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Apoptosis and Antioxidant Activities of *Catharanthus rosues* [L] G.Don Extract on Breast Cancer Cell Line

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Abstract

Tapak dara or Madagascar Periwinkle (*Catharanthus roseus* [L] G.Don), a natural plant, is empirically reported to have promising anticancer activity. To elucidate its mechanism, a research was conducted to investigate the possible ethanol extract of C. *roseus* in inducing apoptosis on breast cancer cell line (T47D). Antioxidant activity of *C. roseus* was investigated as well. Sub-G1 flowcytometric apoptotic analysis result showed that extract of *C. roseus* at 6.25 μg/mL induced apoptosis for 26.365%. Increasing extract concentration resulted an increasing apoptotic level as well, extract at concentration of 12.5 μg/mL induced apoptosis for 22.235%. Meanwhile doxorubicin at concentration of 10 μg/mL induced apoptosis for 36.055%. The antioxidant activity was determined by using *in vitro* assay: inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. Antioxidant activity of *C. roseus* extract were compared to quercetin and butylated hydroxyanisol (BHA), as positive controls. The results showed that DPPH IC_{50} of *C. roseus* extract, quercetin and BHA were 358.411 μg/mL, 19.200 μg/mL and 94.178 μg/mL, respectively. We suggest that *C. roseus* extract had a potential anticancer activity by inducing apoptosis.

Keywords: antioxidant, DPPH, *Catharanthus roseus,* apoptosis, breast cancer, T47D

INTRODUCTION

Breast cancer is the most common cancer among women and the second leading cause of cancer deaths in women after lung cancer (Lopez and Sekharam., 2008). It is estimated that one of eight women will be diagnosed with breast cancer in women (Chen and Yan, 2007). Worldwide, one million women are estimated to be newly diagnosed with breast cancer each year (Hanf and Gonder, 2005; Abdolmohammadi *et al*., 2009). Cancer chemoprevention applies specific natural or synthetic chemical compounds to inhibit or reverse carcinogenesis and to suppress the development of cancer from premalignant lesions (Sarkar and Li, 2007; Abdolmohammadi *et al*., 2009). A major problem with present cancer chemotherapy is the serious deficiency of active drugs for the curative therapy of tumors (Valeriote *et al*., 2002; Kinghorn *et al*., 2003; Abdolmohammadi *et al*., 2009). The chemotherapeutic drugs including etoposide, camptothecin, vincristine, cis-platinum, cyclophosphamide, paclitaxel (Taxol), 5-fluorouracil and doxorubicin have been observed to induce apoptosis in cancer cells (Kaufman *et al*., 2000; Johnstone *et al*., 2002; Abdolmohammadi *et al*., 2008). Among them, the agents that alter the cell cycle have been of particular interest, since cell cycle regulation is the basic mechanism underlying cell fate, i.e., proliferation, differentiation or acquire death (Dobashi *et al*., 2003; Abdolmohammadi *et al*., 2008).

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Apoptosis, a type of programmed cell death, is an active process. It is a normal component of the development and health of multicellular organisms. The study of apoptosis is an important field of biological inquiry since a deficiency or an excess of apoptosis is one of the causes for cancers, autoimmune disorders, and many other diseases. Accordingly, a quick and easy assay for quantification of apoptosis would be very useful for many biological researchers (Ribble *et al*., 2005). Apoptosis was distinguished from necrosis, which was associated with acute injury to cells. Apoptosis is characterized by nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic reticulum, and membrane blebbing. Mitochondria remain unchanged morphologically (Somani *et al*., 2010). Apoptosis is an essential process in both multi-cellular development, and in maintaining cellular homeostasis. Moreover, failure of damaged cells to undergo apoptosis contributes to the progression of cancer as it allows the persistence of DNA damaged cells. Therefore, it is interesting to understand apoptotic signalling pathways in different cancer types. Apoptosis is morphologically defined by cell shrinkage, membrane blebbing, chromatin condensation and formation of apoptotic bodies (Mooney *et al*., 2002). Inducing an apoptotic response is the goal of most current chemotherapeutic interventions against cancer. The main action of anticancer agents is by triggering the apoptotic pathway. However, intrinsic alterations in the apoptotic pathway are a hallmark of cancer cells and are considered to be a major cause of drug efficacy (Lowe and Lin, 2002; Waxman and Schwartz, 2003; Shkreta *et al*., 2008). Clinically, apoptosis has been found to be an important mechanism in the action of radiation and many chemotherapeutic drugs (Kaufmann, 1989; Telford *et al*., 1994). Defects in apoptotic signalling are believed to be an important factor in successfull cancer development (Schwartzman and Cidlowski, 1992; Telford *et al*., 1994).

Lipid peroxidation is a free radical mediated phenomenon in biological tissues where poly unsaturated fatty acids are generally abundant. The Lipid peroxidation content is one of the most frequently used parameters for assessing the involvement of free radicals in cell damage. The probable reason for the elevated level of serum lipid peroxide in breast carcinoma may be due to defective antioxidant system which leads to the accumulation of lipid peroxides in cancer tissue which are released into the blood stream. In breast cancer tissue, the MDA level in stage IV was significantly higher as compared to stage I indicating increased free radical activity with increasing severity of cancer (Sinha *et al*., 2009). Lipid peroxidations are evidenced by the formation of thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH) and conjugated dienes (CD) as well as the status of the antioxidants superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx) and glutathione-*S*-transferase (GST) in breast cancer tissues were enhanced compared to control (Kumaraguruparan *et al*., 2002). Antioxidant CAT, SOD also act as anti-carcinogens and inhibitors at initiation and promotion/transformation stage in carcinogenesis. Mutation caused by potassium superoxide in mammalian cells is blocked by SOD. Plasma DNA strand scission caused by xanthine/xanthine oxidase is prevented by SOD and catalase enzymes (Sinha *et al*., 2009).

One of the approaches used in drug discovery is the ethnomedical data approach, in which the selection of a plant is based on the prior information. *C. rosues* was used as a remedy in cancer related diseases. . *Craceus (L.)* G. Don (Apocynaceae), a perennial plant, commonly exists in tropical countries. It is more known as Madagascar periwinkle, the local name in Indonesia is Tapak dara. Aerial part of the plant contains about 90 different alkaloids. The isolation and purification of these indole alkaloids have been extensively studied (Sapi and Massiot., 1994), the most abundant ones are the monomers like catharanthine and vincloline. Two of the common anti-cancer *drugs* which are derived from this plant are vincristine and vinblastine. Crude extracts of *C. roseus* using 50 and 100% methanol had significant anticancer activity against different cell types *in vitro* at <15 μg/mL) (Ueda *et al.*, 2002). Crude decoction (200 mg and 1 g herb/mL water) showed moderate in vitro anti-angiogenesis effects (Ghosh and Gupta, 1980; Chattopadhyay *et al*, 1991, 1992). In this study, we have examined the antioxidant and apoptosis properties of the *C. roseus* extract.

METHODS

Plant material

C. roseus was collected from Bogor-west Java plantation (May 2009). The aerial part and root

were collected, kept under dry tunnel (40-45^{0}C) and chopped finely using a blender.

Preparation of extract

One kilogram of dried and chopped mateial were extracted with distilled ethanol by maserasi extraction, filtered and evaporated using rotatory evaporator, produced ethanol extract of *C. roseus* 162.5 g (16.25%). The ethanol extract was stored at 4 ^{o}C. The extract was dissolved in DMSO and subsequently diluted to appropriate working concentrations with DMEM culture medium (Tan *et al*., 2005).

Cell culture

The human breast cancer T47D cell line was obtained from the The Indonesian Institute of Sciences of Sciences Research Centre for Chemistry, Natural Products, Food and Pharmaceuticals Division, Bandung (Indonesia). The cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) supplemented with 100 U/mL of penicillin, 100 g/mL of streptomycin at 37^{0}C in a humidified atmosphere of 5% CO_2 (Mooney *et al*., 2002; Tan *et al*., 2005).

Apoptosis assay

Cells were used in apoptotic studies when 80% confluence was reached in T25 flasks. Cells were harvested with trypsin-EDTA (0.25%-0.038%), washed with PBS. T47D cells were seeded into 12 well plate at the density 10^4 cells/well incubated for 24 h with various concentrations of extracts. After 24 h incubated, untreated and treated cell were rinsed with phosphate buffered saline (PBS), fixed with trypsin-EDTA and incubated 37^{0}C for 5 minutes. Afterwards, the medium was added in ratio 3:1 (medium: trypsin-EDTA) and centrifuged at 1500 rpm for 5 minutes. Supernatant was discarded. The pellet was added with 70% ethanol, and then incubated in 4^0 C for 5 minutes. The incubated cells were further centrifuged at 1500 rpm for 5 minutes, and the supernatant was discarded. The cells were stained with Propidium iodide (PI) solution (in PBS) in the final concentration of 2 µg/ml. The cell suspensions were placed in the dark by wrapping up the tubes with aluminium foil for 15 minutes prior to flow cytometric analysis. The apoptosis assay was analyzed by cell cycle analysis using flow cytometer. The apoptotic cells were determined by sub G1 area from cell cycle analysis and it was presented as percentage of cells.

Antioxidant assay

The DPPH assay was carried out as described by Unlu *et al* (2003), Han *et al*. (2004) and Frum and Viljoen (2006). Pipette 50 µL of sample (*C. roseus* extract, quercetin and BHA) of various concentrations of the samples (125; 62.5; 31.25; 15.625; 7.813 and 3.906 µg/mL) entered at the microplate and then were added 200 µL of 0.077 mmol/L methanol solution of DPPH and the reaction mixture was shaken vigorously and kept in the dark for 30 min at room temperature. Furthermore, DPPH scavenging activity was determined by microplate reader at 517 nm. The radical scavenging activity of each sample was expressed by the ratio the of lowering of the absorption of DPPH (%), relative to the absorption (100%) of DPPH solution in the absence of test sample (negative control).

$$\text{scavenging \%} = \frac{A_c - A_s}{A_c} \times 100$$

where A_s and A_c are absorbance at 517 nm of the reaction mixture with samples and without sample, respectively

RESULTS

Morphology

Treatment of cells with *C. roseus* extract at concentrations of 6.25; 12.5; 25; 50 and 100 µg/mL respectively, caused morphological changes compared to normal cells and this showed indication of apoptosis (Figures 1B, 1C, 1D, 1E, 1F). Morphological changes observed in the treated cells included cell shrinkage, lead to membrane rupture. Higher concentrations of *C. roseus* extract cause more morphological changes, indicating that apoptosis occurred in cells.

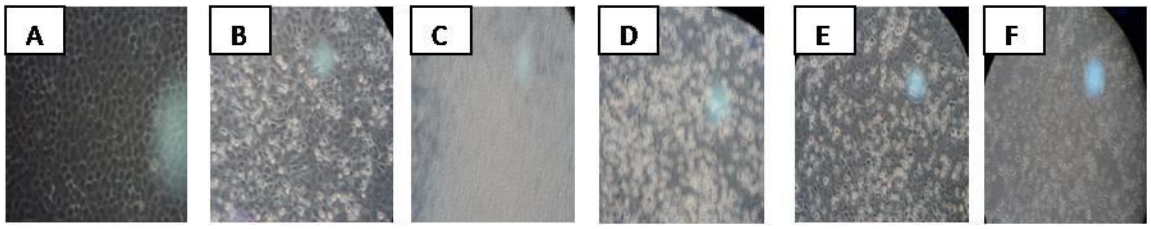

Figure 1: Morphological changes T47D cells treated with *C. roseus* extract. (A = untreated T47D cells (1×10⁴)); B = cells treated with *C. roseus* extract at a concentration of 6.25 µg/ml; C = cells treated 12.5 µg/mL; D = cells treated 25 µg/mL; E = cells treated 50 µg/mL; F = cells treated 100 µg/mL)

Sub G1 apoptosis

The effect of *C. roseus* extract on cell cycle was analyzed by flow cytometry in duplo using concentration of *C. roseus* extract 6.25; 12.5; 25; 50 and 100 µg/mL, respectively. Figure 2 showed that sub G1 apoptotic content was markedly increased when cells were treated with *C. roseus* extract. The sub G1 average of untretaed was formed at 0.465 % (Figure 2), whereas a concentration of 6.25 µg/mL resulted in 6.925 % of cells being in sub G1 phase. This proportion of cells further increased at the extract concentration of 25 µg/mL (26.35%), concentration 50 µg/mL (37.475%) and finally the increased concentration decreased the sub G1 apoptotic content (10.78%).

Antioxidant activity

The DPPH free radical scavenging activity of *C. roseus* extract at various concentrations was measured to know the antioxidant activity. Quercetine and BHA have been used as positive controls. The IC_{50} (median inhibition concentration) is defined as a concentration of samples to scavenge DPPH free radical 50 %. The DPPH free radical scavenging activity of *C. roseus* extract, quercetin and BHA can be found in Table 1. The *C. roseus* extract had a lowest antioxidant activity compared to quercetin and BHA.

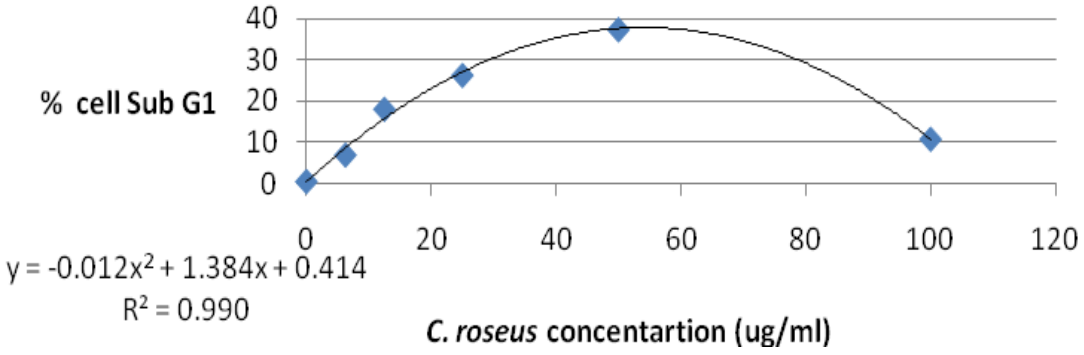

Figure 2A. Effect *C. roseus* extract on T47D cell cycle progression.

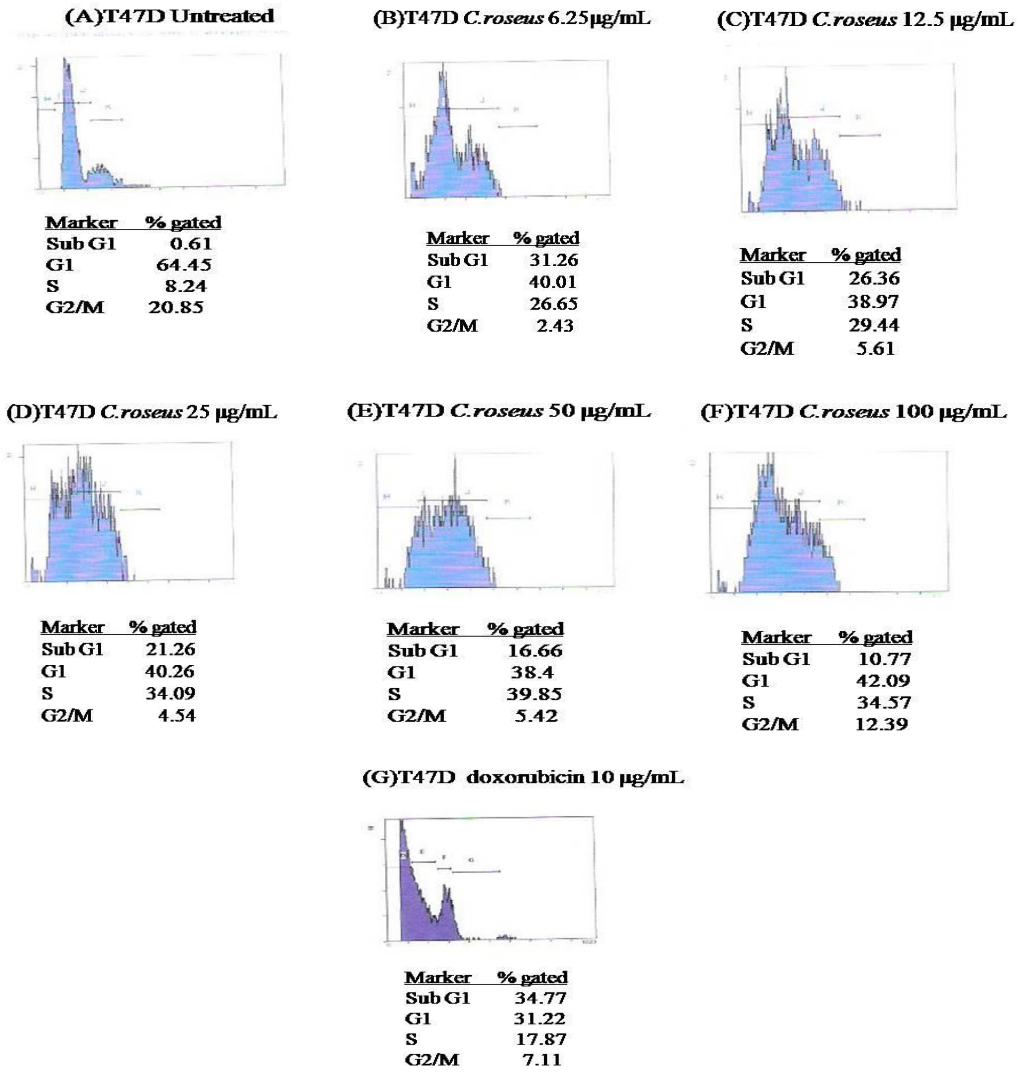

Figure 2B. Effect *C. roseus* extract on T47D cell cycle progression (A=Untreated 1x10⁴cells; B= *C.roseus* 6.25 µg/mL ; C= *C.roseus* 12.5 µg/mL; D= *C.roseus* 25 µg/mL; E= *C.roseus* 50µg/mL; F= *C.roseus* 100 µg/mL; G=doxorubicin 10 µg/mL

Table 1. Antioxidant activity of *C. roseus* extract

No	Antioxidant agents	IC_{50} (µg/mL)
1	*C. roseus* extract	358.411
2	Quercetin	19.200
3	BHA	94.178

DISCUSSION

Apoptosis is characterized by fragmentation of nuclear chromatin into fragments approximately

20 base pairs in length. Apoptosis is known to play a role in maintaining homeostasis and known to occur in mammals (McCloskey *et al*., 1994). The flow cytometric method has several advantages over the electrophoretic method. It analyzes a cell population one cell at a time and thereby allows discrimination of subsets within the main population. By selecting out the apoptotic subset, we can therefore quantitate the percentage of apoptotic cells in each sample.

C. roseus induced apoptosis in T47D cell line. The percentage of apoptotic cells were 6,93%, 26,35% and 37,48% with concentrations of extract 6.25µg/mL, 25 µg/mL and 50 µg/mL, respectively. The more information can be found in Fig 1, 2A and 2B. This result were consistent with previous studies that the *C. roseus* extract is able to induce DNA fragmentation. In each case, DNA fragmentation was characterised by oligonucleosomal size fragments of about 180-200 base pairs (bp), a well-known feature indication of programmed cell death (Compton 1992; Ahmad *et al*., 2010). The apototic effect of *C. roseus* extract were due to the presence of compounds, such as vinblastine which have anti-leukaemic properties *in vitro* (Caron and Herwood 2007). Vinblastine is one of the first vinca alkaloids that identified to have anti-tumour activity (Nobili *et al*., 2009). Further analysis of the *C. roseus* extract should be performed according to the American National Cancer Institute criterion that the IC_{50} limit to consider a crude extract promising for further purification be lower than 30 µg/ml (Suffness and Pezzuto 1990; Ahmad *et al*., 2010). *C. roseus* crude aqueous extract at very low doses inhibited the proliferation of Jurkat cells. The DNA fragmentation result showed that the cytotoxicity effect of the extract was due to programmed cell death. The extract contained compounds that specifically induce cell death in Jurkat cells (Okonogi *et al*. 2007; Nobili *et al*. 2009; Ahmad *et al*., 2010). Two vinca alkaloids from *C. roseus*, vinblastine, and vincristine are widely used in cancer therapy (Comin-Anduix, 2001). Both drugs inhibit the self-assembly of tubulin into microtubules at substoichiometric concentrations by forming a tubulin-drug complex at the end of a growing microtubule and thus blocking self-assembly (Margolis *et al*., 1980; Comin-Anduix, 2001). In addition, it is known that vinblastine induces G2/M arrest and subsequent apoptosis in different cell lines (Fan *et al*., 2001; Comin-Anduix, 2001).

Sample which contain antioxidant compounds will react with DPPH free radical to produce color change from purple to yellow (Kikugawa *et al*., 1999; Gordon, 2001). The changes in colour that was proportional to the concentration of sample were further measured at 517nm (Miliauskas *et al*., 2003). The antioxidant assay showed that sample had lowest antioxidant activity compared to BHA and quercetine with the IC50 values are 358; 94 and 19 µg/mL, respectively. However, *C. roseus* extract induced apoptosis. This result was consistent with previous research. ROS intermediates for apoptosis signaling was demonstrated by the following four observations: (1) Antioxidants could inhibit a certain apoptosis; (2) generation of hydrogen peroxide (ROS) was detected in the cells undergoing apoptosis; (3) extracellular addition of ROS can induce apoptosis and (4) downregulation of intracellular anti-oxidant level can induce apoptosis. The involvement of ROS in apoptosis is also supported by the observation that manganese superoxide dismutase (MnSOD), which inactivates mitochondrial superoxide anion that has leaked from the electron transport (Higuchi *et al*., 1998). ROS Hydrogen peroxide (H_2O_2) is able to induce apoptosis (Ray *et al*., 2000).

Hence, in our current results, we conclude that *C. roseues* extract had potential anticancer property through ROS and apoptosis induction. Investigation to know the apoptotic mechanism induced by *C. roseus* extract should be pursued.

CONCLUSIONS

C. roseus [L] G.Don extract induced apoptosis in T47D cell line, but had no antioxidant property.

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