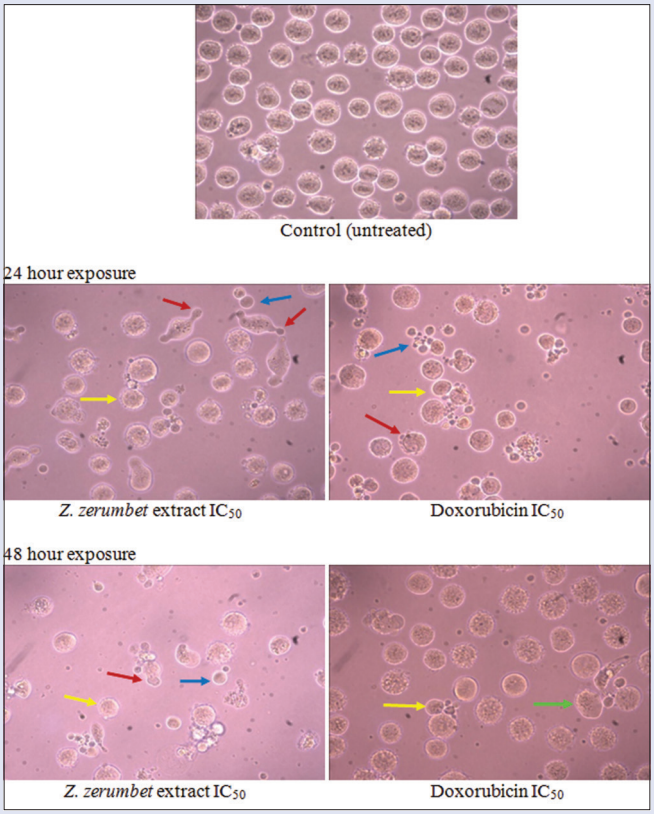

Figure 2: Morphological changes seen in all treated cells with IC_{50} of *Zingiber zerumbet* extract and doxorubicin following 24 and 48 h exposure. Among the features seen are cell shrinkage (yellow arrows), membrane blebbing (red arrows), formation of apoptotic bodies (blue arrows), and cell swelling (green arrows) (×400)

Financial support and sponsorship

This work was financially funded by Ministry of Education Fundamental Research Grant Scheme (UKM-NN-07-FRGS-0152-2010).

Conflicts of interest

There are no conflicts of interest.

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