ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 10, Issue 11, 2017

Online - 2455-3891
Print - 0974-2441

Research Article

# CELL CYCLE ARREST ACTIVITY OF *LITSEA CUBEBA* LOUR: HEARTWOOD AND FRUIT EXTRACTS AGAINST T47D BREAST CANCER CELLS

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***Received: 25 May 2017, Revised and Accepted: 11 July 2017***

**ABSTRACT**

**Objective:** This study was carried out to investigate cell cycle arrest activity toward T47D cells of *Litsea cubeba* heartwoods and fruits extract.

**Methods:** Dry extracts were prepared from dry-grounded heartwoods and fruits by cold maceration using n-hexane, ethylacetate, and ethanol solvent. Cytotoxic activity were measured by [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] methods and interpreted with inhibitory concentration 50% ($IC_{50}$) value. Cell cycle arrest was investigated by flowcytometry method.

**Results:** $IC_{50}$ values for each n-hexane, ethylacetate, and ethanol of *L. cubeba* heartwoods and fruits were 76.34±2.61; 67.52±2.45; 71.23±2.37; 75.59±3.24; 162.58±15.08; 63.70±2.67 µg/mL, respectively. Cell cycle arrest for ethylacetate extract of heartwoods and fruits were accumulated in $G_0/G_1$ phase (56.70% and 65.56%).

**Conclusion:** These results suggest that *L. cubeba* heartwoods and fruits has cell cycle arrest activity.

**Keywords:** Cell cycle arrest, *Litsea cubeba*, Heartwood, Fruit, Extract.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10i11.20204

## INTRODUCTION

The World Health Organization reported that breast cancer is one of the leading cause of death and the most common cancer type among women worldwide in 2012 [1]. Moreover, breast cancer ranks as the 5th cause of death from cancer overall (522,000 deaths) is the most frequent cause of cancer death in women in less developed countries (324,000 deaths, 14.3% of total), and the 2nd cause of cancer death in developed countries (198,000 deaths, 15.4%) after lung cancer. A previous study reported that breast cancer is predicted to be a leading new cancer cases and the 2nd most common death cause of women suffering from cancer in the US [2]. Therefore, research and development in cancer detection and treatment is importantly required to solve those problems.

Attarasa (*Litsea cubeba* Lour.) is a plant from Lauraceae family which contains much essential oils which used as antideppresant, anti-inflammation, antioxidant, pesticide, antimicrobial, anticancer, and neuropharmacology [3]. Methanol extract from attarasa fruits showed to be activeoncervics cancer (HeLa cell lines) which cause apoptosis through activation of caspase 3/7 [3,4]. There are more than forty isoquinolin alkaloids that contained in *Litsea* genus which are active as antibacterial agents against *Staphylococcus aureus* [5]. The heartwoods of *L. cubeba* contained high level of phenolic and flavonoid and found to be active as antioxidant [6]. The aim of this study was to determine cytotoxic activity and cell cycle arrest of *L. cubeba* Lour. heartwoods and fruits on T47D cells.

## METHODS

### Plant and chemicals material

Fresh barks and fruits of *L. cubeba* (Lour.) was collected from Balige subdistrict, Sumatera Utara province. *L. cubeba* (Lour.) was identified in Research Center for Biology, Indonesian Institute of Science, Bogor, and the voucher specimen was deposited in herbarium. Chemicals used were distilled water, dimethyl sulfoxide (Sigma), ethanol (Merck), ethylacetate (Merck), [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) (Sigma), propium iodide (BioLegend), and n-hexane (Merck).

### Preparation of n-hexane, ethylacetate, and ethanol extract

The air-dried and powdered heartwods and fruits of *L. cubeba* (Lour.) (1 kg) were repeatedly extracted by cold maceration with n-hexane (3×3 d, 7.5 L). The powder which has been extracted with n-hexane was dried in the air and extracted with ethylacetate (3×3 d, 7.5 L) and continued with ethanol (3×3 d, 7.5 L) at room temperature with occasional stirring. The filtrate was collected, and then, evaporated under reduced pressure to give a viscous extract and then freeze dried to dry [6-9].

### Cytotoxicity assay

The cells were treated with n-hexane, ethylacetate, and ethanol extract. In this test, T47D cell line was grown in RPMI 1640 medium, medium containing 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin (Gibco), and Fungizone 0.5% (Gibco) in a flask in 5% $CO_2$ at 37°C conditions. The inoculums seeded at $1\times10^4$ cells/mL at an optimal volume of 100 µL per well. After 24 hrs incubation, the medium was discharged and treated with extracts and doxorubicin. After incubation 24 hrs, the T47D cells were incubated with 0.5 mg/mL MTT for 4-6 hrs in 37°C. Viable cells were reacted with MTT to produce purple formazan crystals. After 4 hrs, sodium dodecyl sulfate 10% as stopper (Sigma) in 0.01N HCl (Merck) was added to dissolve the formazan crystals. The cells were incubated for overnight in room temperature and protected from light. After incubation, the cells were shaken, and absorbance was measured using microplate reader at λ 595 nm. The data which were absorbed from each well were converted to percentage of viable cells [8,10-14].

### Cell cycle analysis

T47D cells ($5\times10^5$ cells/well) were seeded into 6-well plate and incubated for 24 hrs. After that, the cells were treated with extract and

then incubated for 24 hrs. Cells were collected in conical tube using tripsin 0.025%. The cells were washed 3 times with cold phosphate-buffered saline (PBS) and centrifuged at 2500 rpm for 5 minutes. The supernatant was separated, while the sediment was collected [7,15]. Cells were fixed in 70% cold ethanol in PBS at −20°C for 2 hr. The cells were washed 3 times with cold PBS and resuspended then centrifuged at 3000 rpm for 3 minutes and PI kit (containing PI 40 µg/mL and RNAse 100 µg/mL) added to sediment and resuspended and incubated at 37°C for 30 minutes. The samples were analyzed using FACScan flow cytometer. Based on DNA content, percentage of cells in each of stage in cell cycle (G1, S, and G2/M) were calculated using ModFit Lt. 3.0.s [12,13,16].

**Statistical analysis**

Data were expressed as mean ± SD. All statistics were analyzed using the SPSS 20 software.

## RESULTS AND DISCUSSION

**Plant authentication**

Plant authentication was identified in Research Center for Biology, Indonesian Institute of Science, Bogor, and the voucher specimen was deposited in herbarium with number 1859/IPH.1.01/If.07/IX/2015 and was showed species of *L. cubeba* (Lour.)

**Inhibitory concentration 50% ($IC_{50}$)**

MTT method was used to determine cell viability after incubation for 24 hrs. Cytotoxic activity of n-hexane, ethylacetate, and ethanol extract of heartwoods and fruits of *L. cubeba* was showed in Table 1.

In every treatment, n-hexane, ethylacetate, ethanol, and doxorubicin were shown to inhibit cells growth. The highest $IC_{50}$ value was obtained from ethanol extract of *L. cubeba* fruits of 63.70±2.67 µg/mL and $IC_{50}$ of doxorubicin as positive control was 0.20±0.18 µg/mL. The estimated cytotoxicity of natural product is related to content of active compound in these plants including *L. cubeba*. Flavonoids, triterpenoids/steroids, volatile oils, and alkaloids estimated as active compounds [17]. Doxorubicin is one of chemotherapeutic agent for breast cancer patient therapy. T47D cells line underwent resistant to doxorubicin due to p53 mutation [18,19].

**Cell cycle arrest activity**

To investigate the activity of ethylacetate extract of heartwoods (EAEH) and fruits (EAEF) is by measuring the cell death by modulating cell cycle, we focused on it on further studies using flow cytometry method. The activity of EAEH and EAEF are presented in Fig. 1 and Table 2. Whereas treatment of EAEH and EAEF with10µg/mL caused cell accumulation at $G_0/G_1$ phase were 56.70% and 65.56% and for control cell was 45.37%. At S phase, the accumulation after EAEH and EAEF treatment were 19.81% and 15.19% and for control cell was 18.57%. This fact indicated that EAEH and EAEF can inhibit cell grow that $G_0/G_1$ phase but EAEH inhibit in S phase as well. In the cell cycle analysis, EAEH and EAEF exhibited higher $G_{0/}G_1$ and S phase accumulation compared to control cells. This analysis also showed that cells underwent apoptosis, indicated by the occurence of apoptosis during inhibition of cell cycle on $G_0$-$G_1$ phase [7,16].

From cell cycle analysis (Table 2) was shown that EAEH and EAEF significantly induced cell cycle arrest on T47D breast cancer cells at $G_0$-$G_1$ phase. EAEH and EAEF contain various secondary metabolites such as alkaloids, flavonoids, saponins, and tannins which affected cell cycle arrest. A number of anticancer agents derived from plants are also reported to inhibit cancer cell growth by blocking cell cycle progression such as quercetine (a polyphenol) arrested breast cancer cells at $G_1$ or $G_2$/M phase [20], curcumin (derived from turmeric of *Curcuma* genus) caused $G_2$/M arrest in bladder cancer cells [21], and gambogic acid (isolated from gamboge resin of *Garcinia hanburryi*) inhibited the growth of gastric carcinoma cells by inducing $G_2$/M arrest [22]. Lycorine is one of alkaloids which inhibits cell cycle progression and induces cell cycle arrest in the G0/G1 phase in K562 cells [23].

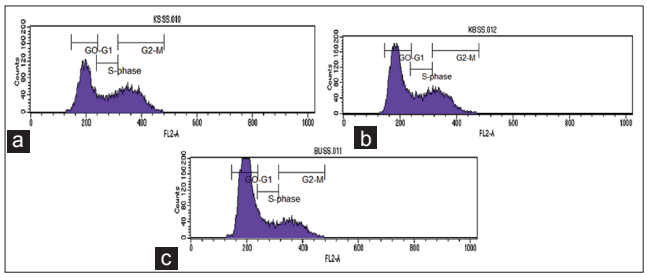


**Fig. 1: Cell cycle analysis using flow cytometry. T47D cells were treated by ethylacetate extract of heartwoods (EAEH) and fruits (EAEF) for 24 hrs and stained using propidium iodide. (a) Control cells, (b) EAEH 10 µg/mL, and (c) EAEF 10 µg/mL. EAEH and EAEF exhibited G0/G1 phase and decreased T47D cell population**

**Table 1: $IC_{50}$ value of n-hexane, ethylacetate, and ethanol extract of *L. cubeba* heartwoods and fruit toward T47D cells**

| Treatment with extract | $IC_{50}$ (µg/mL) | |
|---|---|---|
| | Heartwood | Fruit |
| n-hexane | 76.34±2.61 | 67.52±2.45 |
| Ethylacetate | 71.23±2.37 | 75.59±3.24 |
| Ethanol | 162.58±15.08 | 63.70±2.67 |
| Doxorubicin | 0.20±0.18 | |

$IC_{50}$: Inhibitory concentration 50%, *L. cubeba: Litsea cubeba*

**Table 2: Cell cycle arrest percentage after treatment with EAEH and EAEF**

| Treatment | Concentration (µg/mL) | Phase (%) | | |
|---|---|---|---|---|
| | | $G_0$-$G_1$ | S | $G_2$-M |
| Control | - | 45.37 | 18.57 | 36.06 |
| EAEH | 10 | 56.70 | 19.81 | 24.05 |
| EAEF | 10 | 65.56 | 15.19 | 20.08 |

EAEH: Ethylacetate extract of heartwoods, EAEF: Ethylacetate extract of fruits

## ACKNOWLEDGMENTS

We gratefully thank to Rector University of Sumatera Utara, Indonesia through "TALENTA" Research Grant 2016 for financial support in the study. The authors thank to Prof. Dr. Jansen Silalahi, M.App. Sc., Apt. and Mr. Harald Braun for assistance with the English.

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