

The combination of fraction A3 of *Cyrtostachys renda* Blume with doxorubicin improved cytotoxicity against T47D cell line through cell cycle arrest and apoptotic induction

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ARTICLE HISTORY

Received on: 19/04/2024
Accepted on: 04/08/2024
Available Online: 05/09/2024

Key words:

Cell cycle arrest, apoptosis, T47D cell line, fraction A3, doxorubicin, combination chemotherapy.

ABSTRACT

Currently, natural products of herb medicinal plants are widely reported as a supplement to cancer chemotherapy. The cytotoxic effect of a dichloromethane (DCM) extract of *Cyrtostachys renda* root and its flow cytometric profile against a human breast cancer cell line (T47D) were investigated in this study. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to assess the combined cytotoxic effects of fraction A3 *C. renda* root and doxorubicin. The combination index (CI) criteria was used to analyze the synergistic effect of fraction A3 and doxorubicin. The cell cycle and apoptosis profiles were completed using flow cytometric labeling with propidium iodide/RNase and Annexin V. Cell morphological changes were observed using an inverted microscope. Single cytotoxicity fraction A3 of DCM extract was effective against T47D cells with an IC_{50} value of 43.03 μ g/ml. Fraction A3 and doxorubicin at half of IC_{50} combined had a strong synergistic effect on T47D cells with a CI of 0.2. The cell cycle analysis results showed that this combination caused cell cycle arrest in the G1 and S phases. This observation is consistent with the results of single fraction A3 and doxorubicin treatments, which resulted in cell cycle arrests in the G1 and S phases, respectively. The result of single fraction A3 treatment, combined with doxorubicin for 24 hours, can also induce apoptosis against T47D cells. When the control and treatment groups were compared, the test cell shape altered.

INTRODUCTION

Cancer is a degenerative disease characterized by abnormal cell proliferation. Cancer cells are aberrant cells due to an imbalance in their proliferative and apoptotic properties. Cancer cells proliferate quickly, but their capacity to undergo apoptosis during the cell cycle is limited. The International Agency for Research on Cancer estimates that the global cancer prevalence in 2020 will be 19.2 million cases, with 9.9 million cases (51.6%) resulting in death [1]. Breast cancer is one of the leading causes of the high death rate noted before, followed by cancers of the cervix, lung, colon, prostate, ovary,

liver, nasopharynx, non-Hodgkin lymphoma, and leukemia [2]. Breast cancer is the most frequent type of cancer in women and the main cause of death [3,4]. Currently, the prevalence of cancer in Indonesia has reached 396,914 instances, with a death rate of 234,511 cases (59%), and breast cancer ranks first in terms of new cases, with 65,858 cases (16.6%) when compared to other types of cancer [5].

Various cancer treatment approaches have failed to offer effective and selective medication, leading to cancer therapy failure. Doxorubicin is a commercially available chemotherapeutic agent that is used as a first-line treatment for breast cancer. However, the chemotherapeutic drug doxorubicin's efficiency is limited due to its harmful effect on normal cells [6], hepatotoxicity-induced cardiotoxicity [7], mediated reproductive toxicity [8], and chemoresistance rendering the treatment ineffective [9,10]. Because these side effects are generated by the accumulation of doses, the dose and length of delivery limit doxorubicin's effectiveness

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[11,12]. As a result, the dose can be lowered without causing cytotoxicity or negative effects. Researchers have a huge difficulty in improving the administration of doxorubicin for breast cancer therapy to increase doxorubicin's efficacy as a chemotherapeutic agent. Furthermore, combining doxorubicin with other cytotoxic drugs can be used to improve therapy efficacy while lowering chemotherapeutic agent toxicity. Combination chemotherapy (co-chemotherapy) is a cancer treatment method that combines various chemotherapeutic medicines that are provided concurrently in an attempt to maximize the effectiveness of each chemotherapeutic agent [13]. Co-chemotherapy can use both natural and synthetic products [14]. Doxorubicin can be used with target compounds to improve the sensitivity of chemotherapeutic medicines to target cells. Natural ingredients are used to create compounds suitable for use as co-chemotherapy agents, specifically those that have a synergistic impact with chemotherapeutic drugs. A previous study indicated that combining doxorubicin with ethanol extract of the *Areca catechu* nut has a substantial synergistic effect on MCF-7 breast cancer cells, with a combination index (CI) value of 0.1–0.3 [15].

According to a prior study, several *Areca* plants contain bioactive compounds that could aid in cancer therapy. One of these is *Cyrtostachys renda*, which grows widely in Indonesia and can be found in many community plantations in Jambi Province, but its anti-cancer potential has never been investigated. *Cyrtostachys renda* is designated as the mascot of Jambi Province. It is a Jambi native flora that is utilized as an attractive plant, herbal medicine, and for a variety of other functions due to its midrib fiber. Based on a previous study, the ethanol extract of *A. catechu* seeds, which is related to *C. renda*, contains secondary metabolites with strong cytotoxic activity against the breast cancer cell line (MCF-7) with an IC_{50} value of 75.1 $\mu\text{g/ml}$ and also enhanced the activity of doxorubicin's cytotoxicity [15]. Arecoline, isolated from the *Areca* plant, has been demonstrated to suppress the development of KB epithelial carcinoma cells by up to 58% at a dose of 0.8 mm [16,17]. Alkaloids are found in many medicinal plants and show cytotoxic effects against cancer cell lines. It has also been found in *C. renda* to have a substantial cytotoxic effect and can be combined with doxorubicin [18,19]. The previous study through the DPPH method indicated that the ethyl acetate extract of the fruit and roots of *C. renda* had the highest antioxidant activity, with an IC_{50} value of $11.47 \pm 0.169 \mu\text{g/ml}$ and $10.69 \pm 0.084 \text{ g/ml}$ [20]. According to the brine shrimp lethality test, *C. renda* root dichloromethane (DCM) extract exhibited strong toxicity against *Artemia salina*, with an LC_{50} value of $43.42 \pm 0.659 \mu\text{g/ml}$ [21].

MATERIAL AND METHODS

Experimental design

The T47D breast cancer cell line was used in the *in vitro* assay. The cells were received from Muhammadiyah University's Faculty of Medicine and Health Sciences in Yogyakarta, Indonesia, through the *in vitro* culture laboratory. The cell density is determined using a hemocytometer on confluent developing cells (80%) in a tissue culture dish that is

planted in a 96-well microplate with 1×10^4 cells per well. This study has been granted ethical approval (No. 3486/UN21.8/PT. 01.04/2022, date: 25/Nov/2022). The fractionate of *C. renda* was dissolved with DMSO and cell culture media until the concentration was less than 0.1% v/v. Doxorubicin was utilized as a chemotherapeutic agent in a combination treatment.

Simplicia preparation

Cyrtostachys renda root was collected in Muara Bungo, Jambi Province, and identified at the Herbarium, Plant Taxonomy Laboratory, Padjadjaran University (No.48/HB/04/2022). It was dried for a week before being dried in a 60°C oven for 1×24 hours. The roots were then pulverized with the grinder.

Extraction and fractionation

Cyrtostachys renda root powder (500 g) was macerated in methanol (MeOH) three times for 24 hours. A rotary evaporator was used to evaporate the solvent, yielding 237.7 g dried methanol extract. Liquid–liquid extraction with water was performed to separate the secondary metabolite content based on the level of polarity using the consecutive solvents *n*-hexane, DCM, ethyl acetate (AcOEt), and water (H_2O). After the solvent was removed, the extracts *n*-hexane, DCM, AcOEt, and H_2O were formed. Each extract was evaluated the cytotoxicity against T47D cells. The most promising extract was subjected to SiO_2 vacuum liquid chromatography. The fractionates were divided into five fractions (A1–A5) based on the similarity of the stain patterns based on the thin layer chromatography profile. Each fraction was then tested for the cytotoxicity against T47D cell line.

Preparation of cell cultures

T47D cells were grown in RPMI 1,640 media supplemented with 10% FBS and 2% penicillin/streptomycin. Cells were cultivated and propagated in a CO_2 incubator with 5% CO_2 in a humid atmosphere at 37°C. Once the cells have reached confluence, they are ready to be used as test subjects.

Cytotoxicity assay

The cytotoxic fraction A3 of *C. renda* root, doxorubicin and their combination against T47D breast cancer cells was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as reported in previous studies [22]. Each well of 96-well microplates was filled with 100 μl of T47D cell suspension at a density of 1.5×10^4 cells. The fractionates *C. renda* with a series of concentration variations ranging from 15.63 to 250 $\mu\text{g/ml}$ and doxorubicin with a series of concentration variations ranging from 0.63 to 10 $\mu\text{g/ml}$ added to the cell cultures. Each treatment group was tested three times. After that, the treated cell cultures were incubated for 24 hours at 37°C with 5% CO_2 . The culture medium was discarded at the end of the incubation period, and the cells were washed with phosphate-buffered saline (PBS). A volume of 100 μl MTT solution was then added to each well, followed by 4 hours of incubation at 37°C and 5% CO_2 . Following the completion of the incubation, 100 μl of 10% sodium dodecyl sulfate reagent was added as

a stopper and stored overnight. An ELISA reader was used to measure cell absorbance at 595 nm. The percentage of living cells was calculated by making a curve between the percentage of cell viability and concentration to obtain the IC₅₀ value of the test compound against the T47D cell line. The significance percent test of cell viability was calculated using the following equation:

$$\% \text{ cell viability} = \frac{\text{TC-CM}}{\text{CC-CM}} \times 100$$

where TC is the absorbance of treatment cells, CC is the absorbance of control cells, and CM is the absorbance of control media.

The most cytotoxic fraction A3 was mixed with doxorubicin for the combined cytotoxic test. The combined fraction of A3 and doxorubicin dosage was designed to be less than the A3 and doxorubicin IC₅₀ values, as seen in Table 1. The CI metric was used to quantify the synergistic effect of fraction A3 and doxorubicin.

Cell imaging

Phase-contrast microscopy was used to assess changes in cell morphology after the A3 and doxorubicin were applied in the previous stage. An inverted microscope was used to record images of changes in cell morphology.

Flow cytometry

Flow cytometry (BD FACS Calibur) was used to assess apoptosis and the cell cycle. The T47D cell line was cultured in six-well plates at 5 × 10⁵ cells/well and treated for 24 hours with A3 fraction and doxorubicin. Cells were washed with PBS and centrifuged at 2,000 rpm for 5 minutes. After 3 minutes of separation with 200 µl trypsin-EDTA, 1 ml RPMI was added and centrifuged at 2,000 rpm for 5 minutes. Cell cycle analysis was performed on treated cells using the propidium iodide/ RNase staining kit (BD Pharmingen™) according to the kit instructions. Following that, the treated cells were stained with the FITC Annexin V labeling kit for apoptosis assay (BD Pharmingen™) according to the kit protocol. Next, Cell flow cytometry profiles were measured.

Statistical analysis

A two-way analysis of variance (ANOVA) was utilized to assess whether there was an interaction between fraction A3 and the doxorubicin combination. To compare the effects of fraction A3 and doxorubicin on the T47D cell line, a one-way ANOVA test was used.

RESULT AND DISCUSSION

Bioassay guided fractionation

MeOH extract of *C.renda* root (237.7 g) was successively partitioned into water-*n*-hexane, water-DCM, and water-AcOEt to distribute the content of secondary metabolites based on their polarity level to afford *n*-hexane (1.14g), DCM (36.0 g), AcOEt (4.80 g), and H₂O extract (59.0 g). Based on the cytotoxicity test, only the DCM extract displayed potent cytotoxicity against the T47D cell line (Table 2). Then, the

cytotoxic DCM extract (36.0 g) was subjected to SiO₂ vacuum liquid chromatography by using combination eluent of DCM-MeOH-H₂O = (30:3:1), (15:3:1), (7:3:1), (3:3:1), MeOH 100% to give five fractions, A1(5.2 g), A2 (4.4 g), A3 (7.6 g), A4 (8.3 g), and A5 (10.5 g). Each collected fraction was evaluated for the cytotoxicity against T47D cell line to disclose fraction A3 exhibited the most potent with an IC₅₀ value of 43.03 µg/ml (Table 2).

The phytochemical studies on the methanol extract revealed that alkaloids were the major component, along with flavonoids, tannins, and saponins. The phytochemicals study was carried out using conventional methods [23].

Doxorubicin and fraction A3 possess single cytotoxic effect

The cytotoxic activity of fraction A3 and doxorubicin in suppressing the proliferation of the T47D cell line was assessed using the MTT test. The ability of dehydrogenase enzymes in living cells to convert MTT substrate into purple formazan crystals was used as a cytotoxic parameter. The intensity of the purple color is proportional to the number of living cells. The number of living cells increases as the intensity of the purple color increases [24]. In contrast, mitochondria in dead cells produce the enzyme tetrazolium succinate reductase, which can degrade the MTT reagent into formazan crystals, resulting in slightly purple formazan and a pink color change [25,26].

Table 1. Matrix simulation of the combination of fraction A3 and doxorubicin against T47D cell line.

A3 (IC ₅₀) (µg/ml)	Doxorubicin (IC ₅₀) (µg/ml)			
	1/16	1/8	1/4	1/2
1/16	1/16: 1/16	1/16: 1/8	1/16: 1/4	1/16: 1/2
1/8	1/8: 1/16	1/8: 1/8	1/8: 1/4	1/8: 1/2
1/4	1/4: 1/16	1/4: 1/8	1/4: 1/4	1/4: 1/2
1/2	1/2: 1/16	1/2: 1/8	1/2: 1/4	1/2: 1/2

Table 2. IC₅₀ values and cytotoxicity categories for extracts, fractions, and doxorubicin against the T47D cell line.

Sample	IC ₅₀ (µg/ml)	Cytotoxicity category
Extracts		
MeOH	410.28 ± 7.58	Weakly
<i>n</i> -hexane	1,128.40 ± 3.07	Non-toxic
DCM	109.95 ± 2.56	Weakly
AcOEt	374.10 ± 1.50	Weakly
H ₂ O	1,253.23 ± 2.39	Non-toxic
Fractions		
A1	768.77 ± 1.35	Weakly
A2	224.19 ± 0.92	Weakly
A3	43.03 ± 0.89	Moderately
A4	130.29 ± 1.77	Weakly
A5	90.81 ± 0.83	Weakly
Doxorubicin	2.22 ± 1.02	Potentially

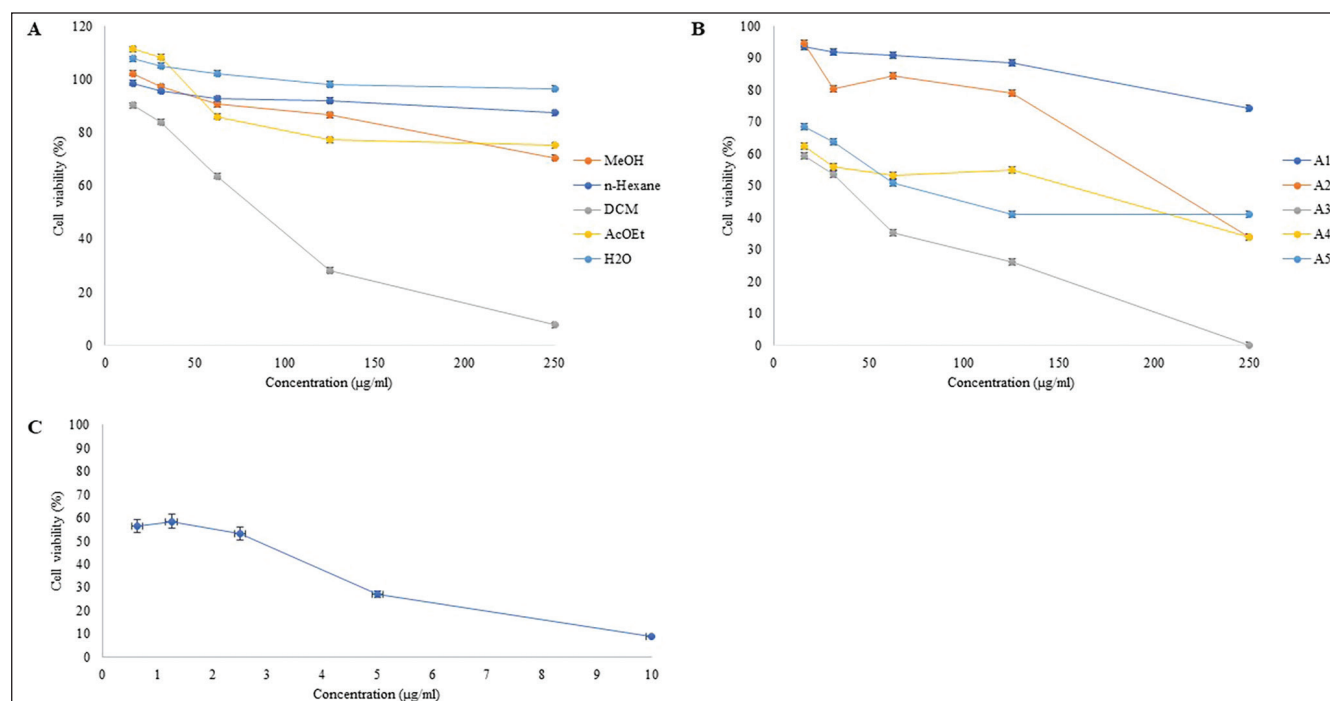


Figure 1. Cytotoxic activity of extracts, fractions and doxorubicin against T47D cell line. The panel showed a decrease in cell viability after treatment for 24 hours. The data were independently carried out for three times. (A) Extracts. (B) Fractions. (C) Doxorubicin.

Based on the data in Figure 1A and B, an IC_{50} value of DCM extract; $109.95 \pm 2.56 \mu\text{g/ml}$ and fraction A3; $43.03 \pm 0.89 \mu\text{g/ml}$ were determined to be the most potent among other extracts, and fractions A1–A5. Meanwhile, doxorubicin, used as a positive control, had an IC_{50} value of $2.22 \pm 1.02 \mu\text{g/ml}$ (Fig. 1C). This was validated by the inhibitory value against T47D cell viability; at the lowest dose ($15.63 \mu\text{g/ml}$), fraction A3 was able to inhibit cell growth up to 38.75% (Fig. 1B), with an IC_{50} value of $43.03 \mu\text{g/ml}$. Cytotoxic activity was divided into three groups based on the IC_{50} value: $IC_{50} 100 \mu\text{g/ml}$ showed potentially cytotoxic, $100 \mu\text{g/ml}$ $IC_{50} 1000 \mu\text{g/ml}$ indicated moderate cytotoxic, and $IC_{50} > 1,000 \mu\text{g/ml}$ indicated nontoxic [27]. In this study, when the cytotoxic effect of fraction A3 was compared to the positive control, doxorubicin exhibited a high cytotoxic effect with an IC_{50} value of $2.22 \mu\text{g/ml}$ as a single evaluation after 24 hours of treatment. Meanwhile, fraction A3 had modest cytotoxic activity (Tabel 2). Both samples demonstrated concentration-dependent effects against the T47D cell line. According to the National Cancer Institute, a compound possesses cytotoxic activity if its IC_{50} value is less than $20 \mu\text{g/ml}$. The most potent cytotoxic compounds can be utilized to treat cancer, while moderately cytotoxic compounds can be used to prevent or inhibit cancer cells or as co-chemoprevention agents [27,28]. As a result, the IC_{50} values of fraction A3 are classified as potentially or strong cytotoxic categories as seen in Table 2. Secondary metabolites, particularly flavonoids, tannins, and saponins, are likely to contribute to fraction A3's cytotoxic activity. Saponins' harmful effects are caused by the induction of apoptosis or non-apoptosis [29,30]. Flavonoids have the ability to reduce hydroxyl radicals, superoxide, and anti-liperoxidants [31].

The cytotoxic effect of fraction A3 in combination with doxorubicin

The combination treatment's concentration was determined using the IC_{50} values of doxorubicin and fraction A3, where the concentrations employed were less than their respective IC_{50} values. Doxorubicin concentrations of 0.14, 0.28, 0.56, and $1.11 \mu\text{g/ml}$ were all low, as were fraction A3 values of 2.69, 5.33, 10.75, and $21.50 \mu\text{g/ml}$. Fraction A3 demonstrated strong cytotoxicity in reducing the development of T47D cancer cells at the given IC_{50} value. At the lowest dose of $2.69 \mu\text{g/ml}$, cell viability reached 47.82%, meaning that fraction A3 may kill 52.20% of T47D cells. Cell viability was 9.63% at the highest dosage of $21.50 \mu\text{g/ml}$, showing that fraction A3 may kill 90.40% more T47D cells (Tabel 3). According to these data, the viability of live T47D cells reduced as the percentage fraction A3 concentration increased. The combined therapy resulted in alterations in shape and decreased cell density. The interaction between fraction A3 and doxorubicin was found to be highly significant ($p < 0.05$) using a two-way ANOVA. The magnitude of this effect was determined using a one-way ANOVA of cytotoxicity with the Scheffé post-hoc comparison. The cytotoxic mean fraction A3 and doxorubicin concentrations varied significantly among the six concentrations. When evaluated with the T47D cell line, the one-way ANOVA of fraction A3 with the Scheffé post-hoc comparison conclusively demonstrated that fraction A3 and doxorubicin were considerably more active doxorubicin than fraction A3 ($p < 0.05$). Combination of fraction A3 and doxorubicin were found to be significantly more cytotoxic than fraction A3 and doxorubicin single.

Table 3. Cell viability profile of fraction A3 and doxorubicin combination at IC₅₀ sub concentration.

Concentration of A3 (µg/ml)	Concentration of doxorubicin (µg/ml)				
	0.00	0.14	0.28	0.56	1.11
0.00	100.00 ± 0.00	80.64 ± 1.99	76.85 ± 0.52	72.25 ± 0.28	62.94 ± 0.98
2.69	47.82 ± 0.29	39.72 ± 0.69	40.54 ± 0.27	31.80 ± 1.37	30.39 ± 0.12
5.38	43.21 ± 1.62	36.36 ± 4.97	34.61 ± 0.52	28.74 ± 0.48	8.55 ± 0.51
10.75	28.89 ± 1.74	30.09 ± 2.30	30.85 ± 2.20	23.87 ± 1.38	8.98 ± 0.97
21.50	9.63 ± 1.64	5.53 ± 2.33	6.56 ± 1.00	4.37 ± 2.30	4.80 ± 0.43

Synergistic effect

The IC₅₀ value for doxorubicin was 2.22 µg/ml. These data hint at T47D cell resistance to doxorubicin. The fraction A3 of *C. renda* root, on the other hand, was insufficient to elicit cytotoxicity in T47D cells. As a result, we employed doxorubicin and the fraction A3 *C. renda* root at various concentrations based on their single cytotoxic IC₅₀ value. This study found out that increasing the concentration in the combination therapy affects cell viability as shown in Table 3. The largest effect was observed at approximately 0.56 µg/ml of doxorubicin and 21.50 µg/ml of A3, with a 95.63% ± 2.30% decrease in cell viability. When doxorubicin with an IC₅₀ value of 0.14 µg/ml was combined with A3 with combined with A3 with an index combination value of 0.07 and 0.08, a very high synergistic effect as in seen in Table 4. These results suggested that fraction A3 could be used to boost doxorubicin’s cytotoxicity against T47D cells.

Cell morphology by treatment with fraction A3 and doxorubicin

Cell morphology was studied using an inverted microscope in addition to quantitative analysis to determine changes in cell morphology before and after therapy. According to the data presented in Figure 2, T47D cells, which were originally elongated, transformed to a round shape, shrank the cell membrane, and became irregular in shape after being treated with fraction A3 and doxorubicin. These findings could be explained by a relationship between the treatment of the test compounds and cell physiological abnormalities, which are characterized by cell death. There was disharmony between the cell groups when tested with doxorubicin and fraction A3 treatment, indicating a change in cell form to the formation of a dead cell that was spherical/globular, and when compared to the cell control, the dead cells are more noticeable with doxorubicin and fraction A3 treatment than those that did not receive treatment (cell control). Significant cell death was demonstrated by changes in cell morphology to be rounder when compared to the cell control, indicating that the cell was dying, on the morphology of cells administered doxorubicin and fraction A3 treatment. Approximately 38% of cells died at a dose of 1.11 µg/ml doxorubicin and 53% died at a dosage of 2.69 µg/ml, and when fraction A3 concentrations were increased in the combination therapy, more cells died.

Flow cytometry profile

Cell cycle modulation effect of fraction A3 and doxorubicin

Flow cytometry was utilized to investigate the association between cytotoxic activity and cell cycle

Table 4. CI analysis of fraction A3 with doxorubicin.

Concentration of A3 (µg/ml)	Concentration of doxorubicin (µg/ml)			
	0.14	0.28	0.56	1.11
2.69	0.07	0.10	0.13	0.22
5.38	0.08	0.11	0.13	0.14
10.75	0.11	0.14	0.15	0.17
21.50	0.11	0.12	0.15	0.20

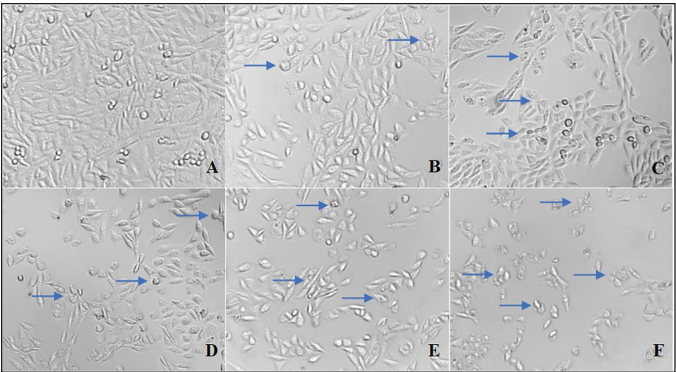


Figure 2. T47D cell morphology. Untreated (A). Doxorubicin 1.11 µg/ml (B). Fraction A3: 21.50 µg/ml (C). Combination of doxorubicin 0.14 and A3: 2.69 µg/ml (D). Combination of doxorubicin 0.14 and A3: 5.38 µg/ml (E). Combination of doxorubicin 1.11 and A3: 21.50 µg/ml (F). Cells with blue arrows have undergone morphological changes.

distribution profile caused by fraction A3, doxorubicin, and their combination. Previous studies have suggested that doxorubicin may cause cell cycle arrest in some cancer cells during the S, G1, and G2/M phases [32]. Simultaneously, the fraction A3 was used to arrest the cell cycle in the G1 and S phases. Fraction A3 and doxorubicin had different cell cycle patterns in the T47D cell line, according to cell cycle analysis. The findings of this study revealed that fraction A3 induced cell accumulation in the G1 phase, whereas doxorubicin promotes cell accumulation in the S phase. Interestingly, treatment with a combination of fraction A3 and doxorubicin contributed to cell accumulation, mainly in the G1 and S phases. In addition to the cell arrest of these two phases, cell accumulation in the G2/M phase increased when fraction A3 and doxorubicin were combined. In contrast, the combination fraction A3 and doxorubicin caused more cells to accumulate in the G2/M phase (22.8 %) than the A3 fraction (15.7 %)

and doxorubicin (10.6%) (Fig. 3). These findings point to the importance of fraction A3 concentration in enhancing doxorubicin's cytotoxic effects through cell cycle arrest.

The synergistic effect of the combination of fraction A3 and doxorubicin against T47D cells indicates be correlation to the induced apoptosis and cell cycle arrest, especially in the G1 and S phases. Previous studies have shown that *Areaceae* active components cause cell cycle arrest in the G1 phase [33,34], while doxorubicin inhibits topoisomerase II, causing cell cycle arrest in the S phase [35,36]. The combination of these two substances alters cell cycle arrest in both phases (G1 and S), causing many cells to fail to divide and decrease cell viability. This experiment was carried out in 24 hours, which is the normal period for one round of cell division. Based on these findings, the A3 fraction was capable of inducing cell death; however, apoptosis assays were required for more information.

Effect of fraction A3 and doxorubicin on apoptosis

Flow cytometry analysis employing the FITC dye Annexin V confirmed decreased cell viability despite cell cycle arrest and subsequently cell death. The treatment results showed that the single A3 fraction and its combination with doxorubicin increased cell death through inducing apoptosis. However, the number of necrotic cell deaths due to a single fraction of A3 and its combination with doxorubicin was higher than that caused by a single doxorubicin treatment (Fig. 4). Overall, flow cytometry analysis showed no significant difference in the evidence of cell death caused by the single fraction A3 or its combination with doxorubicin through apoptosis induction. However, when compared to a single treatment of doxorubicin, the results were significantly different.

The inhibitory effect of *C. renda* active components on cell cycle progression appears to be typical for many kinds of cancer cells, which can generally promote apoptosis. The cell cycle arrest effect of the fraction A3 combined with doxorubicin

against T47D cells appears to be correlated with apoptosis induction. Flow cytometry analysis (Fig. 4) showed a significant difference in the number of cells that experience mortality between a single treatment of doxorubicin and a combination with fraction A3. The fraction A3 and doxorubicin might act sequentially, despite having been shown that cell population arrest is much reduced in the S phase. This occurrence might be due to the antioxidant properties of the fraction A3, which may counteract the increase in intracellular ROS caused by doxorubicin. However, cells that pass through the S phase have the process inhibited by the fraction A3 and arrest at the G2/M phase. This is consistent with the findings of the cell population arrest at the G2/M phase, which showed an increase. This is interesting to explore further in future studies.

A previous study found that alkaloid-containing fractions promoted apoptosis in T47D cells [37], and that arecoline extracted from *Areca catechu* may induce apoptosis in HeLa cells [38]. Alkaloids' potential to trigger apoptosis by boosting caspase-3 activation [39,40]. Alkaloid compounds can also impact the cell cycle [38], and impede the function of P-glycoprotein [41]. As a result, it was conceivable that compounds from the alkaloid group identified in the A3 fraction promote apoptosis in T47D cells, and the mechanism of apoptosis needs to be investigated further. In the T47D cell line, there were two primary apoptotic mechanisms: intrinsic and extrinsic routes. This apoptotic pathway involves the production of regulatory proteins such as p53, the Bcl-2 family, and caspase activation [42]. Because the T47D cell line lacks p53, apoptosis proceeds via a p53-independent route. Alkaloid compounds block the ERK phosphorylation pathway, which is important in breast cancer cell proliferation [43]. Alkaloid components have also been shown to suppress the proliferation of cancer cells by reducing the production of cyclin D1 and CDK4/6, which are essential regulators in the G1 phase [44,45]. The combination of fraction A3 and doxorubicin may cause an

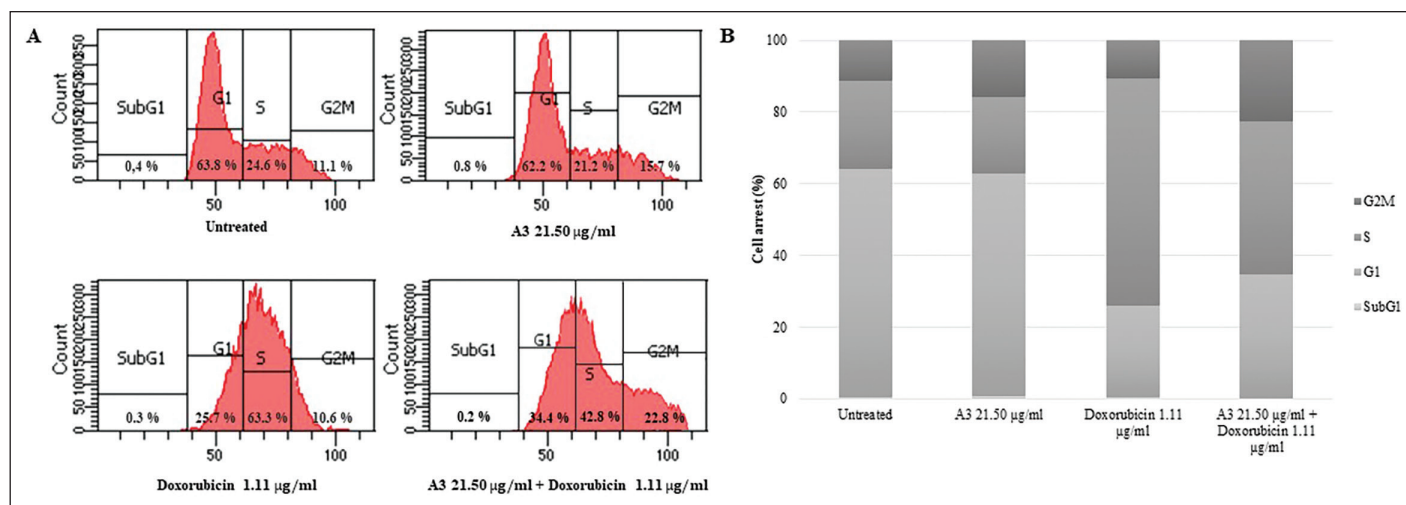


Figure 3. The cell cycle distribution of the T47D cell line was altered by fraction A3, doxorubicin, and their combination. Cells were treated for 24 hours with fraction A3 and doxorubicin, subjected to flow cytometric analysis after stained with propidium iodide/RNase. (A) Flow cytogram of cell cycle profiles; (B) Quantification of cell cycle distribution.

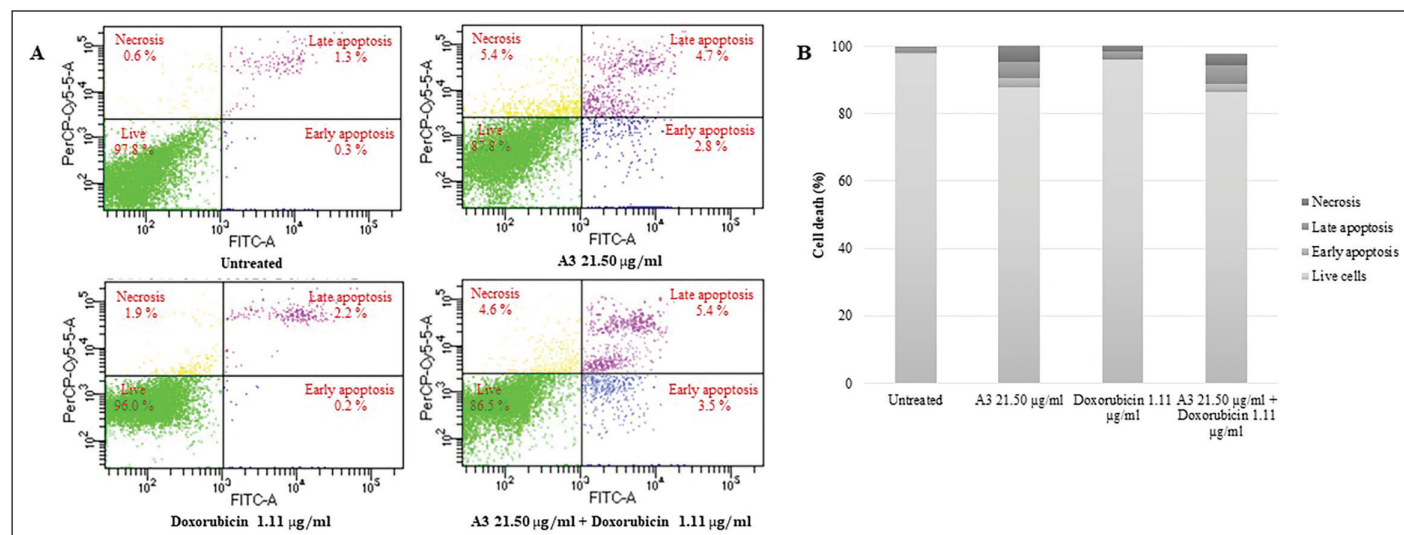


Figure 4. Fraction A3, doxorubicin and their combination induced apoptosis against T47D cell line. Cells were treated for 24 hours with fraction A3 and doxorubicin and at the appropriate concentrations before being stained with FITC Annexin V for flow cytometric analysis. (A) Flow cytogram profiles of treated cells; (B) Quantification of treated cell death population.

increase in apoptotic cells. However, the precise process needs to be investigated further.

CONCLUSION

The fraction A3 of *C. renda* root contains alkaloids as the major component, followed by flavonoids, tannins, and saponins as minor components, which contribute to potential cytotoxicity (43.03 g/ml) against T47D cells and could be used to increase the cytotoxic activity of doxorubicin with a very strong synergistic CI. The fraction A3 can enhance doxorubicin cytotoxic activity by enhancing cell cycle arrest in the G1 phase and triggering apoptosis, thus providing a potential co-chemotherapy strategy for inhibiting breast cancer cell proliferation.

ACKNOWLEDGMENT

The authors are gratefully acknowledges due to Faculty of Medicine, Public Health and Nursing (FKMKM) Universitas Gadjah Mada, Indonesia for collected the test cells and the use of facilities in the Department of Parasitology and Physiology.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published. and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

FINANCIAL SUPPORT

This research was supported by The PNB Institute for Research and Community Service at the University of Jambi, Indonesia (Number: 1392/UN21.11/PT.01.05/SPK/2022).

ETHICAL APPROVALS

The study protocol was approved by the ethics committee of the Faculty of Medicine and Health Science, Universitas Jambi, Indonesia (Approval No. 3486/un21.8/pt. 01.04/2022, date: 25 november 2022).

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

PUBLISHER'S NOTE

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How to cite this article:

Syamsurizal S, Utami DT. The combination of fraction A3 of *Cyrtostachys renda* Blume with doxorubicin improved cytotoxicity against T47D cell line through cell cycle arrest and apoptotic induction. *J Appl Pharm Sci*. 2024;14(09):270–278.