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 antidepressant, anti-inflammation, antioxidant, pesticide, antimicrobial, anticancer, and neuropharmacology [3]. Methanol extract from attarasa fruits showed to be acteonecrosis cancer (HeLa cell lines) which cause apoptosis through activation of caspase 3/7 [3,4]. There are more than forty isoquinolinol 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 Sciences, 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 heartwoods 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% CO2 at 37°C conditions. The inoculums seeded at 1×10^4 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. After 4 hrs, the mixture was incubated at 37°C for 15 min. The absorbance was measured using microplate reader at A 595 nm. The data which was obtained from each well were converted to percentage of viable cells [8,10-14].

Cell cycle analysis

T47D cells (5×10^3 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 tripins 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 [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/II/07/IX/2015 and was showed species of L. cubeba (Lour.).

Inhibitory concentration 50% (IC50)

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 IC50 value was obtained from ethanol extract of L. cubeba fruits of 63.70±2.67 µg/mL and IC50 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 with 10µg/mL caused cell accumulation at G0/G1 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 growth at G0/G1 phase but EAEH inhibit in S phase as well. In the cell cycle analysis, EAEH and EAEF exhibited higher G0/G1 and S phase accumulation compared to control cells. This analysis also showed that cells underwent apoptosis, indicated by the occurrence of apoptosis during inhibition of cell cycle on G0/G1 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 G0/G1 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 quercetin (a polyphenol) arrested breast cancer cells at G0 or G/M phase [20], curcumin (derived from turmeric of Curcuma genus) caused G/M arrest in bladder cancer cells [21], and gambogenic acid (isolated from gamboge resin of Garcinia hanburyi) inhibited the growth of gastric carcinoma cells by inducing G0/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 treatment were showed species of L. cubeba: Litsea cubeba.

Table 1: IC50 value of n-hexane, ethylacetate, and ethanol extract of L. cubeba heartwoods and fruit toward T47D cells

<table>
<thead>
<tr>
<th>Treatment with extract</th>
<th>IC50 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heartwood</td>
<td>Fruit</td>
</tr>
<tr>
<td>n-hexane</td>
<td>76.34±2.61</td>
</tr>
<tr>
<td>Ethylacetate</td>
<td>71.23±2.37</td>
</tr>
<tr>
<td>Ethanol</td>
<td>162.58±15.08</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.20±0.18</td>
</tr>
</tbody>
</table>

IC50: Inhibitory concentration 50%. L. cubeba: Litsea cubeba

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

<table>
<thead>
<tr>
<th>Treatment Concentration (µg/mL)</th>
<th>Phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G0/G1</td>
</tr>
<tr>
<td>Control</td>
<td>45.37</td>
</tr>
<tr>
<td>EAEH 10</td>
<td>56.70</td>
</tr>
<tr>
<td>EAEF 10</td>
<td>65.56</td>
</tr>
</tbody>
</table>

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.

REFERENCES


