

Enhanced cytotoxic effects of *Clinacanthus nutans* and doxorubicin in combination toward breast cancer cell lines

Sry Suryani Widjaja, Rusdiana,
M. Ichwan¹

Departments of Biochemistry and
¹Pharmacology, Medical Faculty
Universitas Sumatera Utara, Indonesia

J. Adv. Pharm. Technol. Res.

ABSTRACT

Breast cancer is one of the most common cancers with a relatively high mortality rate. Despite the advancement of its medical treatments, many patients are still seeking complementary alternative medicines, namely *Clinacanthus nutans* which is found mainly in South-East Asian countries. We aim to find the antioxidant properties and cytotoxic activity of the plant extract toward breast cancer cell lines Michigan Cancer Foundation-7 (MCF7) and T47D individually and in combination with doxorubicin. Extractions of *C. nutans* with ethanol, n-hexane, and ethyl acetate were done using rotatory vacuum evaporators with the reflux method. Screening of biochemical properties was conducted. Antioxidant activity was measured toward α , α -diphenyl- β -picrylhydrazyl (DPPH) with IC₅₀ scores were shown to be highest in ethyl acetate extract. Cytotoxic effects of all three extracts were shown to be low in both MCF7 and T47D cells. However, combinations of 125 μ g/ml n-hexane extract of *C. nutans*, and 0.1 μ g/ml doxorubicin in T47D cancer cells showed further proliferation reduction compared to the single administration. The results suggested possible synergisms of the treatment combination.

Key words: Antioxidant, breast cancer, *Clinacanthus nutans*, cytotoxic, doxorubicin

INTRODUCTION

Breast cancer has consistently been the most common type of cancer ranks 3rd to 4th as the cause of mortality.^[1] GLOBOCAN 2018 showed that the number of new breast cancer cases is 2,088,849 with the number of deaths 626,679.^[2] Despite its high incidence, the survival rates of breast cancer have increased to nearly >90% and

89% in 5-year, respectively.^[1] Global initiatives have established therapeutic intervention guidelines for surgery, chemotherapy, and radiation therapy for breast cancer patients. However, there are more or less of 55% of cancer patients who are still searching for alternative treatments to be used in combination to or even substituting their primary medical therapies.^[3] A study by Akhtar *et al.*^[4] showed that 98% of patients believe that there is no harm in taking herbal medicine therapies and often think of combining it with their primary medical treatment.

Some of the herbs commonly used to treat breast cancer are *Echinacea*, *Allium sativum* or garlic, *Curcuma longa* or turmeric, *Camellia sinensis* or green tea, *Panax ginseng* or

Address for correspondence:

Dr. Sry Suryani Widjaja,
Department of Biochemistry, Medical Faculty, Universitas
Sumatera Utara, Medan, Indonesia.
E-mail: srysuryani@gmail.com

Submitted: 22-Nov-2020

Revised: 19-Jan-2021

Accepted: 24-Feb-2021

Published: 27-Apr-2021

Access this article online

Quick Response Code:



Website:

www.japtr.org

DOI:

10.4103/japtr.JAPTR_251_20

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How to cite this article: Widjaja SS, Rusdiana, Ichwan M. Enhanced cytotoxic effects of *Clinacanthus nutans* and doxorubicin in combination toward breast cancer cell lines. *J Adv Pharm Technol Res* 2021;12:152-6.

ginseng, flaxseed, and *Arctium lappa* or burdock. They contain substances with antioxidant, antimutagenic, and antiproliferative activity, which were proven to inhibit cancer cell growth.^[5]

One of the rising potential alternative treatments is *Clinacanthus nutans* from the family *Acanthaceae* or commonly termed elephant trunk leaf (“daun belalai gajah” [Indonesia]), Sabah snake grass (Malaysia), “you dun cao” (China), and “sloed pang pon” (Thai).^[6,7] The plant itself has gained its popularity as an alternative therapy for viral skin lesions such as herpes simplex virus infection types 1 and 2, snake venom, and other insect bites antidote, antifever, and even as a diuretic agent. It possesses anti-inflammatory actions as well as significant inhibitory effects toward superoxide anion and elastase.^[6] Ethanol extract of *C. nutans* was shown to have an immunomodulating effect with apoptotic expressions toward human neutrophil cells and porcine peripheral blood mononuclear cells. In addition, inhibition of cell proliferation and cytotoxic effects have been found *in vitro*, particularly in HeLa and K-562 cells with a tested dose of 18.0 and 20.0 µg/mL, respectively. Moreover, antioxidant activity has also been shown using a free radical assay.^[7]

It is still, however, limited evidence of the potential anticancerous effect of *C. nutans* toward breast cancer cells.^[6,7] Some of the most frequently used breast cancer cell lines are the Michigan Cancer Foundation-7 (MCF7) and T47D, mainly due to their hormone-dependent characteristics and their expression of estrogen receptors.^[8] In this study, we aim to find the potency of antioxidant and cytotoxic activity of *C. nutans* leaf extract toward breast cancer cell (MCF7 and T47D) growth, individually and/or in combination with doxorubicin.

MATERIALS AND METHODS

Extraction of *Clinacanthus nutans* using ethanol, N-hexane, and ethyl acetate

Extraction of *C. nutans* with ethanol, N-hexane, and ethyl acetate was carried out through a reflux method by mixing 50 g of finely dry-powdered leaves with 500 mL 100% ethanol/N-hexane/ethyl acetate.

Biochemical properties of *Clinacanthus nutans*

The chemical components contained in the plant ethanolic extract were analyzed using the high-performance liquid chromatography with tandem mass-spectrometry and two mass analyzers (MS/MS). The results were shown using the software Compound Discoverer with mzCloud MS/MS Library.

Antioxidant property measurement

Our method in measuring the antioxidant property of *C. nutans*

was adapted from Molyneux,^[9] which is to find the IC₅₀ score or the inhibitory concentration (concentration of samples sufficient to attenuate 50% of a free radical agent's oxidative process) toward α, α-diphenyl-β-picrylhydrazyl (DPPH; C₁₈H₁₂N₅O₆, M = 394.33)).

Analysis of IC₅₀

The rate of color intensity reduction of DPPH is calculated by the formula below.

$$\% \text{ of DPPH attenuation} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100\%$$

Abs control = absorbance reading of DPPH before the addition of samples

Abs sample = absorbance reading of a mixture of DPPH and sample extract

The value of 0% is defined as no antioxidant activity, while 100% score means a total reduction of the violet color. The results are plotted to a linear regression equation, such as the concentrations of the *C. nutans* extract (µg/mL) as the X-axis and the rate of color intensity reduction or the antioxidant activity as the Y ordinate using the software Statistical Package for Social Sciences (SPSS) V 21.0.^[10] The classifications of the compounds according to the strength of the antioxidant property were as follows: very strong (IC₅₀ < 50 parts per million (ppm = µg/mL), strong (IC₅₀ 50–100 ppm), moderate (IC₅₀ 100–150 ppm), and weak (IC₅₀ > 150 ppm).^[11]

Total phenol content measurement with Folin-Ciocalteu method

Measurement of sample's phenol component was prepared by diluting 50 mg of the extract with methanol (1000 ppm). 0.1 mL of the diluted extract was also added to 7.9 mL aquadest and 0.5 mL of Folin-Ciocalteu reagent, the absorbance of the solution is measured at wavelength 775 nm using spectrophotometry for three times.

Total flavonoid content measurement with a colorimetric method

About 50 mg of quercetin (used as standard) and 50 mg of the extract were each diluted with 50 mL of methanol (100 ppm). About 2 mL of each concentration of the quercetin (100 ppm, 50 ppm, 25 ppm, 12.5 ppm, and 6.25 ppm) and 2 mL of the diluted extract were individually pipetted to 0.1 mL. The absorbances were measured for three times at 432 nm using the spectrophotometer.

Cell lines and culture

Breast cancer cell lines MCF-7 and T47D were purchased from ATCC; the cells were grown and maintained in DMEM-F12 complete media supplemented with 10% fetal



Table 1: Analysis of the biochemical compounds found in the ethanol extract of *Clinacanthus nutans* with liquid chromatography with tandem mass spectrometry method

Secondary Metabolites	Name	Formula	Molecular Weight (g/mol)	Retention Time (minute)
Flavonoids	Corymboside	C ₂₆ H ₂₈ O ₁₄	564.1490	6.735
Flavonoids	Orientin	C ₂₁ H ₂₀ O ₁₁	448.1010	6.898
Cinnamic acid	4-methoxycinnamic acid	C ₁₀ H ₁₀ O ₃	178.0630	3.347
Flavonoids	Apigenin	C ₁₅ H ₁₀ O ₅	270.0530	8.544
Coumarins	4-coumaric acid	C ₉ H ₈ O ₃	164.0480	5.000
Hydroxycoumarins	4-methylumbelliferone	C ₁₀ H ₈ O ₃	176.0480	1.681
Coumarins	6,8-dimethoxy-2-oxo-2H-chromen-8-yl β-D-glucopyranoside	C ₁₇ H ₂₀ O ₁₀	384.1040	1.610
Hydroxycoumarins	7-hydroxycoumarin	C ₉ H ₆ O ₃	162.0320	7.627
Cinnamic acid	Sinapinic acid	C ₁₁ H ₁₂ O ₅	206.0584	6.007
Flavonoids	(+)-dihydromyricetin	C ₁₅ H ₁₂ O ₈	320.0542	1.417

bovine serum, gentamycin in a 5% CO₂ incubator with the environmental temperature of 37°C.

Cytostatic effect measurement

We used MTT assay to test the *in vitro* cytotoxic effect of the ethanol 96% extract, ethyl acetate, and n-hexane extract of *C. nutans* toward cancer cell MCF7 and T47D with and/or without doxorubicin.^[12] Cancer cells were retrieved from the CO₂ incubator and were examined for their conditions. When the cells reached 80% of confluence, they would be harvested. The cells were incubated for a night to enhance recovery after the harvesting process.^[13] About 100 μL of MTT reagent was added to each well, including the controls. We use an ELISA reader with 550–600 nm and the results were converted to cell-survival rate (%) where relative viability = (experimental absorbance – background absorbance)/(absorbance of untreated controls – background absorbance) × 100.^[14]

RESULTS AND DISCUSSION

The lists of the primary chemical metabolites found in the extract are shown in Table 1.

We find some primary metabolites contained in *C. nutans*, which were not mentioned in previous studies such as a hydroxycoumarin derivative called 4-methylumbelliferone, cinnamic acids like 4-methoxycinnamic acid and sinapinic acid, and a flavonoid, namely (+)-dihydromyricetin. Scientific investigations conducted before this study have shown extensive primary metabolites in *C. nutans* stem and leaves extracts, including the vital phytochemicals of this plant, namely stigmaterol, lupeol, b-sitosterol, belutin, and myricyl alcohol as well as the six C-glycosyl flavones, five glucosides, and finally, eight compounds related to chlorophyll a and chlorophyll b.^[6,7]

To ensure that the extraction of the leaf does not cause any structural damage to the essential metabolites, a qualitative assessment of the phytochemical properties in the ethanol

Table 2: Comparison of the phytochemical contents in the simplicial powder and ethanol extract of *Clinacanthus nutans*

Secondary metabolites	Simplicial powder	Ethanol extract
Alkaloid	+	+
Steroid/triterpenoid	+	+
Tannin	+	+
Glycoside	+	+
Flavonoid	+	+
Saponin	+	+

(+) Contain the secondary metabolite compounds, (-) No secondary metabolite compound observed

Table 3: Total flavonoid content of the ethanol extract, N-hexane fraction, and ethyl acetate fraction of *Clinacanthus nutans*

Samples	Total flavonoid compound (QE mg/g)		
	I	II	III
Ethanol extract	413,124	414,149	412,100
N-hexane fraction	434,641	435,666	434,641
Ethyl acetate fraction	133,405	133,405	132,380

QE: Quercetin equivalent

Table 4: Total phenolic content of the ethanol extract, N-hexane fraction, and ethyl acetate fraction of *Clinacanthus nutans*

Samples	Total phenolic total (mg/g GAE)		
	I	II	III
Ethanol extract	128	129.66	129.66
N-hexane fraction	1.33	3	1.33
Ethyl acetate fraction	91.33	93	91.33

GAE: Gallic acid equivalent

extract form and in dry powder form of the *C. nutans* was carried out using specific tests to visually confirm the existence of each secondary metabolite as shown in Table 2.

Different solvents used for extraction will give a different value of phenolic and flavonoid as shown in Table 3 and 4, also reported by Wakeel *et al.*^[15]

The amount of phenolic and flavonoid content, as well as the antioxidant activity of *C. nutans* extracts (1 mg/mL), varied with the range of 17.12–44.13 mg/g GAE (TPC), 14.08–30.80 mg/g QE (TFC), and DPPH inhibition of 38.1%–58%.

We also compare the activity of *C. nutans* with quercetin, a type of flavonols, in measuring IC₅₀ toward DPPH; it was reported in Table 5.

We found that ethyl acetate fraction, which had the highest antioxidant activity compared to the other two extraction methods, still had an efficacy of ten times less than quercetin against DPPH. However, the ethyl acetate fraction did not have the highest TPC and TFC in this study compared to ethanolic and N-hexane extracts. In a previous study by Yuann *et al.*,^[16] antioxidant activity against DPPH, superoxide dismutase activity, and the TPC of green tea was found to be higher than *C. nutans* with the latter were almost reaching 10-fold.

Table 5: Comparison of half maximal inhibitory concentration between all *Clinacanthus nutans* extracts and quercetin toward α , α -diphenyl- β -picrylhydrazyl

Samples	IC ₅₀ toward DPPH (μ g/mL)		
	I	II	III
Ethanolic extract	69.62	67.55	70.62
Quercetin	4.9	4.73	4.99
N-hexane fraction	163.83	160.84	164.88
Ethyl acetate fraction	49.74	51.28	47.34

DPPH: α , α -diphenyl- β -picrylhydrazyl, IC₅₀: Half maximal inhibitory concentration

Table 6: Half maximal inhibitory concentration data of the cytotoxic effect on Michigan Cancer Foundation-7 and T47D cancer cells without doxorubicin

Samples	MCF7 (μ g/mL)	T47D (μ g/mL)
Ethanol extract	610.86 \pm 4.62	343.81 \pm 1.85
N-hexane fraction	284.98 \pm 2.56	229.63 \pm 2.67
Ethyl acetate fraction	1133.16 \pm 6.26	384.43 \pm 2.26

MF7: Michigan Cancer Foundation-7, IC₅₀: Half maximal inhibitory concentration

Table 7: Reduction of T47D cell viability postadministering N-hexane extract of *Clinacanthus nutans* and doxorubicin separately

N-hexane (μ g/mL)	Absorbance (%)			Doxorubicin (μ g/mL)	Absorbance (%)		
125	44.64	43.42	44.02	0.1	47.67	46.46	44.64
93.75	78.95	76.9	75	0.075	61.64	59.88	58.6
62.5	79.86	82.28	82.89	0.05	77.73	76.82	76.21
31.25	87.75	85.93	85.32	0.025	85.02	84.41	85.93

Phenolic compounds have a hydroxyl-bearing aromatic ring skeleton that could exert anti-inflammatory, antioxidant, cytotoxicity, antimicrobial, and antiallergic actions.^[17] Flavonoids and their glycosides are the main components of phenolic compound and it consists of a C₆-C₃-C₆ skeleton, which is derived from 1,3-diphenylpropane.^[17] Many plants such as the ones from the *Moraceae* family have isoprenylated flavonoids which were proven to show cytotoxic activity in MCF7.^[18] There are six known C-glycosyl flavones, which were isolated in previous studies from butanol and methanol solvents of *C. nutans*, namely shaftoside, isomollupentin 7-O--glucopyranoside, orientin, isoorientin, vitexin, and isovitexin.^[19] These flavones are considered rare with suspected antimicrobial, hepatoprotective, and antioxidant activity.^[19]

The IC₅₀ of cytotoxic activity toward breast cancer cell lines T47D were measured between all extracts and chemotherapeutic agent doxorubicin individually and in combination to one another. The cytotoxic effect of the *C. nutans* extracts showed weak cytotoxic activity toward both breast cancer cell lines [Table 6]; the concentrations of all three extracts were found to be lower in T47D compared to MCF7 cells. In addition, N-hexane extract of *C. nutans* showed the lowest concentration required to obtain 50% inhibition of both cancer cell lines' growth. N-hexane extract of *C. nutans* showed the lowest IC₅₀, in another previous experiment by Pannangpetch *et al.*,^[20] ethanolic extract of *C. nutans* showed antitumor activity with IC₅₀ of 110.40 \pm 6.59 μ g/mL, which was 59 times less potent compared to ascorbic acid with IC₅₀ of 9.72 \pm 0.56 μ g/mL. These findings suggest that tumor-specific activity might be exhibited by the ethanolic extract of the plant. However, it is not considered as an active anticancer agent according to the National Cancer Institute due to the IC₅₀ value being >20 μ g/mL.

The reduction of T47D cell viability after administering N-hexane extract and doxorubicin separately is shown in Table 7.

When administered separately, we obtained 44.64% and 47.67% viability of the T47D breast cancer cells, but when administered combined with 0.1 μ g/ml doxorubicin, the average reduction of cancer cell viability is 22.87%.

There are still minimal findings on synergistic effects yielded by a combination of *C. nutans* and doxorubicin.

However, a study conducted by Hii *et al.*^[21] found synergies of nonpolar stem extracts of *C. nutans* and gemcitabine toward the inhibition of pancreatic ductal adenocarcinoma cell lines AsPC1, BxPC3, and SW1990 up to reducing the later dose by 2.38–5.28 folds. Doxorubicin was also found to have additive cytotoxic effects when combined with aqueous *Mucuna pruriens* leaf extracts.^[22]

CONCLUSION

C. nutans showed a minimum level of antioxidants as well as antitumor activity compared to other herbal alternatives. However, we found that the combination of *C. nutans* and doxorubicin yields more reduction of cancer cell viability hence suggesting synergistic cytotoxic effect.

Acknowledgment

This study was supported by the Ministry of Research and Technology and Higher Education Republic Indonesia, under research grant DRPM.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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