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Secang (*Caesalpinia sappan* L.) Heartwood Ethanolic Extract Shows Activity as Doxorubicin Co-chemotherapeutic Agent by Apoptotis Induction on T47D Breast Cancer Cells

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Abstract

Doxorubicin, primary chemoteurapeutic agent used for breast cancer treatment, is known to have various side effects included multi drug resistance (MDR) phenomenon. Therefore, exploration of co-chemotherapeutic agent is important to be conducted in order to prevent MDR. Secang (*Caesalpinia sappan* L.) which contains active compounds brazilin and brazilein, is proven to have activity as anticancer. The aim of this study is to determine the potency of *Caesalpinia sappan* L. ethanolic extract (CEE) as co-chemotherapeutic agent of doxorubicin and its mechanism through apoptosis induction on T47D breast cancer cells. *Caesalpinia sappan* L. heartwood powder was macerated with ethanol 70%. The cytotoxic effect of CEE alone and its combination with doxorubicin was analyzed using MTT assay. Apoptosis assay was done by flowcytometry-annexin V method. CEE showed cytotoxic activity on T47D cells with IC_{50} value of 35 µg/ml, while combinatorial test showed that all of combination doses of CEE and doxorubicin gave synergistic effect. Flowcytometry-annexin V assay proved that treatment of CEE induced apoptosis of doxorubicin. Based on these results, we conclude that *Caesalpinia sappan* L. heartwood ethanolic extract is potential to be developed as co-chemotherapeutic agent of doxorubicin.

Keywords : *Caesalpinia sappan* L., doxorubicin, apoptosis, T47D cells

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and the leading cause of death in women (Fang *et al*., 2011). *Sistem Informasi Rumah Sakit* (SIRS)/Hospital Information System mentions that breast cancer ranks first in hospitalized patients in all hospitals in Indonesia (16.85%) (Dinkes, 2010). Breast cancer shows complex pathological mechanisms, structural changes at the molecular level to determine various aspects that affect the nature of the disease (Dong, 2006). Malignant nature of cancer, a major challenge in the clinical world, primarily because it has the ability to independently provide sufficient signal growth, inhibits anti-growth signals, capable invaded other tissues and metastasis, has potency to divide in an unlimited amount, capable to form new blood vessels (angiogenesis), and has a mechanism of evasion of apoptosis (programmed cell death) that causes cancer cells to be able to proliferate rapidly (Hanahan and Weinberg, 2011). Mechanisms in cancer cells avoid apoptosis has become a serious attention in the clinical world, and influenced researcher to aim at developing drugs that can induce apoptosis in cancer cells (Wong, 2011).

Primary treatment of breast cancer is done with chemotherapy if surgery is not able to cope with cancer that has metastasis. Chemotherapy is a treatment step with a chemical substance that showed cytotoxic activity and work directly on cancer cells (Staerk *et al*., 2002).

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Doxorubicin is one of many chemotherapeutic agents used in cancer treatment, works by inhibiting the cell cycle at the G2/M phase by inducing DNA damage (Potter *et al*., 2002). However, long term used of doxorubicin caused adverse side effects, such as heart problems, nausea, diarrhea, alopecia (Gunawan, 2007) and resistance (Lee, 2010). Dose reduction is done to reduce the side effects of doxorubicin (Wattanapitayakul *et al*., 2005), but it may also reduce its efficacy against cancer cells. Therefore, it is necessary to explore a co-chemotherapy agent which combined with doxorubicin to improve doxorubicin efficacy with lower toxicity to normal tissue.

Secang (*Caesalpinia sappan* L.) is one of the plants potential to be developed as a co-chemotherapeutic agent with doxorubicin. Empirically, secang's heartwood used as the main ingredient in Indonesian traditional beverage such as *wedang secang* and *wedang uwuh*. Pharmacologically, it was reported that secang induces cell death in neck cancer and this effect is associated with the increase of p53 and $p21^{WAF1/CIP1}$ protein expression at the cellular level (Kim *et al*., 2005). Secang also induces apoptosis in several cancer cell types such as human promyelocytic leukemia HL-60 cells (Zhang *et al*., 2002), and human leukemia K562 cells (Wang *et al*., 2001). The scientific evidence indicates that secang is potential to be developed as a co-chemotherapeutic agent with doxorubicin.

This study was conducted in a series of tests to determine the effect of Secang (*Caesalpinia sappan* L.) heartwood ethanolic extract alone and its combination with doxorubicin in increasing cytotoxic activity and apoptosis induction of doxorubicin on T47D breast cancer cells. Scientific data obtained from this research can be used as the basic of the application of the *Caesalpinia sappan* L. heartwood ethanolic extract in the treatment of breast cancer.

MATERIALS AND METHODS

Sample Preparation

Dried *Caesalpinia sappan* L. shavings heartwood were obtain from Wonosari Yogyakarta, Indonesia. The heartwood then powdered and standardized by Laboratory of Biology, Faculty of Pharmacy Universitas Gadjah Mada. Dried powder was then macerated for 5 days with 70% ethanol. Filtrate was collected then concentrated using rotary evaporator (Heidolph WB2000). For application on cells, *Caesalpinia sappan* L. ethanolic extract (CEE) was dissolved in dimethyl sulfoxide as co-solvent with concentration 5 mg/ml, and then diluted in DMEM cell culture medium in various concentrations.

Cells Culture

Human breast cancer T47D culture cells were a collection of Cancer Chemoprevention Research Center (CCRC), Universitas Gadjah Mada. The cell line was kindly given by Prof. Kawaichi, Nara Institute of Science and Technology (NAIST), Japan.

Chemicals

Making 100 ml cell culture medium was prepared by mix Dulbecco's Modified Eagle Medium (DMEM) (Gibco) medium stock contain HEPES (Sigma) and sodium bicarbonate (Sigma), 10% v/v Fetal Bovine Serum (FBS) (Gibco), 1% Penicillin-Streptomycin (Gibco), and 0,5% Fungizone (Gibco). Tripsin-EDTA 0,25% (Gibco) were used for cell preparation. For cytotoxicity assay, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) reagent were used which was dissolved in PBS with concentration 5 mg/ml. Phosphate Buffer Saline (PBS) pH 7.4 containing KCl (HPLC grade, Sigma), NaCl (HPLC grade, Sigma), Na_2HPO_4 (HPLC grade, Sigma), and KH_2PO_4 (HPLC grade, Sigma) dissolved in aquadest as washing reagent and 10% b/v Sodium Dodecyl Sulphate (SDS) (Merck) dissolved in 0.01 N HCl (Merck) as stopper reagent. Annexin-FITC was diluted in binding buffer (10mM HEPES, 100 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) which is contained propidium iodide (50 µg/ml).

Cytotoxic Assay

T47D cells were planted in 96-well plates (Iwaki) with $5x10^3$ cells/well and divided into control and treatment group. Final concentrations of CEE that treaten to cells were 10, 25, 50, 100, and 150 µg/mL For combinatorial assay, final concentrations of CEE were $^1/_8$, $^1/_4$, $^2/_5$, $^1/_2$ IC_{50} and doxorubicin were $^1/_{10}$, $^2/_{10}$, $^3/_{10}$, $^4/_{10}$ IC_{50}. Based on Fitriasari *et al*. (2009) doxorubicin has IC_{50} value of 15 nM on T47D cells. After 24 h of incubation,

culture medium was removed and cells were washed using PBS (Sigma). 5 mg/mL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) on PBS (Sigma) was diluted by DMEM culture medium (1:10) and 100 µl of it was added into each well. After incubated for 3-4 h the reaction was stopped by Sodium Dodecyl Sulfate (SDS) 10% in HCL 0,01 N. After that, the plate was incubated for one night in room condition at dark place. To make sure the formazan was dissolved, the plate was shaked for 10 minutes and the absorbance was then measured using ELISA reader at wave length of 595 nm.

Apoptosis Assay

Cells ($5x10^5$ cells/well) were transferred in to six well tissue culture plate (Iwaki) and incubated for 24 h. Cells were treated with CEE, doxorubicin, and their combination with concentration ½ IC_{50} and then incubated for 24 h. After incubation, adherent and detached cells were collected and centrifugated at 2000 rpm for 3 min, then washed twice with cold PBS. Cells were resuspended in 500 µl of Annexin V buffer (Biovision) and then treated with annexin V and propidium iodide for 10 min at 37^{o}C. The treated cells were then subjected to FACS flowcytometry. Bivariant analysis of FITC-fluoresence (FL-1) and PI-fluoresence (FL-3) gave different cell populations where FITC (-) and PI (-) were designated as viable cells; FITC (+) and PI (-) phenotype as apoptotic cells; FITC (-) and PI (+) as necrotic cells; and FITC (+) and PI (+) as late apoptotic cells.

Data Analysis

Single cytotoxicity assay. To determine cell viability, percent viability was calculated as [(absorbance of treated-drug) - (absorbance of medium)/ (absorbance of control (untreated) cells - absorbance of medium)] × 100%. Linier regression between log concentration and % cell viability giving the equation Y = Bx + A were used to calculate IC_{50} value. The IC_{50} values are defined as the drug concentrations required to reduce the absorbance by 50% of the control.

Combinatorial cytotoxic assay. Combinatorial treatment was evaluated by calculating Combination Index (CI) value and interpretation was done based on Reynolds and Maurer (2005), which has the formula as follows.

$$CI = \frac{D_1}{D_{x1}} + \frac{D_2}{D_{x2}} + \frac{D_1.D_2}{D_{x1}.D_{x2}}$$

RESULTS AND DISCUSSION

In order to determine cytotoxic effect of CEE on T47D cells, MTT assay was done with various concentrations of CEE treatments. As shown in Fig. 1, T47D cells viability was decreased after CEE treatment for 24 h in dose dependent manner. The MTT results demonstrated that CEE has potent cytotoxicity against T47D with IC_{50} value of 35 µg/ml.

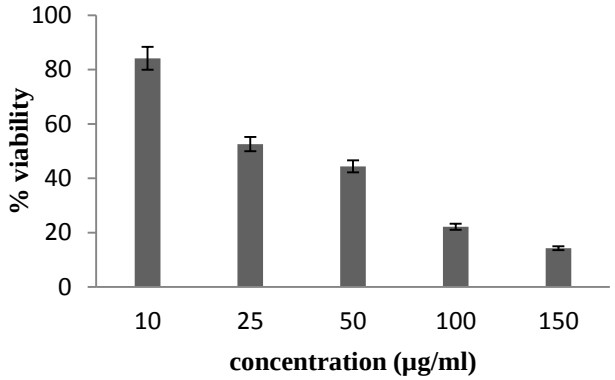

Figure 1. Cytotoxic effect of CEE on T47D cells. T47D cells (5000 cells/well) were seeded in 96 wellplate and treated with CEE 10, 25, 50, 100, and 150 µg/mL. Plate was then incubated for 24 hours and cells viability was determined by using MTT assay as described in methods. The higher concentration of CEE caused lower cells viability. Data were mean of two replications $\bar{x}$ ± SD ($p<0,05$). IC_{50} value was calculated using linier regression and CEE perform cytotoxicity with IC_{50} value of 35 µg/ml.

Moreover, the present study observed potency of CEE increase doxorubicin's cytotoxicity. Cell morphology after treatment was observed (Fig. 2(A)-2(D)). Treatment of CEE and doxorubicin alone led to cells' morphological changing, (Fig. 2(B) and 2(C)) compared to control cells (2A). Combination of them caused more changes compared to single treated cells (Fig. 2(D)). Combination of CEE and doxorubicin with various concentrations reduced percent cells viability as shown in Fig. 2(E). Combination treatment of CEE and doxorubicin on T47D cells gave synergistic effect in all concentration that tested with CI values less than 0.9, shown in Table I.

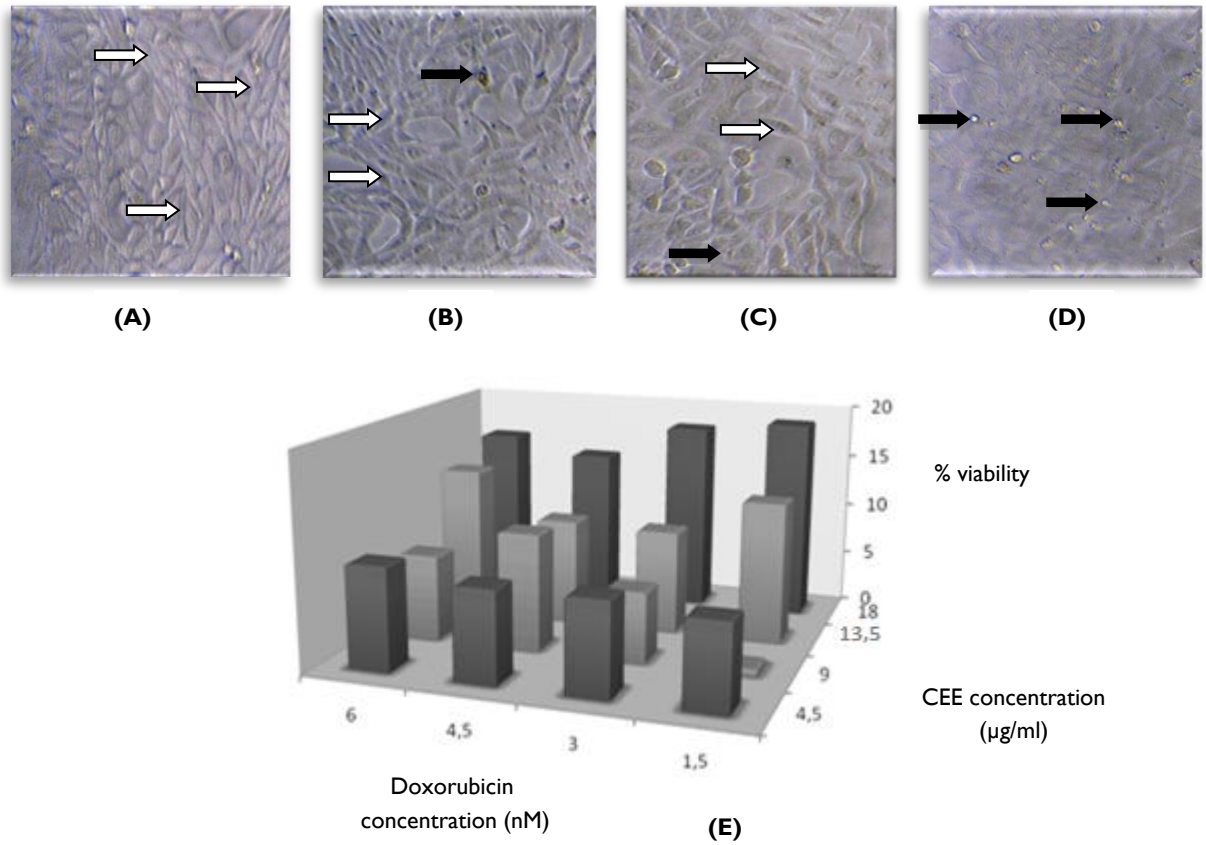

Figure 2. Combinational cytotoxic effect of CEE and doxorubicin on T47D cells. T47D cells (5000 cells/well) were seeded in 96 wellplate and treated with CEE $^{1}/_{8}$, $^{1}/_{4}$, $^{2}/_{5}$, $^{1}/_{2}$ IC_{50} and doxorubicin $^{1}/_{10}$, $^{2}/_{10}$, $^{3}/_{10}$, $^{4}/_{10}$ IC_{50}. Plate was then incubated for 24 hours and cells viability was determined by using MTT assay as described in methods. Combinational treatment altered cells morphology. Cells treated with (A) control cells, (B) CEE, (C) doxorubicin, and (D) combination of CEE and doxorubicin. White arrows (⇨) showed viable cells while black arrows (➡) showed death cells were observed under light microscope with 400x magnification. Graph (E) showed that combination of CEE and doxorubicin decreased cells viability compared to doxorubicin solely.

Table I. Combination Index of Combination of CEE and Doxorubicin

CEE (µg/ml)	Dox (nM)			
	1,5	3	4,5	6
4,5	$3,9 \times 10^{-2}$	$3,1 \times 10^{-2}$	$4,6 \times 10^{-2}$	$6,4 \times 10^{-2}$
9	**$3,7 \times 10^{-2}$**	$4,7 \times 10^{-4}$	$5,2 \times 10^{-4}$	$6,4 \times 10^{-4}$
13,5	$9,0 \times 10^{-2}$	$5,3 \times 10^{-4}$	$8,7 \times 10^{-6}$	$8,4 \times 10^{-6}$
18	$1,5 \times 10^{-1}$	$1,3 \times 10^{-3}$	$8,7 \times 10^{-6}$	$2,0 \times 10^{-7}$

Synergistic effect caused by combinational treatment of CEE and doxorubicin could be related with apoptosis and/or cell cycle arrest. To evaluate the impact of CEE and doxorubicin induce apoptosis on T47D cells, apoptosis phenomenon were detected using flowcytometric. This method is detected translocation of phosphatidyl serine residues which are normally located in the internal phospholipid layer to external layer in apoptotic cells. The result showed that combination of doxorubicin with CEE induced apoptosis rates of late apoptotic cells, but did not induced early apoptotic cells on T47D cells (Fig. 3). The percentage of late apoptotic cells were increased after treatment with CEE alone (25.80%) or its combination with doxorubicin (39.88 %) in comparison to the control cells and doxorubicin treated cells (1.12 % and 1.27 %, respectively). While treatment of CEE alone led to increase early apoptotic cells compare with control or doxorubicin treated cells (29.86 %; 2.82 %; and 16 %, respectively). However, treatment of CEE alone gave unsignificant differences percentage of apoptotic cells compare with CEE combination with doxorubicin (55.67 % and 54.55 %, respectively).

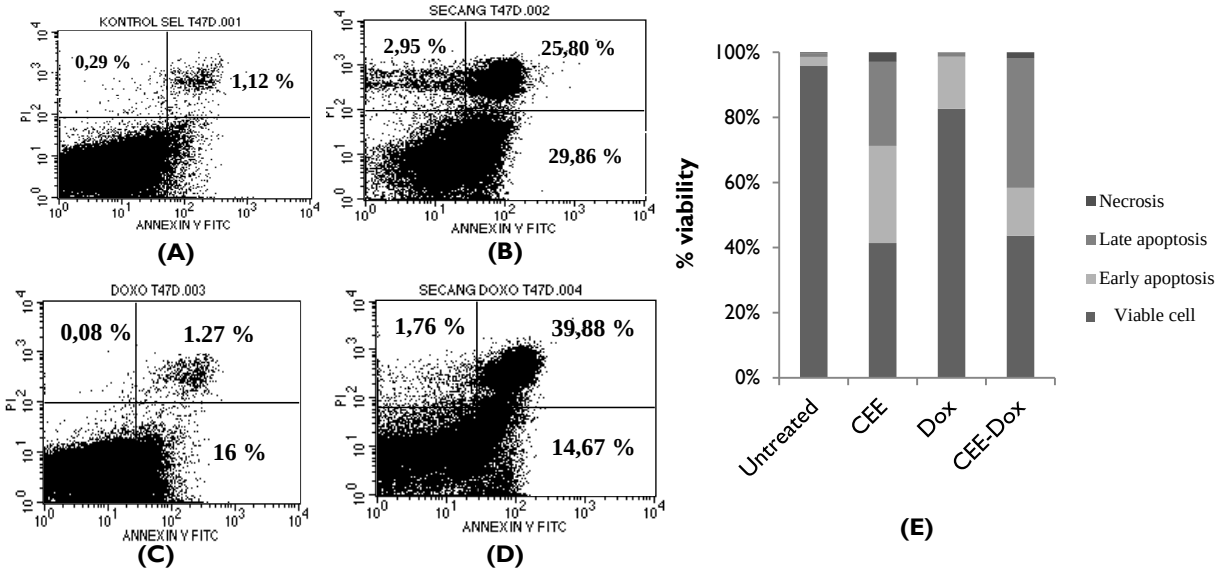

Figure 3. Effect of CEE-doxorubicin on T47D cells apoptosis. T47D cells were seeded at 5x10^5 cells/well on six wells tissue culture plate, then treated with CEE ½ IC$_{50}$ alone and its combination with doxorubicin ½ IC$_{50}$. After 24 hours of incubation, cells were harvested as described in methods, added with AnnexinV and PI reagent, then subjected to FACS flowcytometry. Flowcytometric profile of cells treated with (A) control, (B) CEE, (C) doxorubicin, and (D) combination of CEE and doxorubicin. There are 4 quadrans: lower left (LL) indicates viable cells, lower right (LR) indicating early apoptotic cells, upper left (UL) indicating late apoptotic cell, upper right (UR) indicating necrotic cells. Graph (E) showed that combination of CEE and doxorubicin induced apoptosis.

Doxorubicin is the most widely used drug in the treatment of various human neoplasms, espessially breast cancer. However, long term use of doxorubicin causes several side effects such as cardiotoxicity and multi drug resistance, that leading to cause cancer treatment failure. Many of cancer patients would seek other complementary or alternative medicines. Indonesia, as a country which has large biodiversity, has become one of the sources of herbal product that used for cancer treatment. However, very little of them is known about their action and possible interaction with other anticancer drugs. Thus, exploration about medicinal plant's activity on cancer cells is helpful as basic of their used.

Caesalpinia sappan L. has been used as traditional medicine in various countries including Indonesia. The results of current study demonstrated potentially ability of CEE on inhibiting T47D cells proliferation with IC_{50} value of 35 µg/ml in dose dependent manner. While, when combine with doxorubicin, CEE could increase cytotoxic effects of doxorubicin in all tested concentration. This indicates that CEE might perform dominant effect on combination compared to doxorubicin. Combination therapy is a rationale strategy to increase response and tolerability and to decrease resistance. This result indicates that CEE has a potency to be developed as co-chemotherapeutic agent for doxorubicin in breast cancer.

Mechanism that mediates cytotoxic activity of CEE on T47D cells can be undergoes by apoptosis and or cell cycle arrest. Kim *et al*. (2005) reported that *Caesalpinia sappan* was able to induce expression of p53 and $p21^{WAF1/CIP1}$ at cellular level. *Caesalpinia sappan* has been reported to induce apoptosis in several cancer cell types such as human promyelocytic leukemia HL-60 cells (Zhang *et al*., 2002), and human leukemia K562 cells (Wang *et al*., 2001). Nurulita and Muflih (2006) reported that secang's methanolic extract has cytotoxic effect on T47D cells with IC_{50} value of 150.9 µg/ml, and observations on the morphology of cells after double staining method with ethidium bromide-acridyn orange showed apoptosis phenomenon. The ability to induce apoptosis in cancer cells is an important property of a candidate anticancer drug because cancer cells are capable to evading apoptosis.

The major finding of the present study is an effect of CEE in inducing apoptosis of doxorubicin on T47D cells. Human breast cancer T47D cells are ER/PR-positive cells that express wildtype caspase-3, wildtype caspase-7, and mutant p53 (Bouker *et al*., 2005; Schafer *et al*., 2000). Mutations in *p53* gene lead to inhibition of doxorubicin induced apoptosis in T47D cells. Mechanisms that mediate doxorubicin induced apoptosis in T47D cells only through extrinsic pathway by tumor necrosis factor (TNF), so induction of apoptosis is less effective (Keane *et al*., 1999). Our study represented that CEE induced apoptosis on T47D cells while treatment alone or combination with doxorubicin compare with control and treatment of doxorubicin alone. However, compare with the combination treatment, administration of CEE alone exhibit no differences percentage of apoptotic cells of T47D cells. This result indicates that CEE might has stronger effect than doxorubicin on T47D cells.

Various studies about *C. sappan*'s active compound have been reported on its anticancer activity. Brazilein, a flavonoid compound of *Caesalpinia sappan* has been known to cause down-regulation of survivin, and increase activation of caspase-9 and caspase-3 and induce PARP (Poly ADP ribose polymerase) cleavage in HepG2 liver cancer cells (Zhong *et al*., 2009). Tao *et al*. (2011) reported that brazilein is able to improve the sensitivity of K562 and K562/AO2 leukemia cells resistant to doxorubicin chemotherapeutic agents, because brazilein is not a substrate of P-gp protein. Previous study by Ren *et al*. (2011) mentioned that brazilin, another compound of *Caesalpinia sappan*, induced apoptosis of T24 bladder cancer cells, but the mechanisms of this activity not fully understand. These mechanisms might be responsible in apoptosis induction of *Caesalpinia sappan* in T47D cells. Thus, further study is needed to be conducted.

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