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Activity of α-terpineol as a potential anticancer candidate: cytotoxicity, proapoptotic and antiproliferative evaluation in TD47 cell lines

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ABSTRACT

Previous study has shown that α-terpineol has a cytotoxic effect on NCI-H69 cancer cell line. This study aimed to evaluate cytotoxicity, proapoptotic, and antiproliferative activities of α-terpineol in T47D cell lines. The cytotoxic activity of α-terpineol was determined using MTT cell viability assay, while apoptosis was tested by the acridine orange-ethidium bromide staining method. The cell cycle was evaluated by flowcytometry method. The results showed that α-terpineol had cytotoxic effect on the T47D cells with the IC_{50} value of 135.00 ± 8.74 μM. Furthermore, α-terpineol induced the cells apoptosis in a dose-dependent manner. Flowcytometry analysis showed that α-terpineol induced the T47D cell distribution in G_o/G_1 phase, however inhibited cell distribution in S phase. This study suggests that α-terpineol has a potential anticancer activity.

ABSTRAK

Penelitian sebelumnya menunjukkan bahwa α-terpineol mempunyai efek sitotoksik pada sel kanker NCI-H69. Penelitian ini bertujuan mengkaji aktivitas sitotoksisitas, proapoptotik dan antiproliferatif dari alpfa terpinol in sel kanker T47D. Aktivitas sitotoksisitas α-terpineol ditentukan dengan MTT *cell viability assay*, sedangkan apoptosis diuji dengan metode pewarnaan akridin oranye-etidium bromida. Siklus sel dievaluasi dengan metode flowsitometri. Hasil penelitian menunjukkan α-terpineol mempunyai efek sitotokik pada sel T47D dengan nilai IC_{50} 135,00 ± 8.74 μM. Selain itu α-terpineol menginduksi apoptosis yang bergantung konsentrasi. Analisis flowsitometri menunjukkan α-terpineol menginduksi distribusi sel T47D pada fase G_o/G_1, namun demikian menghambat distribusi sel pada fase S. Penelitian ini menunjukkan bahwa α-terpineol mempunyai aktivitas antikanker potensial.

Keywords: α-terpineol - T47D cell line - cytotoxicity - proapoptotic - antiproliferative

INTRODUCTION

Cancer remains as a major health problem in the world. In the United States, 1,529,560 cases of cancer were reported with 569,490 deaths in 2010.[1] These cases have increased over the previous year amounting to 562,341.[2] Furthermore, it was estimated that by 2030, the cancer cases will reach approximately 26

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million cases and 17 million deaths around the world.[3]

Breast cancer is the most common cancer in women.[1] In the 2007 data obtained from Dharmais Hospital, National Cancer Center, Jakarta, it was indicated that 437 breast cancer patients had been hospitalized among a total of 1,264 outpatients.[4] Gerber *et al.*[5] reported that 40% of patients experienced recurrence of breast cancer and most of them died from it. Breast cancer is one of the cancers with the highest incidence of death in the world. In 2004, it was reported that there were 519,000 deaths due to breast cancer around the world.[6]

Chemotherapy is one of the breast cancer managements which is normally performed in conjunction with surgery and radiotherapy.[7] However, chemotherapy owns disadvantages ranging from adverse or toxic effects of anticancer to patients' death.[8] Moreover, resistance to anticancer remains a major problem in chemotherapy. The problems in chemotherapy make the availability of a new anticancer that is more sensitive and specific urgently needed. One of the strategies in the search for new anticancer molecules is exploration of active constituents from natural resources.[9]

α-Terpineol is one potential natural anticancer isolated from *Pinus merkusii,* an endogenous Indonesian plant. The potency of α-terpineol as anticancer candidate has been reported by some authors. α-Terpineol and two other active constituents namely linlyl acetate and camphor isolated from *Salvia libanotica* have been proven to be able to induce cell cycle and apoptosis of colon cancer HCT-116 cells *in vitro* through caspase activation, cytochrome-c release and PARP cleavage.[10] In addition, the study of Hassan *et al.*[11] proved that α-terpineol is able to prevent the MCF-7 and HeLa cells growth by inhibiting NF-kB signaling pathway.

α-Terpineol, which is derived from the sap processing of *P. merkusii*, is relatively abundant.[12] α-Terpineol is the major component of terpineol which is usually present in a mixture of three isomers namely α-, β- and γ-terpineol.[13] Terpineol can be synthesized from turpentine oil which is a non timber product of *P. merkusii* obtained from the sap without having to chop down the tree. This study was conducted to evaluate the cytotoxicity of the α-terpineol synthesized from turpentine oil in TD47D cancer cell lines. The effect of the α-terpineol on the apoptosis and proliferation of the T47D cells were also evaluated.

MATERIALS AND METHODS

Chemicals

α-Terpineol was obtained and synthesized by Prof Arief Budiman from Department of Chemical Engineering, Faculty of Engineering, Universitas Gadjah Mada. Doxorubicin (Ebedoxo, Ebewe Pharma), RPMI 1640 medium (Gibco), fetal bovine serum (FBS) (Gibco), amphotericin B (Gibco), L-glutamine (Sigma-Aldrich), penicillin-streptomycin (Penstrep®-Gibco), trypsin EDTA (Gibco), 4-(2-hydrocyethyl)-piperazine-ethane) sulphonic acid (HEPES) (Sigma-Aldrich), sodium bicarbonat (Nacalai Tesque), phosphate buffer saline (PBS) (Invitrogen), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Bio Basic Inc.), sodium dodesil sulfat (SDS) (Merck), acid chloride (Merck), ethidium bromide (Promega Corp.), acridine orange (Sigma-Aldrich), and propidium iodide reagent (Becton-Dickinson) were used in this study.

Cell cultures

T47D cells were cultured in culture flasks containing complete RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/mL of streptomycin, and 100 mg/mL of penicillin. Cultures were maintained in 5% CO_2 incubator at 37°C and fed every 3

days with complete RPMI 1640 medium. Confluent cells were trypsinized, and harvested cells were used for experiments.

MTT assay

Cytotoxicity of α-terpineol was evaluated on TD47D cells using the MTT assay as reported by Mosmann after modification.[14] Cells were distributed in 96-wells microplates at 1 x 10^4 cells per well in 100 mL and then 100 mL of complete RPMI 1640 medium were added. The cell cultures were then incubated in 5% CO_2 incubator at 37°C for 24 hours. After incubation, the medium was removed and replaced with new complete RPMI 1640 medium containing various concentrations of α-terpineol. Cells culture and α-terpineol were then incubated again in 5% CO_2 incubator at 37°C for 24 hours. After the incubation, the medium was removed and the cells were resuspended in RPMI 1640 medium, 10 mL of 5 mg/mL MTT [3-9,4,5-dimethylthiazole-2-yl-2,5-diphenyltetrazolium bromide] and then further incubated for 4 hours. The reaction was stopped by adding 100 mL of 10% sodium dodecyl sulfate (SDS) in 0.01N HCl. Microculture plates were then shaken gently for 5 minutes, covered with aluminium foil and incubated at room temperature overnight. Absorbance of the microculture plates was measured in an ELISA plate reader at λ_{max} 595 nm. The absorbance values were directly proportional to the number of live cells. The absorbance values in the presence of α-terpineol were compared with that of control cultures without α-terpeniol to obtain cells growth inhibition. For this MTT method, IC_{50} values were determined by probit analysis based on the relationship between log concentrations versus the percentage of cells growth inhibition. Doxorubicin was used as positive control.

Apoptotic assay

The acridine orange-ethidium bromide staining method was used to observe the apoptotic morphologic changes as reported by Ribble, *et al.*[15] T47D cells were suspended at a final concentration of 5 x 10^4/mL in complete RPMI 1640 medium and distributed in 24-wells plates coated with coverslip at the bottom. The cells were then treated with 1000 µL of α-terpineol at a concentration equivalent with the IC_{50} value (135 µM) and incubated in 5% CO_2 incubator at 37°C for 15 hours. Doxorubicin at a concentration of 17.89 µM was used as control. After incubation, the medium was removed and the cells were washed gently with PBS. Coverslip containing the treated cells were removed to glass object and then stained with 100 µL of mixture of acridine orange-ethidium bromide in PBS solution. The cells were observed under a fluorescence microscope. The apoptotic cells would emit orange fluorescence and the viable cells would emit green fluorescence. The number of cells undergoing apoptosis was observed in at least three microscopes field of view and the percentage of apoptotic was calculated.

Cells cycle analysis

Cells cycle analysis was performed by flowcytometry. Two small tubes were prepared for each treatment. T47D cell line were seeded onto 6-well plates at density of 7 x 10^5 cells per well and incubated in 5% CO_2 incubator at 37°C overnight. After overnight incubation, the T47D cells culture were treated with 500 µL of α-terpineol at 2 different concentrations which were equivalent to the value of ½ IC_{50} (67.5 µM) and IC_{50} (135 µM) for 24 hours. After incubation, the cells were collected and harvested. After centrifugation, cell pellets were washed twice with 500 µL of cold PBS. Cells were then incubated with 400 µL of propidium iodide reagent at 37°C for 10 minutes and

transferred to flowcytotube. The cells were immediately analyzed by FACS Calibur flowcytometer to evaluate cell cycle profile. Flowcytometric data were analyzed using Cell Quest to evaluate the cells distribution at each phase of the cell cycle namely the sub G_1 (apoptosis), S, G_2/M, and the cells undergoing polyploidy. The cell cycle inhibition was observed by comparing the cells distribution at G_0/G_1 and G_2/M phases of treated and untreated cells. The protocol of the study has been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Statistical comparisons were performed using Student's t-test, and differences between groups were considered significant at a value of <0.05.

RESULTS

The growth inhibition of TD47D cells *in vitro* after incubation with α-terpineol or doxorubicin in various different concentrations for 24 hours in 5% CO_2 incubator at 37° C is presented in TABLE 1. This study showed that α-terpineol or doxorubicin inhibited the T47D cells growth in a dose-dependent manner. In the highest concentration of α-terpineol (450 μM), the growth inhibition reached 96.03%, while for doxorubicin (172.40 μM), the growth inhibition reached 83.3%. Statistical analysis showed that the IC_{50} value of α-terpineol (135.00 ± 8.74 μM) was significantly higher than doxorubicin (17.89 ± 0.45 μM) ($p<0.05$).

TABLE 1. The average value of growth inhibition of T47D cells (% ± SD) after 24 hours incubation with the a-terpineol and doxorubicin and its IC_{50} value (μM)

Compound	Concentration (μM)	Inhibition (%± SD)	IC_{50} (μM)
α-Terpineol	450	96.03 ± 0.74	135.00±8.74
	400	92.78 ± 0.68	
	350	89.66 ±1.22	
	300	77.25 ± 0.96	
	250	62.68 ± 2.33	
	200	58.91 ± 0.74	
	150	52.48 ± 1.66	
	100	41.04 ± 9.03	
	50	17.89 ± 6.79	
Doxorubicin	172.40	83.38 ± 1.03	17.89±0.45
	86.20	69.30 ± 1.23	
	43.10	60.57 ± 0.89	
	21.60	44.67 ± 1.44	
	10.80	42.25 ± 0.67	
	5.40	39.87 ± 1.62	

To evaluate the effect of α-terpineol on the apoptosis, T47D cells were analyzed in the presence of acridine orange-ethidium bromide staining. Morphologic observation after staining is presented in FIGURE 1. Green-stained T47D cells represent viable cells, whereas orange staining represents apoptotic cells. α-Terpineol at concentration of 135 μM induced 50.53% apoptosis of the T47D cells, whereas doxorubicine at concentration of 17.89 μM induced 51.33% apoptosis of the cells (TABLE 2).

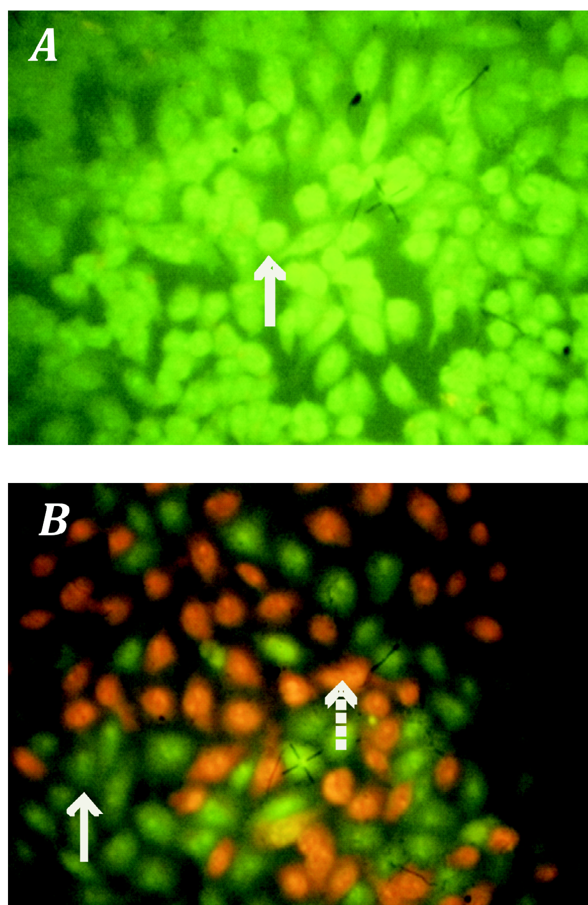

FIGURE 1. Morphologic observation with ethidium bromide-acridine orange staining T47D cells were treated without (A) and with a-terpineol (B). Viable cells are indicated by green color (↑) and apoptotic cells are indicated by orange color (↑). The observations used a fluorescent microscope with magnification of 100x.

TABLE 2 The percentage of apoptotic T47D cells after treated with and without b-terpineol or doxorubicin.

Compound	Concentration (μM)	% Mean ± SD
	135.00	50.53±13.19
α-Terpineol	67.50	9.83±0.97
	33.75	5.5±4.93
Doxorubicin	17.80	51.33±1.6

The effect of α-terpineol on the T47D cell cycle progression was evaluated by cell flowcytometry. FIGURE 1 and TABLE 3 show the T47D cell cycle profile after treatment with á-terpineol at 2 different concentrations (135.00 and 67.50 μM) and doxorubicin at 17.89 μM. TABLE 3 shows that there was a significant increase in the number of cell distributions in G_0/G_1 phase after treatment with α-terpineol from 38.17± 1.05 % to 50.98±1.60% at concentration of 67.50 μM and 56.89±1.93% at concentration 135.00 μM. In contrast, significant decrease was observed in S phase, from 19.35±0.21% to 18.39±0.35% and 15.34±0.72% at concentration 67.50 and 135.00 μM, respectively. Moreover, significant decrease in G_2/M was also observed to be from 33.05± 1.46% to 24.62±0.96% and 18.84±0.68% at concentration 67.50 and 135.00 μM, respectively.

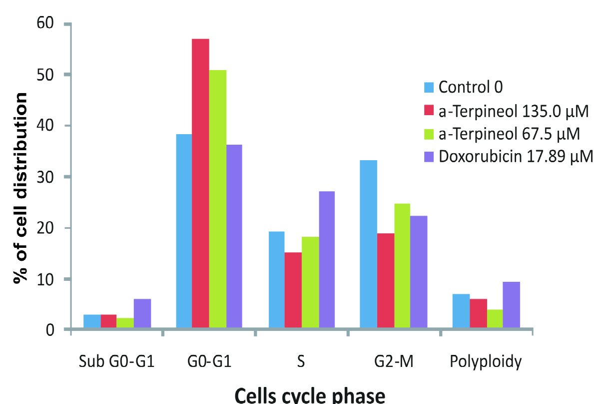

FIGURE 2. The percentage of T47D cell distribution (mean ± SD) in each cells cycle phase after treated with a-terpineol at concentration 67.5 and 135.0 μM and doxorubicin at concentration 17.89 μM for 24 hours after analyzed by flowcytometry

TABLE 3. The percentage of T47D cell distribution (mean ± SD) in each cells cycle phase after treated with α-terpineol at concentration 67.5 and 135.0 μM and doxorubicin at concentration 17.89 μM for 24 hours after analyzed by flowcytometry

Treatment	Percentage of cell distributions (mean ± SD)				
	Sub G_0/G_1	G_0/G_1	S	G_2/M	Polyploidy
Control (0 μM)	3.06±0.67	38.17± 1.05	19.35±0.21	33.05±1.46	6.95±0.44
α-Terpineol (135.0 μM)	3.11±0.32	56.89±1.93	15.34±0.72	18.84±0.68	6.19±0.53
α-Terpineol (67.5 μM)	2.37±0.20	50.98±1.60	18.39±0.35	24.62±0.96	4.15±0.29
Doxorubicin (17.89 μM)	6.10±0.39	36.12±1.27	26.97±0.42	22.28±0.59	9.28±0.82

DISCUSSION

This study showed that α-terpineol inhibited the T47D cell growth in a dose-dependent manner. In the lowest concentration, α-terpineol (50 μM) yielded the inhibition of cell growth of 17.89 ± 6.79%, while in highest concentration, it yielded 96.03 ± 0.74%. Further analysis demonstrated that the cytotoxicity of α-terpineol on the T47D cells (IC_{50}:135.00 ± 8.74 μM) was significantly lower than doxorubicin (IC_{50}: 17.89±0.45 μM) as positive control. However, the cytotoxicity of α-terpineol on T47D cells obtained from this study was higher than the cytotoxicity on NCI-H69 lung cancer cells (IC_{50}: 260 μM) as reported by Hassan *et al.*[11] America National Cancer Institute classifies a compound as a potential candidate for anticancer if it has IC_{50} value less than 30 μg/mL.[16] Based on this criteria, α-terpineol that has IC_{50} 135 μM or equivalent with 20.28 μg/mL, can be categorized as a potential anticancer compound.

Apoptosis is a cellular mechanism that is useful for eliminating permanent damage of the cells without causing inflammation.[17,18] It is an important homeostatic mechanism that balances cell division and cell death and maintains the appropriate cell number in the body.[19] Some anticancers cause the death of sensitive cells through the induction of apoptosis. Therefore, induction of apoptosis is one of mechanisms expected in the development of new anticancers.[20] In this study, the effect of α-terpineol on induction of apoptosis in T47D cells has been also evaluated.

Double staining test with ethidium bromide-acridine orange was selected in this study to evaluate the α-terpineol-induced apotosis because of its ease, speed and accuracy.[15] The T47D cells staining green represented viable cells, whereas orange staining represented apoptosis cells. The T47D cells treated with 135.00 μM of α-terpineol showed changes in cellular morphology including chromatin condensation, membrane blebbing, and fragmented nuclei (FIGURE 1B) as reported by Ribble *et al.*[15] and Renvoize *et al.*[21] In contrast, in the untreated T47D cells or cells control (FIGURE 1A), all cells stained green and no orange staining cells were observed indicating no apoptotic cells.

The fragmentation of nuclei represents DNA fragmentation which is a marker of the end of apoptosis after activation of caspase proteins.

DFF40 is responsible for DNA fragmentation after DFF45 (DFF40 binding) protein cleaved by caspase 3/7 protein.[22] T47D cell consists of caspase 3 protein (a major mediator of apoptosis). Activation of caspase pathways are also triggered by cutting other essential proteins such as PARP (poly-ADP-ribose polymerase), which functions as a DNA repair enzyme.[23]

The regulation of cell cycle is crucial in the growth and development of cancer. In this study the effect of α-terpineol on the T47D cell cycle progression was evaluated by flowcytometry method. The results showed that the α-terpineol induced the T47D cell distribution in G_o/G_1 phase and inhibited cell distribution in S phase. The effect of α-terpineol on cell cycle has been also reported by Hassan *et al.*[11] α-Terpineol inhibiting the proliferation of lymphoma U937-GTB cancer cells in G_0/G_1 phase leads to a reduction in the number of cells in the later stages of cell cycle (S, G_2 and M) as shown in this study.

Hassan *et al.*[11] also demonstrated that α-terpineol exhibited a potential anticancer which acts by suppressing NF-κB which signals various cancer cells line. NF-κB protein is one of transcription factors that are involved in the control of inflammatory responses, developmental processes, cellular growth and apoptosis.[24] α-Terpineol inhibits NF-κB translocation and activity and down-regulates the expression of several NF-κB-related genes such as IL-1β and IL1R1 resulting in the inhibition of cancer cells growth.[11]

CONCLUSION

In conclusion, these findings suggest that á-terpineol has a potential anticancer activity. Further study concerning the mechanism of the anticancer action and specificity of α-terpineol will be conducted. In addition, the *in vivo* anticancer activity of α-terpineol on animal cancer model should be the subject of further study.

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