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Original Article

Anticancer and free radical scavenging potency of *Catharanthus roseus, Dendrophthoe petandra, Piper betle* and *Curcuma mangga* extracts in breast cancer cell lines

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Key Words
Anticancer; Apoptosis; Catharanthus roseus; Curcuma mangga; Dendrophtoe petandra; Piper betle; T47D cell line

Abstract
This research was conducted to identify the anticancer and antioxidant activity of *Catharanthus roseus* [L] G.Don, *Dendrophtoe petandra* L, *Piper betle* L and *Curcuma mangga* Val aqueous extracts in T47D human ductal breast epithelial tumor cell line. The anticancer potency was determined via the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay while the apoptotic activity was determined with Sub-G1 flow cytometric analysis. The antioxidant activity was determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. *C.roseus* was able to reduce T47D cell proliferation with a median inhibition concentration (IC_{50}) of 2.8%; *D.petandra* 1.2%; *P.betle* 2.8%; and *C.mangga* 74.8%. The apoptotic analysis result showed that *C.roseus* induced apoptosis for 37.67%; *D.petandra* 24.03%; *P.betle* 9.45%; and *C.mangga* 0.41%. Meanwhile doxorubicin at 10 µg/ml induced apoptosis for 36.06%. The highest DPPH scavenging activity was recorded for *P.betle* extract as to be 83%; *D.petandra* 75.11%; *C.roseus* 71.87%; and the lowest for *C.mangga* as 38.45%. *C.moseus* and *D.petandra* aqueous extracts presented the highest anticancer activity by means of cell proliferation inhibition and inducing apoptosis. *P.betle* extract represented the strongest antioxidant activity. *C.mangga* extract exhibited no anticancer and only low antioxidant activity.

INTRODUCTION

Breast cancer is the most common cancer among women and the second leading cause of cancer deaths in women after lung cancer [1, 2]. The etiology of breast cancer involves genetic, hormonal, and dietary factors. Cancer prevention by using dietary or natural substances is considered as an approach to reduce the increasing incidence of cancer [3]. In spite of many advances in cancer treatment, chemotherapy of solid tumors is still greatly limited by the lack of selectivity and recurrence of drug resistant tumors. Finding novel chemotherapeutic agents, especially from natural substances, continue to be a focus of effort. Diets rich in grains, fruits, and vegetables are able to reduce cancer risk, and to implicate edible plants as potential sources of anticancer agents. A variety of compounds produced by edible plants has demonstrated anticancer activity [4, 5]. The dietary polyphenols are great interest due to their antioxidant and anticarcinogenic activities [6, 7]. Polyphenols may inhibit carcinogenesis by affecting the molecular pathway in the initiation, promotion and progression stages [6]. Fruits and vegetables contain high polyphenols and significantly reduce risks for cancers in many types. The leading effects of polyphenols on cancer cells are concentrated on growth, differentiation and apoptosis [8].

Lipid peroxidation is a product of free radical mediated injury in biological tissues; it is widely been used for evaluating the free radical involvement in cell damage [9]. Lipid peroxidation can be detected by the formation of thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH) and

conjugated dienes (CD). The status of the antioxidants such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) also reflect the oxidative state of the tissue. Lipid peroxidation in breast cancer tissues is reported to be significantly higher than control [10]. The antioxidants CAT and SOD are able to act as anticarcinogens, *i.e.* inhibitor at the initiation and promotion/transformation stage of carcinogenesis. Cellular injury caused by superoxide as well as DNA strand scission caused by the xanthine/xanthine oxidase can be prevented by SOD and CAT [9]. The chemotherapeutic drugs including etoposide, camptothecin, vincristine, cisplatinum, cyclophosphamide, paclitaxel, 5-fluorouracil and doxorubicin have been observed to induce apoptosis in cancer cells [2, 11-13]. Apoptosis, a type of programmed cell death, is an active process; deficiency or an excess of apoptosis can trigger cancers, autoimmune disorders, and many other diseases [14]. Primary intracellular targets, drug-induced cytotoxicity ultimately converges on a common pathway, causing apoptosis. Cells exposed to anticancer agents exhibit apoptotic alterations, such as cell shrinkage, chromatin condensation, and internucleosomal DNA fragmentation [15].

Catharanthus roseus is used as a remedy in cancer. Ethanolic extract of *C.roseus* is able to inhibit proliferatin in T47D human ductal breast epithelial tumor cell lines with a median inhibition concentration (IC_{50}) of 26.22 µg/ml [2]. *Dendrophtoe petandra* is traditionally used as cancer medicine. *Curcuma mangga* oil inhibits growth of cells and induce apoptosis by increasing expression of p53 [16]. The objective of this research is to elucidate anticancer, apoptosis inducing and antioxidant activities of aqueous extracts of *C.roseus*' whole parts, *D.petandra'*s leaves and small branches, *Piper betle*'s leaves and *C.mangga'*s rhizomes.

MATERIALS AND METHODS

The materials were aerials and roots of *C.roseus* [L] G.Don, small branches of *D.petandra* L, rhizomes of *C.mangga* Val, leaves of *P.betle* L collected from plantations located in Bogor, West Java, Indonesia. The plants were identified by staff of herbarium of the Department of Biology, School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. The aerials and roots, leaves, branches and rhizomes of the materials were collected, chopped finely and kept under drier tunnel (40-45ºC).

Preparation of extract

Ten grams of dried and chopped materials were boiled with 100 ml distilled water with 75-90ºC until the water remained 50 ml and filtrated. The aqueous extracts were stored at 4ºC. The aqueous extracts of *C.roseus*, *D. petandra*, *P.betle* and *C.mangga* were dissolved in 10% dimethysulfoxide (DMSO; Merck) and subsequently diluted to appropriate working concentrations with Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich) culture for proliferation inhibitor proliferative [2, 17]. The aqueous extracts were dissolved in water for determining 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity.

Cell culture

The human breast cancer T47D cell line was obtained from Research Center for Chemistry, Indonesian Institute of Sciences, Division of Natural Products Bandung, West Java, Indonesia. The cells were grown and maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich) and 100 µg/ml streptomycin (Sigma-Aldrich), and incubated at 37ºC in a humidified atmosphere and 5% CO_2 [17, 18].

Cell viability assay

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Madison, WI, USA) was used in order to determine cell viability. This assay uses an optimized reagent containing resazurin converted to fluorescent resorufin by viable cells that absorbs the light at 490 nm. Briefly, the cells were seeded into a 96-well plate ($5x10^4$ cells per well) [19]. After 24 h incubation, cells was supplemented by *C.roseus*, *D.petandra*, *P.betle* and *C.mangga* extracts with various concentrations, then incubated for 24 h. Cells not supplemented with extract were used as negative control. MTS was added to each well at a ratio of 1:5. The plate was incubated at 5% CO_2, 37ºC for 2-4 h. The absorbance of the cells was measured at 515 nm with a microplate reader. The data were presented as percent of viable cells (%) and analyzed by calculating the IC_{50} using probit analysis (SPSS 20).

Apoptosis assay

Cells were used in apoptotic studies when 80% confluence was reached in T25 tissue culture treated flasks. Cells were harvested with trypsin-EDTA (0.25-0.038%) and washed with phosphate buffered saline (PBS). T47D cells were seeded into 12-well plate at the density 10^4 cells per well incubated for 24 h with various concentrations of extracts. After 24 h of incubation, untreated and treated cell were rinsed with PBS, fixed with trypsin-EDTA and incubated at 37ºC for 5 min. Afterwards, the medium was added with a ratio of 3:1 (medium:trypsin-EDTA) and centrifuged at 1500 rpm for 5 min. The supernatant was discarded, while the pellet was added with 70% ethanol, and then

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incubated at 4ºC for 5 min. The incubated cells were centrifuged afterwards at 1500 rpm for 5 min, and the supernatants were 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 aluminum foil for 15 min 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 the percentage of cells.

DPPH scavenging activity assay

50 µl of *C.roseues, D.petandra, P.betle* and *C.mangga* aqueous extracts. To obtain the IC_{50} value, a range of various final concentrations were used; *e.g.* 10%, 5%, 2.5% and 1% introduced at the microplate and then were added 200 µl of 0.077 mmol/l DPPH in methanol. The reaction mixture was shaken vigorously and kept in the dark for 30 min at room temperature, furthermore DPPH scavenging activity was determined by a microplate reader at 517 nm [2, 20]. The radical scavenging activity of each sample was calculated by the ratio of lowering of the absorption of DPPH (%) and by the relative of the absorption (100%) of the DPPH solution in the absence of test sample (negative control) [20]:

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

-**A_s:** absorbance of samples
-**A_c:** negative control absorbance (without sample)

RESULTS

Antioxidant activity of *C.roseus, D.petandra, P.betle* and *C.mangga* extracts

The DPPH free radical scavenging activity of *C.roseus*, *D.petandra*, *P.betle* and *C.mangga* aqueous extracts of various concentrations were measured to examine the antioxidant activity. The IC_{50} is the concentration of antioxidant activity to scavenge DPPH free radical 50%. DPPH scavenging activity of *C.mangga* extract showed the lowest activity compared to *C.roseus*, *D.petandra* and *P.betle* aqueous extracts (Fig.1, Table 1).

Cytotoxic activity of *C.roseus, D.petandra, P.betle* and *C.mangga* extracts

The *C.roseus* and *D.petandra* extracts exhibited a decrease of viability in T47D cell lines in a concentration dependent-manner. The IC_{50} value (concentration of anticancer candidate which could inhibit 50% cell proliferation) was found to be 2.8%, 1.2%, 2.8% and 74.8% for *C.roseus*, *D.petandra*, *P.betle* and *C.mangga* extracts, respectively (Table 2).

Apoptosis inducing activity of *C.roseus, D.petandra, P.betle* and *C.mangga* extracts

Table 3 shows the apoptosis rate of T47D cells treated by *C.roseus*, *D.petandra*, *P.betle* and *C.mangga* extracts. As a main result, *C.roseus* and *D.petandra* extract were clearly able to induce apoptosis in T47D cell line. With 10% concentration the apoptosis rate was found to be 37.67%, 24.025% and 9.445% for *roseus*, *D.petandra* and *P.betle* extracts, respectively. *C.mangga* extract was not able to induce apoptosis in T47D cell line at any concentration.

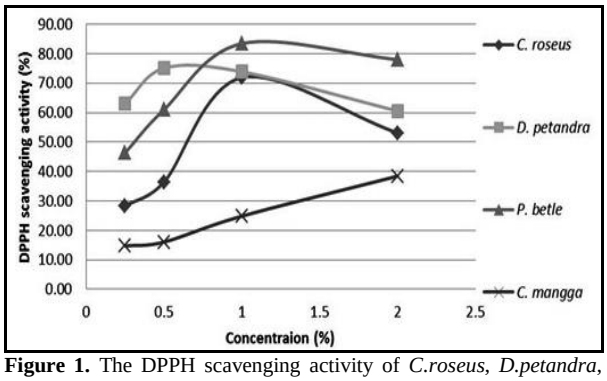

Figure 1. The DPPH scavenging activity of *C.roseus, D.petandra, P.betle* and *C.mangga* aqueous extracts diluted in concentrations of 10, 5, 2.5 and 1%.

Table 1. The highest DPPH scavenging activity, linear equation, coefficient of regression (R^2) and IC_{50} of *C.roseus*, *D.petandra*, *C.mangga* and *P.betle* aqueous extracts.

Samples (extracts)	The highest DPPH scavenging activity (%)	Linear equation	R^2	IC_{50} (%)
Catharanthus roseus	71.87	Y=59.907X+10.591	0.975	0.66%
Dendrophthoe petandra	75.11	Y=67.031X+43.132	0.973	0.1%
Piper betle	83.46	Y=48.812X+35.14	0.995	0.3%
Curcuma mangga	38.46	Y=14.063X+10.337	0.993	2.82%

The DPPH scavenging activity assay was performed three times for each extract.

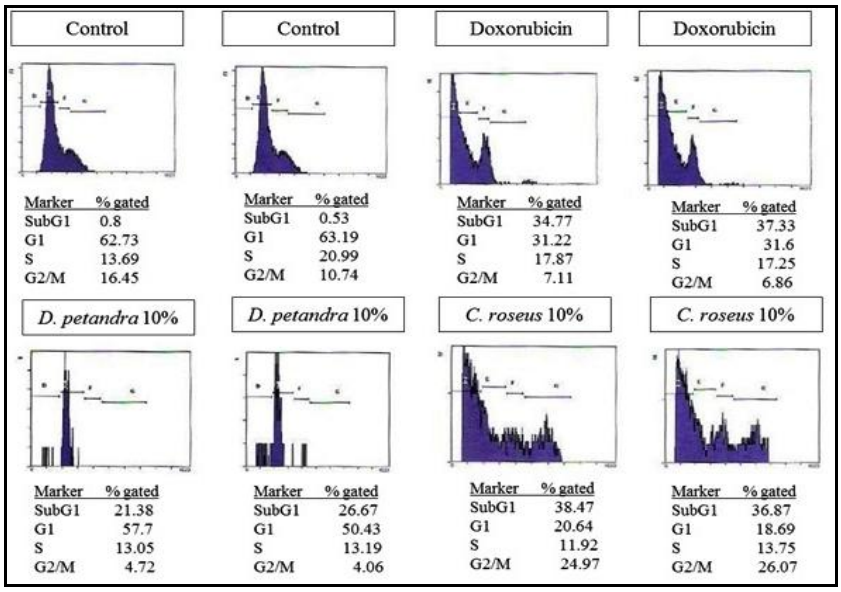

Figure 2.
Dot blot of control, doxorubicin, *D.petandra* (10%) and *C. roseus* (10%) extracts towards cell population on Sub-G1, G1, S and G2/M (%) phase for 24 h incubation.
Each treatment were replicated two times. The apoptotic cells were determined by Sub-G1 area from cell cycle analysis and it was presented as the percentage of cells.

Table 2. The IC_{50} of *C.roseus*, *D.petandra*, *P.betle* and *C.mangga* aqueous extracts in T47D cell viability for 24 hours incubation.

Samples (extracts)	IC_{50}
Catharanthus roseus	2.8%
Dendrophthoe petandra	1.2 %
Piper betle	2.8%
Curcuma mangga	74.8%

Each extract was replicated three times and inhibition data towards cell growth were analyzed using probit analysis.

Table 3. Effect various extracts towards cell population on Sub-G1 (%) for 24 hours incubation.

Samples (extracts)	Concentration		
	0	**10 %**	**1%**	**0.01%**
Normal cells	0.8			
	0.53			
Average	0.44			
Doxorubicin 10 µg/m1	34.77			
	37.33			
Average	36.05			
Catharanthus roseus		36.87	2.86	0.65
		38.47	3.4	0.5
Average		37.67	3.13	0.58
Dendrophthoe petandra		26.67	0.43	0.88
		21.38	0.56	1.2
Average		24.03	0.5	1.04
Piper betle		9.6	0.66	0.56
		9.29	0.91	0.5
Average		9.45	0.79	0.53
Curcuma mangga		0.41	0.36	1
		0.41	0.34	0.86
Average		0.41	0.35	0.93

The apoptosis assay was analyzed by cell cycle using a flow cytometer. The apoptotic cells were determined by Sub-G1 area from cell cycle analysis and it was presented as the percentage of cells.

DISCUSSION

The present data showing that *D.petandra* aqueous extract has the highest antioxidant activity with an IC_{50} of 0.1% (Fig.1, Table 1) verifies the previous studies reporting that ethanolic extract of *D.petandra* has high antioxidant potency [2], its crude aqueous ethanol extract has high antioxidant activity [21], and water and ethanolic extracts exhibit DPPH free radical scavenging activity [22]. *P.betle* aqueous extract presented antioxidant activity with an IC_{50} of 0.3%; this result is in line with the previous research reporting that *P.betle* ethanolic extract has high antioxidant properties [2]. The lowest antioxidant activity in the present study was seen for *C.mangga* with an IC_{50} of 2.82%; this data is similar to our previous research showing that *C.mangga* ethanolic extract has no antioxidant activity [2]. However, the present IC_{50} of *C.roseus* was 0.66%; this result was not verified with our previous data representing that *C.roseus* ethanolic extract has low antioxidant activity [2]. A different solvent would have a different compound and bioactivity; for instance, two previous reports presented that aqueous extract of *Forsythia koreana* flowers have higher phenolic content than its ethanol extract [23, 24].

D.petandra aqueous extract showed anticancer activity both cytotoxic and apoptotic inducing activities (Tables 2&3). This results was validated by previous research that *D.petandra* is traditionally used as cancer medicine and its flavonoid content inhibits growth of *Artemia salina* Leach as an anticancer activity assay *in vivo* [25]. *D.petandra* contains quercitrine, β-sitosterol [26], flavonoids, tannins, amino acids, carbohydrates, alkaloids, saponins [27], L-asparaginase which lower

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asparagin blood level and inhibit cancer cells growth [27, 28], and quercetin which regulate cell cycle, induce estrogen receptor II (ESR2, ER-beta) expression for growth inhibition and inhibit tyrosine kinase to be involved in oncogenesis via an ability to override normal regulatory growth control, inhibit heat shock protein (HSP) production on cancer cells, and down regulate expression of mutant p53 [29]. On the other hand, the high anticancer activity of *D.petandra* aqueous extract was not validated by previous studies reporting that water and methanolic extracts of *D.petandra* leaves from various host shows that these extract were not toxic in brine shrimp [30], and that its ethanolic extract has no cytotoxic activity in breast cancer T47D cell line [2].

The anticancer activity of aqueous extracts of *C.roseus* was comparable with *P.betle*. These results were validated with our previous study showing that *C.roseus* and *P.betle* ethanolic extracts do have cytotoxic activity [2]. This result was also consistent with previous studies reporting that *C.roseus* extract is able to induce DNA fragmentation by gel electrophoresis [31, 32]. Crude extract of *C.roseus* using 50 and 100% methanol has significant anticancer activity against different cell types *in vitro* [33]. Crude decoction shows moderate *in vitro* antiangiogenetic effect [34-36]. Vincristine and vinblastine, alkaloid compounds in *C.roseus*, are effectively used against a number of cancers [37, 38]. These alkaloids are able to inhibit cancer cell growth during metaphase leading to cell death [39]; they cause apoptosis rather than necrosis in human neuroblastoma cell line SH-SY5Y [40]. The NF-κB/IκB signaling pathway may contribute to the mediation of vinca alkaloid-induced apoptosis in human tumor cells [41]. Vinca alkaloids increase apoptosis by increasing concentrations of the cellular tumor antigen p53 and cyclin-dependent kinase inhibitor 1 (p21), and by inhibiting Bcl-2 activity. Increasing concentrations of p53 and p21 lead to changes in protein kinase activity [42]. *P.betle* aqueous extract presented clearly anticancer activity in the present study (Table 2.) which is a consistent result with our previous study using its ethanolic extract in the same cell line [2]. Hydroxycavichol, a *P.betle* leaf component, is reported to exhibit anticancer activity in KB cells (mouth epidermal carcinoma cells) as revealed by detection of Sub-G0/G1phase, and depletion of reactive oxygen species production [43].

In our study, *C.mangga* aqueous extract exhibited no anticancer activity in T47D cell line. This result is also consistent with our previous research showing that ethanolic extract of *C.mangga* has no anticancer activity [2]. In addition, the low antioxidant activity seen for *C.mangga* aqueous extract in the present work is also similar to the low DPPH scavenging activity of its ethanolic extract in the earlier study. [2].

In conclusion, *C.roseus*, *D.petandra* and *P.betle* aqueous extracts have potential anticancer activity by inducing apoptosis, as well potential antioxidant activity, while *C.mangga* aqueous extract have no anticancer activity and only low antioxidant activity.

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COMPETING INTERESTS

The authors declare that they have no conflict of interest.

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