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Ethanolic periwinkle leaf extract reduces telomerase expression in T47D cancer cells

Endang Purwaningsih*, Etty Widayanti*, and Yulia Suciati**

ABSTRACT

BACKGROUND

Cancer cells have a relatively high telomerase activity and a lower p53 protein expression than normal cells, so that cancer cells have the ability to continue to proliferate and do not undergo apoptosis. One of the cancer treatments is chemotherapy using bioactive ingredients from synthesis or isolation of natural materials. One of the plants that have potential as anticancer agent is periwinkle (*Catharanthus roseus* L). The research objective was to evaluate the effect of ethanolic periwinkle leaf extract against p53 protein and telomerase expression in T47D cancer cells.

METHODS

An experimental study with controls was conducted involving T47D breast cancer cells. They were divided into 3 groups (control, ½ dose of IC50/ 26.849 µg/mL, and one dose of IC50/53.699 µg/mL) at a cell density of 1 x 10^4 cells/well. Expression of p53 and telomerase was measured by the immunohistochemistry method. Data were analyzed using one-way ANOVA followed by a multiple comparison test.

RESULTS

Periwinkle leaf extract significantly increased p53 protein expression ($p<0.05$) at both treatment doses, ½ IC50 and IC50, compared to the control group and it highly significantly reduced telomerase expression ($p<0.01$), in comparison with the control group at both treatment doses.

CONCLUSION

Periwinkle leaf extract has potential as an anti-breast cancer agent by increasing p53 protein expression and inhibiting telomerase expression.

Keywords: Periwinkle, p53, telomerase, T47D cells

*Department of Anatomy, Faculty of Medicine, YARSI University, Jakarta
**Department of Biochemistry, Faculty of Medicine, YARSI University, Jakarta

Correspondence:
Prof. Dr. Endang Purwaningsih, MS, PA
Department of Anatomy, Faculty of Medicine, University of YARSI
Jln. Letjend Suprapto, Cempaka Putih, Jakarta10510
Phone: +6221-4206674/76
Email: endpurwaning@gmail.com/ endang.purwaningsih@yarsi.ac.id

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Ekstrak etanol daun tapak dara menurunkan ekspresi telomerase pada sel kanker T47D

ABSTRAK

LATAR BELAKANG

Kanker merupakan penyakit yang mendapat perhatian serius di dunia kedokteran. Hal ini disebabkan oleh jumlah penderitanya yang semakin meningkat dan belum ada cara efektif untuk mengobatinya. Sel kanker memiliki aktivitas telomerase yang relatif tinggi dan menunjukkan ekspresi protein p53 yang lebih rendah dibandingkan sel normal sehingga sel kanker memiliki kemampuan untuk terus berproliferasi dan tidak mengalami apoptosis. Salah satu pengobatan kanker adalah dengan kemoterapi menggunakan bahan-bahan bioaktif dari hasil sintesis atau isolasi bahan alam. Salah satu tanaman yang memiliki potensi sebagai antikanker adalah tapak dara (Catharanthus roseus L). Tujuan penelitian adalah untuk menilai efek ekstrak etanol daun tapak dara terhadap ekspresi protein p53 dan telomerase pada kultur sel kanker T47D.

METODE

Sebuah penelitian eksperimental dengan kontrol dilakukan deengan mengikut sertakan kultur sel kanker payudara T47D (cell line). Mereka secara acak dibagi menjadi 3 kelompok (kontrol, ½ dosis IC50/26.849 µg/mL, dan satu dosis IC50/53.699 µg/mL) dengan kepadatan sel 1 x 10^4 sel/sumuran. Ekspresi p53 dan telomerase diukur dengan metode immunohistochemistry. *Data dianalisis menggunakan Anova satu jalan dilanjutkan dengan uji perbandingan berganda.*

HASIL

Ekstrak daun tapak dara meningkatkan ekspresi protein p53 secara bermakna pada kedua dosis perlakuan ($p<0,05$) baik ½ IC 50 maupun 1 IC50 dibandingkan kelompok kontrol ($p<0,05$), dan menurunkan ekspresi telomerase secara sangat bermakna pada kedua dosis perlakuan ($p<0,01$).

KESIMPULAN

Ekstrak daun tapak dara memiliki potensi sebagai anti kanker payudara melalui peningkatan ekspresi protein p53 dan hambatan telomerase.

***Kata kunci** : Tapak dara, p53, telomerase, sel T47D*

INTRODUCTION

Cancer is a health problem in various countries throughout the world and is a disease that attracts serious attention in the medical field. This is because the number of victims is increasing from year to year and no effective treatment method has been found. Medical cancer treatments that have been currently used are surgery, irradiation, and chemotherapy. The latter, which is currently of interest, comprises the use of synthetic or natural bioactive substances such as those from the rat tail rhizome (*Typhonium flagelliforme*) and periwinkle (*Catharanthus roseus*).[1-4]

According to data from the World Health Organization (WHO), 80% of the world population still depends on plant-derived traditional medications for its health needs. A total of 25% of the commercially available modern drugs in the world are derived from active substances that have been isolated and developed from plants. At present the use of traditional medications is on the increase, despite the

substantial advances in modern medical therapeutics. The Indonesian community has recognized various medicinal plants, because Indonesia is known as one of the countries with the greatest biodiversity and as such has potential for the development of plant-based medications, particularly anticancer drugs.[5] One of the plants that contain potential anticancer compounds is the periwinkle plant (*Catharanthus roseus* (L) G Don), formerly known as *Vinca rosea* L. The leaves of this plant contain a number of alkaloids that are potential anticancer agents, i.e. vincristine, vinblastine, vindesine, vindoline, tabersonine, leurosine, catharanthine, and lochnerine.[4,6]

Periwinkle leaf extract may be used as anticancer or antitumor agent, according to a number of previous in vivo studies on rats as well as in vitro studies using cultures of the MCF7 cancer cell line.[7,8] In addition, periwinkle leaf extract at a dose of 1.0 mL/kg has been shown to be able to significantly reduce serum HDL (high density lipoprotein) and LDL (low density lipoprotein) cholesterols and triglycerides in normal rats.[7] Furthermore it is known that there are more than 70 types of alkaloid in the roots, stems, leaves, and seeds of the periwinkle plant, including 28 bi-indole alkaloids. In addition to anticancer alkaloids, the periwinkle plant also contains blood glucose reducing alkaloids, i.e. leurosine, lochnerine, tetrahydroalstonine, vindoline and vindolinine.[6,9] An extract of its flowers also has hyperglycemic effects in diabetic rats and functions as antioxidant, antibacterial, anthelminthic, and antidiarrheal agent.[9-11]

A number of cancer cells have a high telomerase activity and each type of cancer cell may have different degrees of telomerase expression according to the type or location of the cancer. This can be used as a basis for the treatment of cancer cells if there are substances that can inhibit the activity of the telomerase enzyme. In addition, cancer cells possess a high mitotic activity since they undergo uncontrolled cell division as a result of low p53 tumor suppressor protein expression. The p53 protein is responsible for the mechanism of apoptosis of cancer cells and has become the latest cancer treatment strategy.[12,13]

A number of scientific data have demonstrated the benefits of periwinkle, such as inhibition of proliferation and triggering of apoptosis in cancer cells [11,14,15] as well as cytotoxicity against cancer cells.[8] The anti-apoptotic effect may be determined by counting the percentage of cells undergoing apoptosis by flow cytometer. The effect of the periwinkle plant on p53 or telomerase protein expression in cancer cells is still unknown. The objective of this study was to evaluate the effect of periwinkle (*Catharanthus roseus* L) leaf extract on p53 and telomerase expression in T47D breast cancer cells.

METHODS

Design of study

This laboratory experimental study was carried out from June 2013 until November 2013 at the Department of Biochemistry, Faculty of Medicine, YARSI University.

Study subjects

The study subjects were cultures of the T47D cancer cell line, obtained from the Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University. The cells were cultured on 24-well microplates using RPMI (Roswell Park Memorial Institute) medium, with two replications for each group. The cultures were incubated for 72 hours or until confluence was reached, with a density of 1×10^4 cells/well. Upon confluence, the cultures were treated with the respective doses.

Preparation of periwinkle leaf extract

Periwinkle leaf extract was prepared as follows: fresh leaves were cleansed, cut into small strips, and air-dried. The dried strips were then blenderized into a powder. The powder was macerated in 80% ethanol in an Erlenmeyer flask, under continuous stirring. The ethanolic extract was then transferred to a rotary flask to

be concentrated in a rotary evaporator. The concentrated periwinkle leaf extract was dissolved in DMSO, diluted to give a number of concentrations, and filtered using a 0.2 µm filter before being placed on the 24-well plates.

Intervention

The cultures of the T47D cancer cell line were assigned to 3 groups, i.e. one control group that did not receive extract, one group receiving extract at a dose of one half the maximal inhibitory concentration (IC50) (53.699 µg/mL), and one group receiving a dose of ½ IC50 (26.849 µg/mL). Before treatment with the extract, a test was performed to determine the IC50 of the periwinkle leaf extract against T47D cell cultures. The result of IC50 testing was used to determine the treatment dose to be used. The treated cell cultures on coverslips were incubated under 5% CO_2 at 37°C for 24 hours.

Preparation of cell culture and cell growth media

Preparation of Roswell Park Memorial Institute (RPMI) culture medium was by dissolving RPMI 1640 powder for one liter into approximately 800 ml of twice distilled water. After adding 2.0 grams of sodium bicarbonate and 2.0 grams of HEPES, twice distilled water was added to make up a volume of one liter. The solution was mixed with a magnetic stirrer for about 10 minutes until homogenous, then neutralized with 1 N HCl to give a pH of 7.2 – 7.4. Growth medium was prepared by mixing FBS 19 mL, penicilline streptomycin 2 mL, fungizone 0.5 mL, then diluted with RPMI 1640 culture medium to a volume of 100 mL. The solution was then aseptically filtered using a sterile 0.2 µm polyethylene sulfone filter and afterwards stored in the refrigerator in stoppered bottles.

Immunohistochemical determination of p53 protein and telomerase expression

T47D cell cultures at a density of 1x 10^4/well that had attached to the coverslips were stained immunohistochemically according to standard procedures of the Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University. Immunohistochemical determination of p53 protein and telomerase expression was as follows: the cell cultures on the coverslips, after being given the extract and incubated under 5% CO_2 at 37°C for 24 hours, were placed on poly-l-lysine slides and fixed in acetone or methanol for 10 minutes at -20°C. The slides were then washed with PBS for 3 x 5 minutes, then 0.3% H_2O_2 was dripped 20 minutes. Normal mouse serum (1:50) was dripped for 15 minutes and the liquid was discarded (without washing). Then p53 or telomerase antibody was dripped for 60 minutes. The slides were then washed with PBS for 3 x 5 minutes, incubated with biotinylated secondary antibody for 5-10 minutes, and again washed with PBS for 3 x 5 minutes.

This was followed by incubating the slides with streptavidin-peroxidase for 5-10 minutes, washing with PBS for 3 x 5 minutes, incubating with the chromogen deaminobenzidine tetrahydrochloride (DAB) for 5-10 minutes (at a chromogen to substrate ratio of 1:20), and washing with distilled water. The slides were immersed in hematoxyllin for 3-5 minutes for counterstaining, washed with distilled water, dehydrated in 95% ethanol and immersed in xylene, for 10 minutes respectively. The slides were given a drop of Canada balsam as mounting medium and covered with a coverslip. The expression of p53 protein and telomerase was observed under the light microscope. Cells expressing p53 protein or telomerase showed brownish colored nuclei and cytoplasms, whereas cells without protein expression showed blue-violet nuclei and cytoplasms. Expression-positive cells were counted per 100 observed cells and counting was done in triplicate. The results were expressed as percentages.

Data analysis

The p53 protein expression and telomerase percentages were analyzed by one-way ANOVA, followed by least significant difference (LSD) multiple comparisons, using SPSS version 17.

Table 1. Expression of p53 protein and telomerase in T47D cell cultures by treatment group

	Treatment group			p
	½ IC50	1 IC50	Control	
Protein 53 expression (% positive)	23.67 ± 5.51	36.00 ± 3.61	12.67 ± 1.15	0.000
Telomerase expression (% positive)	33.33 ± 2.08	16.67 ± 2.08	59.33 ± 3.06	0.000

IC50 : one half the maximal inhibitory concentration

RESULTS

Before evaluating the effect of the periwinkle leaf extract on the expression of p53 and telomerase, toxicity testing was performed on periwinkle leaf extract against cultures of T47D breast cancer cells. The toxicity tests yielded an IC50 value of 53.699 µg/mL. Assessment of periwinkle leaf extract effects on p53 protein and telomerase expression by immunohistochemistry was done by counting the percentages of expression-positive cells. The percentages of p53 and telomerase positive cells in the treatment groups receiving extract (at doses of ½ IC50 or 26.849 µg/mL and 1 IC50 or 53.699 µg/mL, respectively) are shown in Table 1. From the percentages of the expression-positive cells it is apparent that periwinkle leaf extract tended to increase p53 protein expression and to reduce telomerase expression.

According to the results of Anova analysis on the expression of p53 protein and telomerase, there were substantially significant differences (p=0.000) between groups. Between-group LSD multiple comparison test results on the expression of p53 and telomerase are presented in Table 2.

The LSD multiple comparison test results on p53 protein expression showed significant differences ($p<0.05$) between the control group and the group receiving a dose of one-half IC50, and also between the group receiving a dose of one-half IC50 and the group receiving a dose of one IC50. There were also highly significant differences ($p<0.01$) between the control group and the group receiving a dose of one IC50. The LSD multiple comparison test results on telomerase expression showed highly significant differences between the control group and the one-half IC50 group, between the control group and the one IC50 group, and between the one-half IC50 group and the one IC50 group.

DISCUSSION

From the results of this study, it is apparent that ethanolic periwinkle leaf extract is able to reduce telomerase expression and increase p53 protein expression in T47D breast cancer cells. Therefore the compounds contained in periwinkle leaf extract are able to inhibit telomerase expression in breast cancer cells, or in other words, the extract may act as a telomerase inhibitor.

Previous investigators have reported that chloroform and methanolic extracts of the periwinkle (*Catharanthus roseus* L) possess cytotoxic effects against HCT-116 colon cancer cells.[16] In addition crude aqueous periwinkle extract also has cytotoxic effects against leukemic T cells and induces the proliferation of normal blood cells.[17] There have been no reports on the effect of periwinkle extract on telomerase expression in colon cancer cells and leukemic T cells. It has also been reported that

Table 2. Results of LSD test on expression of p53 and telomerase, by treatment group

	Mean differences	p
Protein-53 expression		
Control ½ IC50	11.00	< 0.05
IC50	23.33	< 0.01
½ IC50 IC50	12.33	< 0.01
Tekomerase expression		
Control ½ IC50	26.00	< 0.01
IC50	42.66	< 0.01
½ IC50 IC50	16.66	< 0.01

IC50: one half the maximal inhibitory concentration

curcumin, a component of turmeric (*Curcuma longa* L) rhizomes, has in vitro anti-proliferative activity against Bel7402, HL60 and SGC7901 cancer cell lines and demonstrates in vivo antitumor effects in laboratory rats. In addition, curcumin at a dose of 1 ìM is able to inhibit telomerase activity in cancer cells in vitro and to induce apoptosis.[18]

It is known that various cancer cells have relatively higher telomerase activities than normal cells and that there is a correlation between telomerase activity and the development of cancer cells. Telomerase is the principal key to cellular immortality and tumorogenesis. Telomerase is activated in 80–90% of human cancer cells, but not in somatic cells (normal cells). Furthermore it has been reported that telomerase may be a biomarker for early detection of cancers and cancer monitoring, and has sufficient potential for a basis of development of cancer treatment.[19]

The p53 protein is a tumor suppressor protein that is responsible for the mechanism of apoptosis in cancer cells. The mechanism comprises the repair of cells that have been induced to become cancerous. Expression of specific p53 protein occurs in abnormally replicating cells and requires programmed cell death called apoptosis. Higher p53 protein expression is associated with a higher tendency to apoptosis. The p53 protein is expressed upon the occurrence of DNA damage.[12,13]

The p53 protein is a tumor suppressor protein, with a molecular weight of 53 kilodalton, that is activated upon the occurrence of DNA damage or certain types of stress in the cells. This protein can promote apoptosis by increased expression of the *Bax* gene, which codes for the Bax protein that plays a role in apoptosis. Detection of damaged DNA is regulated by the p53 tumor suppressor. When DNA damage occurs, p53 prevent the cells from entering the next phase of cell division and gives time to the DNA to repair itself, or if the damage is sufficiently severe, p53 will initiate programmed cell death (apoptosis). Loss of a number of molecular checkpoints are to be found in the development of several tumors or cancers.[20]

Administration of the leaf extract of periwinkle (*Catharantus roseus*) can increase p53 protein expression, such that this extract may be used to increase apoptosis of breast cancer cells. The periwinkle plant produces secondary metabolites that are active as alkaloid anticancer agents.[9,14] On the other hand, periwinkle extract has been shown to reduce telomerase expression, as has been shown in T47D cell cultures in the treatment group at ½ IC50 and 1 IC50. Telomerase is a ribonucleoprotein enzyme that maintains the protective structures at the ends of eukaryotic chromosomes, called telomers. Telomerase is a structure that is responsible for the maintenance of telomers, being a TTAGGGn nucleotide replication. In most human somatic cells, such as fibroblasts, telomerase expression is repressed, the telomers become progressively shorter and at each somatic cell division the cells undergo programmed cell death or apoptosis. In contrast, the majority of human tumor or cancer cells express telomerase, such that the telomer length is stable and the cells do not die by apoptosis. This observation shows that telomer maintenance is essential for tumor or cancer cell proliferation.[21] Telomerase activity determines cell proliferation, both of cancer and normal cells, in vitro as well as in vivo conditions.[22,23]

Telomerase activity in cancer cells increase after certain stages, i.e. when there is a loss of a number of substances or telomer subunits in the cells. Finally the telomerase is activated to stabilize the telomers, the cancer cells become immortal and proliferate continuously.[24] Telomerase plays a role in cell replication, since it is able to maintain telomer length in the cell progeny. Therefore cancer cells without sufficient telomerase will cease to grow when this enzyme cannot any longer continuously maintain telomer length as a result of abnormal control of proliferation. Lack of telomerase may inhibit

cancer growth by continuous cell division such that the telomers become shorter and the cells die before the occurrence of further damage. However, if the cancer cells are able to synthesize telomerase then these cells will proliferate continuously and become immortal.[23]

Periwinkle leaf extract contains more than 100 monoterpenoids that exert cytotoxic effects and are used in cancer chemotherapy. Alkaloids such as vincristin and vinblastin in periwinkle leaf extract may function as potent antineoplastic agents [25] by inhibiting DNA synthesis and blocking mitosis in metaphase or anaphase.[26] A study using periwinkle leaf extract on T47 D cancer cells found that the extract had anticancer effects through induction of apoptosis, but that it did not show antioxidant activity in T47D cells.[11] One limitation of this study is that it did not investigate other proteins that play a role in apoptosis, such as Bax and BcL-2, after administration of periwinkle leaf extract to T47D breast cancer cells. As a result of this study, a natural raw material with chemopreventive actions against breast cancer cells will be available from the periwinkle plant (*Catharanthus roseus*).

CONFLICT OF INTEREST

There are no conflict of interest.

CONCLUSION

Compounds in periwinkle leaf extract are potential agents against breast cancer by increasing p53 protein expression leading to apoptosis and by telomerase inhibition.

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