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Research Article

Anti-Cancer Properties of Diethylether Extract of Wood from Sukun (*Artocarpus altilis*) in Human Breast Cancer (T47D) Cells

Enos Tangke Arung^{1,2}, Britanto Dani Wicaksono¹, Yohana Ayupriyanti Handoko¹, Irawan Wijaya Kusuma², Dina Yulia¹ and Ferry Sandra^{1*}

¹Stem Cell and Cancer Institute, Jalan Ahmad Yani no. 2 Pulomas, Jakarta, 13210, ²Wood Chemistry Laboratory, Forest Product Department, Forestry Faculty, Mulawarman University; Jalan KH. Dewantara, Kampus Gn. Kelua, Samarinda, East Kalimantan 75123, Indonesia.

Abstract

Purpose: To evaluate the anti-cancer properties of the diethylether extract of Sukun (*Artocarpus altilis*) wood.

Methods: The extract was tested in human T47D breast cancer cells and examined for its effect on cell viability, nuclear morphology and sub-G1 formation. Cell viability was determined by microculture tetrazolium technique (MTT), nuclear morphology investigated using 4'-6-diamidino-2-phenylindole (DAPI) staining technique, and cell cycle progression monitored by sub-G1 apoptosis assay using flow cytometry.

Results: The results showed decreasing cell viability in a concentration-dependent manner. Altered cell morphology after treatment with the extract demonstrated that cells experienced apoptosis. Cell cycle analysis indicated that the number of cells in sub-G1 phase rose with increasing concentrations of the extract.

Conclusion: The data demonstrate that Sukun wood extract induced apoptosis and sub-G1 phase formation in breast cancer (T47D) cells, and therefore, has a potential as an anti-cancer agent.

Keywords: *Artocarpus altilis*; Diethylether extract; T47D cells; Anti-cancer; Apoptosis; Sub-G1 formation

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*Corresponding author: **Email:** fsandra@sci-indonesia.org; **Tel:** +62-21-47860173; **Fax:** +62-21-47860180

INTRODUCTION

For centuries, people have been using herbs for medicinal purposes. In fact, the use of herbal medicine can be traced back to at least 5000 years. Today, more than 85,000 plant species have been documented for medical use globally¹. The World Health Organization (WHO) estimates that almost 75% of the world's population has therapeutic experience with herbal remedies¹.

Artocarpus altilis, known as breadfruit, is a widely known food source but is also commonly used as a folk medicine in Indonesia where it is locally called Sukun. Traditionally, the leaves of Sukun are used for the treatment of various kinds of diseases such as liver cirrhosis, hypertension and diabetes². Scientifically, some biological activities of the extract of this plant have been reported. The methanol/dichloromethane extract from bud covers of sukun was shown to have activity in a cathepsin K inhibition assay³. A study has reported that cathepsin K inhibitors are very effective in preventing bone resorption, and may, therefore, be a potential treatment option for osteoporosis⁴. The acetone extract of the leaves showed an inhibitory effect on 5α -reductase activity which might make it useful for the selective treatment of benign prostate hyperplasia and prostate cancer⁵. Sukun extract has also been shown to alleviate the symptoms of diabetes mellitus and urinary problems⁶. Recently, a study showed the ethylacetate extract of the leaves had cytotoxic effects on some human cancer cell lines, including human lung adenocarcinoma (SPC-A-1 cells), human colon carcinoma (SW-480 cells), and human hepatocellular carcinoma (SMMC-7721 cells)⁷, thus indicating that the extract might be a potential anti-cancer agent.

In this study, we have examined the anti-cancer properties of the diethylether (DE) extract of Sukun (*A. altilis*) wood. Previous studies have shown that the DE extracts of the wood contain artocarpin, a prenylated

flavonoid compound, which has various biological activities such as inhibition of 5α -reductase^{8,9} and melanin biosynthesis in B16 melanoma cells¹⁰. The activity of Sukun wood extract was assessed by investigating cell viability, observing the nuclear morphology of cells and examining the cell cycle process after treatment with Sukun wood extract.

EXPERIMENTAL

Plant materials

The wood of *Artocarpus altilis* was collected at Samarinda City, Indonesia in 2006. The plant was identified by Raharjo, BSc. in the Dendrology Laboratory, Forestry Faculty, Mulawarman University, Indonesia and the voucher specimen (AK-CW-2) was deposited in the Wood Chemistry Laboratory, Forestry Faculty, Mulawarman University, Indonesia.

Preparation of diethylether (DE) extract

The DE extract of *A. altilis* wood was prepared according to the method previously described by Arung *et al.*¹⁰. The dried heartwood of *A. altilis* (2.3 kg) was cut into small pieces (approx. 0.5 cm³) and repeatedly extracted with methanol (MeOH) at room temperature. The MeOH extract was concentrated using rotary evaporator under vacuum at room temperature to obtain a final residue (60.6 g). A portion of the extract (43.1 g) was suspended in MeOH/water (1:2) and partitioned with n-hexane, diethyl ether (DE) and ethylacetate (EtOAc). The DE soluble fraction was concentrated under vacuum at room temperature to obtain a final residue (16.3 g) for further tests. The extraction was conducted at the Wood Chemistry Laboratory, Forestry Faculty, Mulawarman University, Samarinda, East Kalimantan, Indonesia.

Cell culture

T47D cells were obtained from the Indonesian Institute 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) with L-glutamine supplemented with 10% v/v foetal bovine serum, sodium bicarbonate, 100 µg/ml streptomycin and 100 U/ml penicillin at 37°C in a humidified atmosphere of 5 % CO₂.

Cell viability assay

To determine cell viability, a microculture tetrazolium technique (MTT) assay was performed according to the method previously described by Arung *et al*¹¹, with minor modifications. The MTT assay provides a quantitative measurement of viable cells by determining the amount of formazan crystals produced by metabolically active cells. Briefly, the cells were seeded into a 96-well plate (5 x 10³ cells per well). After 24 h incubation, the medium was replaced with fresh medium containing Sukun wood extract, dimethyl sulfoxide (1 %v/v DMSO in water), hydrogen peroxide (0.07 %v/v H₂O₂ in water) or medium alone and the cells incubated for a further 24 h. DMSO (1 %v/v, in water) was used as a negative control since this was used to re-suspend the extract. MTT reagent [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (10 µl) in PBS (5 mg/ml)] was added to each well. The plate was incubated in a humidified atmosphere of 5% CO₂ at 37 °C for 3 h, and then the medium was discarded and formazan crystals were dissolved in 1.0 ml of 0.04N HCl. The absorbance of cells was measured at 570 nm with a microplate reader. Control cells were counted with a haemocytometer and used for interpolating the absorbance of MTT assay results. The data were presented as percent of viable cells (%). The data were analyzed with the two-tailed student's *t*-test against samples treated with DMSO.

Apoptosis measurements and DAPI staining

In order to determine the level of apoptosis in cells, DAPI staining was performed as

described by Sandra *et al*¹². Briefly, the cells were seeded onto glass slides and treated with Sukun wood extract for 24 h. Untreated and treated cells were rinsed with phosphate buffered saline (PBS), fixed with ice-cold 10% trichloroacetic acid, and further washed with cold 70, 80, 90% and absolute ethanol. The cells were permeabilized with Triton-X (10 %v/v) and stained with 1 µg/ml 4'-6-diamidino-2-phenylindole (DAPI) for 3 min. To reduce the background, the stained cells were washed with PBS, cover-slipped with 90% glycerol and observed under a fluorescence microscope (Zeiss Axio Observer Z1, Göttingen, Germany).

Sub-G1 apoptosis assay and flow cytometry

The cells were separated into four treatment groups as stated for the cell viability assay above. The cells were analyzed for sub-G1 apoptosis using the method described by Sandra *et al*¹². Briefly, the cells were seeded into a 24-well plate (25 x 10⁴ cells per well) and incubated for 24 h. The medium was then replaced with fresh medium containing Sukun wood extract, DMSO, H₂O₂ or medium alone. After 24 h, the cells were harvested and suspended in 1 ml of hypotonic fluorochrome solution (50 µg/ml propidium iodide in 0.1% sodium citrate containing 0.1 % Triton X-100). The cell suspensions were placed in the dark by wrapping up the tubes with aluminium foil and incubated at 4 °C for 1-2 h prior to flow cytometric analysis. The propidium iodide fluorescence of individual nuclei was measured using a FACS Calibur apparatus (Benton Dickinson, San Jose, California).

High performance liquid chromatographic (HPLC) analysis

The wood extract was filtered through a 0.22 µm syringe filter prior to analysis. Spectra were generated on a WatersTM 2487 series HPLC (USA) equipped with a WatersTM 1525 binary HPLC pump and Millenium³² Photodiode Array Detector (PDA). Data

acquisition and treatment were performed with Empower 2 software. The separation was carried out at 25 °C on a 200 mm x 4.6 mm column packed with 5 µm C18 (Symmetry, Waters). The mobile phase was methanol (85%) and water (15%) and delivered isocratically with a flow rate of 1.0 ml/min. The sample volume injected was 20 µl. UV absorbance was measured at 254 nm.

Statistical analysis

The IC₅₀ (median inhibition concentration) is the concentration of toxic compound that reduces the biological activity by 50 %. The IC₅₀ value was obtained from the MTT assay and calculated using non-linear regression analysis in Microsoft Excel software. The value was expressed as a geometric mean. Differences were considered to be statistically significant when $p < 0.05$ and $p < 0.01$.

RESULTS

Cytotoxic effects of Sukun wood extract in T47D cells

Figure 1 shows the cell viability data obtained. There was a moderate decrease (< 20 %) in cell viability when cells were treated with DMSO, the solvent used to re-suspend the extract. T47D cells treated with Sukun wood extract showed a decrease in viability in a concentration dependent-manner. Compared with those treated with DMSO, a significant decrease in cell viability ($p < 0.01$) was observed for T47D cells treated with 7.5 and 10 µg/ml of extract. IC₅₀ was reached at a concentration of 6.19 µg/ml.

Nuclear morphology

Based on the MTT assay results, DAPI staining was conducted to investigate whether Sukun wood extract induced apoptosis. Treatment of cells with Sukun wood extract at concentrations of 2.5 and 7.5 µg/ml, respectively, caused nuclear morphological changes compared to normal

cells and this may be indicative of apoptosis (compare Figures 2B and C with Figure 2A). Morphological changes observed in the treated cells included cell shrinkage, nuclei that were broken into discrete fragments and cell budding (indicated by arrow) that resulted in cells of various sizes. Higher concentrations of Sukun wood extract appeared to cause more morphological changes, indicating that apoptosis occurred in a concentration-dependent fashion.

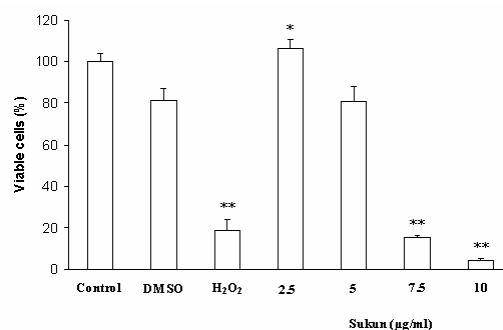


Figure 1: Effect of Sukun wood extract on T47D cell viability. (Treatments significantly differed from DMSO at * $p < 0.05$ and ** $p < 0.01$).

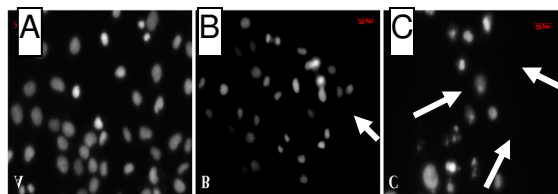


Figure 2: DAPI-stained T47D cells treated with Sukun extract. (A = untreated T47D cells (1×10^4); B = cells treated with Sukun wood extract at a concentration of 2.5 µg/ml; and C = cells treated with Sukun wood extract at 7.5 µg/ml. Arrows indicate apoptotic cells.)

Sub-G1 apoptosis

To understand the mechanism of inhibition of the Sukun wood extract, the effect of Sukun wood extract on cell cycle progression was analyzed by flow cytometry. In this experiment, 2.5, 7.5 and 10 µg/ml of Sukun wood extracts were used. Figure 3 shows that sub-G1 apoptotic content was markedly

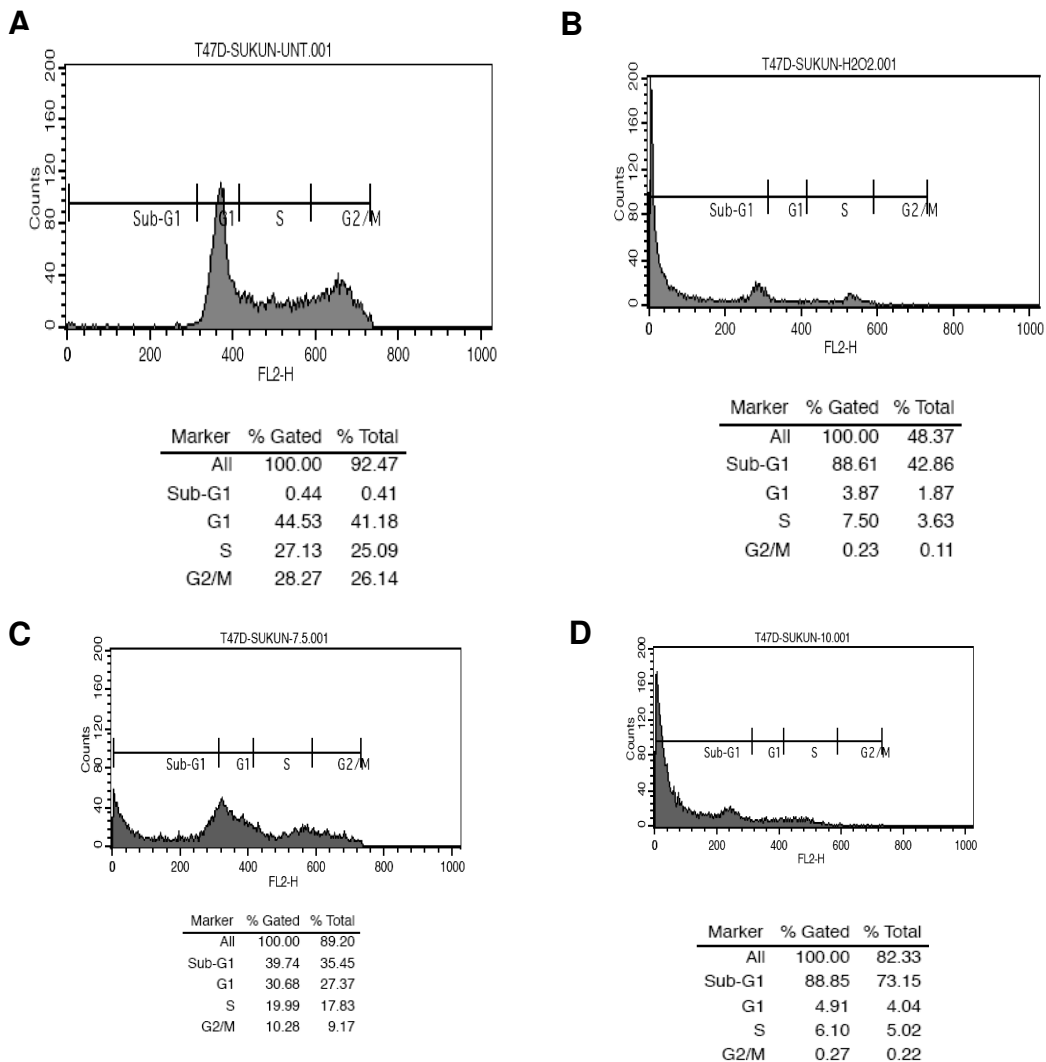


Figure 3: Effect of Sukun wood extract on T47D cell cycle progression. (A = untreated T47D cells (25×10^4); B = cells treated with H_2O_2 ; C= Sukun wood extract at a concentration of $7.5 \mu\text{g/ml}$; and D = Sukun wood extract at a concentration of $10 \mu\text{g/ml}$).

increased when cells were treated with Sukun wood extract. In the absence of the extract, sub-G1 was formed at 0.44 % (Figure 3A), whereas a concentration of $7.5 \mu\text{g/ml}$ resulted in 39.74 % of cells being in sub-G1 phase (Figure 3C). This proportion of cells further increased at the extract concentration

of $10 \mu\text{g/ml}$ (88.85%, Figure 3D). This is similar to of the value for cells treated with H_2O_2 (88.61 %, Figure 3B). Therefore, an increase in the sub-G1 apoptotic fraction may be the major cause of reduced viability of T47D cells.

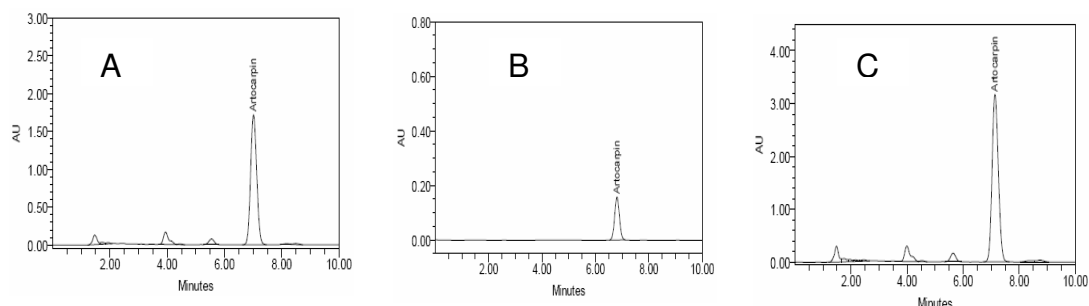


Figure 4: HPLC chromatogram of Sukun wood extract (A), artocarpin (B) and Sukun wood extract spiked with artocarpin (C)

HPLC analysis

To investigate the components present in Sukun wood extract, the extract was run in a HPLC. Four peaks were present in the extract and the primary peak appeared at approximately 7 min (Figure 4A). Previous studies have shown that the DE extract of *A. altilis* contained artocarpin⁸⁻¹⁰. In this study, therefore, pure artocarpin, from the compound collections at Wood Chemistry Laboratory, Forest Product Department, Forestry Faculty, Mulawarman University, Samarinda, East Kalimantan, Indonesia, was run on a HPLC for comparison. Figure 4B shows that artocarpin peak was also produced at approximately 7 min. When the Sukun wood extract was spiked with pure artocarpin (Figure 4C), the main Sukun peak overlapped with the peak of pure artocarpin, indicating that the identity of the main Sukun peak was artocarpin.

DISCUSSION

Various plant extracts have been evaluated and shown to have cytotoxic or cytostatic effects in cancer cell lines. They include those of *Solanum lyratum* tested on human colon adenocarcinoma cell line (colo 205)¹³, *Annona glabra* on human leukemia cell lines (CEM/VLB)¹⁴, *Gynostemma pentaphyllum* on human lung cancer (A549)¹⁵ and *Blumea balsamifera* on rat and human hepatocellular carcinoma cells (McA-RH7777 and HepG2)¹⁶.

Previous studies on *Artocarpus* plants have shown various responses to extracts. Wang *et al*⁷ reported that five geranyl dihydrochalcones from the ethylacetate extract of *A. altilis* leaves had cytotoxic effects on some human cancer cell lines, such as human lung adenocarcinoma (SPC-A-1 cells), human colon carcinoma (SW-480 cells), and human hepatocellular carcinoma (SMMC-7721 cells). The results of our study show that Sukun wood extract has a cytotoxic effect on breast cancer cells (T47D) in a concentration-dependent manner, with an extract IC₅₀ 6.19 µg/ml. Our observations of altered nuclear morphology after treatment with the Sukun wood extract (Figure 2B and C) are consistent with previous reports of cells undergoing apoptosis. Kerr *et al*¹⁷ stated that characteristics of cells undergoing apoptosis include the formation of sharply delineated, uniformly fine granular masses adjacent to the nuclear envelope and cytoplasmic condensation; breaking up of the nucleus into discrete fragments surrounded by a double layered envelope; and cell budding to produce membrane-bounded apoptotic bodies. In line with morphological analysis, a sub-G1 apoptosis assay also confirmed that Sukun wood extract induced apoptosis in T47D cells in a concentration-dependent fashion (Figure 3B, C and D).

The apoptotic effect of Sukun wood extract may be due to the presence of artocarpin, since this component was found to be the

main compound in the extract (Figure 4). A similar finding that artocarpin, which was tested on other breast cancer cells, MCF-7 and MDA-MB-231, showed produced cytotoxicity effect with an IC₅₀ of 3.3 and 3.8 µg/ml, respectively, has also been reported by Wang *et al*¹⁸.

CONCLUSION

In this study, we found that Sukun wood extract, which primarily contains artocarpin, reduced cell viability by inducing apoptosis and sub-G1 phase formation in human breast T47D cells *in vitro*. This extract, therefore, has the potential of an anti-cancer agent. However, further study may still be necessary to elucidate the mechanism of apoptosis induction in T47D cells by Sukun wood extract.

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