IN VITRO CYTOTOXIC ANTICANCER POTENTIAL OF BIOACTIVE FRACTION ISOLATED FROM INDONESIAN TIDAL SPONGE CALTHROPELLA SP.

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ABSTRACT

Objective: This study was taken to examine the cytotoxicity of the bioactive fraction isolated from marine sponge Calthroppella sp. as a preliminary anticancer assay and identify its bioactive compounds.

Methods: The cytotoxic activity was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay against three human cancer cell lines, namely human breast (MCF-7), human lung (H-460), and human liver (HepG-2). The bioactive compounds were identified using a high-resolution liquid chromatography-mass spectrometry (LC–MS).

Results: The active fraction 7 showed moderate to strong cytotoxic activity on all cell lines tested and promising a strong potent cytotoxicity against MCF-7 cell lines with IC_{50} value as low as 1.925 µg/mL comparable to control cisplatin (IC_{50} 0.977 µg/mL). In regard to the promising bioactive compounds, the high-resolution LC–MS predicted the existing of several known compounds such as benzamide Q, dapeciptine A, 4'-N-methyl-5'-hydroxystaurosporine, carteriofenone A, and one strong possibility of a new compound.

Conclusion: This study has revealed that the isolated bioactive fraction of Indonesian tidal sponge, Calthroppella sp., possesses potential anticancer properties with a promising significant cytotoxicity on MCF-7 cell lines (IC_{50} 1.925 µg/mL).

Keywords: Calthroppella sp., 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, MCF-7, H-460, HepG-2.

INTRODUCTION

Cancer is a leading cause of deaths worldwide, accounting for approximately 7.6 million deaths (13% of all deaths) in 2008. The WHO predicted that cancer mortality will increase up to 11 million deaths in 2030 [1]. In Indonesia, cancer becomes the seventh leading cause of death (5.7%), with the prevalence of 1.4/1000 populations or equal to 347 thousand of people [2]. This growing trend indicates deficiency in the present cancer therapies; moreover, the increasing number of multidrug-resistance had worsened the situation [3]. In this context, natural products derived have gained significant interest in the treatment of cancer.

In the past decades, a dramatic increase in the number of preclinical anticancer lead compounds from diverse marine life enters human clinical trials [4-6]. Among thousands of marine organism, sponges, in particular, are responsible for >30% isolated potent compounds from marine origin [7]. Marine sponges continue to attract wider attention in the way of discovery attempt for new natural leads of anticancer candidates.

Cytotoxicity has been defined as the cell killing property of a chemical compound independent from the mechanism of death [8]. Cytotoxic assay is an appropriate method for screening new substances within a short time to determine cytotoxicity on cancer cells [9]. In oncology research and clinical practices, in vitro testing is preferred before in vivo testing. In vitro methods like 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay have been described as rapid, simple, and reproducible method, widely used in the screening of anticancer drugs and to measure the cytotoxic properties. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate MTT into a dark blue water-insoluble formazan. This formation production is proportionate to the viable cell number and inversely proportional to the degree of cytotoxicity.

Our present purpose is to assess the in vitro cytotoxic anticancer activity of bioactive fractions of Indonesian tidal sponge Calthroppella sp. on the three human cancer cell lines, namely breast adenocarcinoma (MCF-7), hepatocellular carcinoma (HepG-2), and lung carcinoma (H-460). The active fraction was then analyzed by high-resolution liquid chromatography-mass spectrometry (LC–MS) to predict the bioactive compounds.

MATERIALS AND METHODS

Materials

The sample used was fresh marine sponge Calthroppella sp. The solvent used for general purposes were commercial grade: Merck proanalysis solvent, for example, hexane, dichloromethane (DCM), ethyl acetate (EtOAc), methanol (MeOH), high-performance liquid chromatography (HPLC)-grade acetoniitrile (MeCN), and formic acid (FA). The flash column chromatography was done using silica gel 60 (0.040–0.063 mm). Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} (0.20 mm thickness). The In vitro preliminary MTT cytotoxic assay was carried out with Roswell Park Memorial Institute (RPMI-1640), Wako cell medium, fetal bovine serum (FBS) BioWest, trypsin blue, dimethyl sulfoxide (DMSO), Dulbecco’s phosphate-buffered saline Biochrom, ethanol, trypsin-ethylenediaminetetraacetic acid, cisplatin (Sigma-Aldrich), and MTT reagent (Sigma). Three human cancer cell lines were used: MCF-7 breast adenocarcinoma cancer cells (ECACC), H-460 lung cancer cells (ATCC HTB-177, Summit Pharma), and hepatocellular carcinoma liver cancer cells, HepG-2 (ATCC, HB-8065).

Instrumentations

Analytical balance (Nettler Toledo AB204-S), Ultrasonic bath SIBATA, Rotavapor B-720 Büchi, CAMAG UV lamp (254 and 366 nm), vortex
mixer (Thermoline), centrifuge (Himac CR5B HITACHI), autoclave, incubator with 5% CO₂ humidity at 37°C (Mementr), microscope (Olympus CX-3), TC-10 automated cell counter (BioRad), and Thermo Labsystems Multiskan JX microplate reader were used.

Sample collection
The sea sponge Calthropella sp. was collected by handpicking during low tide from Krapak Beach, Gunungkidul (8° 42.66’N and 110° 36’ 3.15” E), on August 2015. The sponges were kept frozen from collection until the extraction process. The marine sponge Calthropella sp. had earlier been identified at the Laboratory of Animal Taxonomy, Faculty of Biology, Universitas Gadjah Mada.

Extraction and isolation
The frozen sponge (0.8 kg, total) was cut and macerated with a mixture of solvent MeOH/DCM (1:2, v/v). The two layers were separated using a separatory funnel, and DCM layer was concentrated under reduced pressure. DCM extract was then partitioned between EtOAc/H₂O (3:2, v/v). The interesting EtOAc layer was dried and evaporated to yield a viscous extract of EtOAc. EtOAc extract was then fractionated by flash column chromatography (Si gel 60) by stepped gradient elution. The hexane, EtOAc, and MeOH were added to enhance polarity [10]. The elution was driven through the column by applying pressure. Fractions of a standard volume were collected and then combined based on the spots similarity using TLC. The resulted fractions were then concentrated under reduced pressure.

In vitro MTT cytotoxic assay
A standard MTT assay was performed based on the method reported by Mosmann [11]. Briefly, MCF-7, H-460 lung, and HepG-2 were maintained in RPMI-1640 medium with 10% FBS. Cancer cells were seeded into 96-well plates at a density of 1.0 × 10⁴ cells per well. After 24 h at 37°C with 5% CO₂, the cells were treated with sample solutions containing different concentrations of the test material and incubated for 72 h under the same conditions. The medium was removed and replaced with 100 µL of MTT in RPMI-1640 with 10% FBS (0.5 mg/mL), and the cells were then incubated for 3 h at 37°C with 5% CO₂. MTT solution was aspirated, and the formazan crystals were dissolved in DMSO. After 10 min of incubation, the optical density at 570 nm was measured. Cisplatin was used as the positive control.

Compounds identification by LC–MS analysis
LC–MS analysis was conducted on an Agilent 1100 Series HPLC system coupled with a Bruker Daltonics microOTOF-HS mass spectrometer (ESI). HPLC system was equipped with a Cadenza CD-C18 column (2×150 mm, 3 μm, 25°C, 0.2 mL/min) under the following condition: 0–40 min, isocratic elution of 80% MeCN with 0.1% (v/v) FA in H₂O.

RESULTS AND DISCUSSION
Extraction and isolation
EtOAc extract obtained (217.5 mg, weight) was yellowish-brown in color. The fractionation by column chromatography afforded eight fractions after combining the spots similarity based on TLC.

Preliminary in vitro MTT cytotoxic assay
The preliminary in vitro MTT cytotoxic assay was done to screen the most active fraction out of eight fractions. The MCF-7 cell lines were incubated for 72 h with the fractions at concentrations of 10 and 100 µg/mL; then, the cell viability was assessed by MTT assay. Then, loads for further assay were selected on the fraction with highest inhibition, which is close to cisplatin as positive control.

Overall cytotoxicity varied between fractions, yet it exhibited concentration-dependent growth inhibition (Fig. 1). Of all fractions, maximum inhibition showed by the fraction 7, satisfactory inhibited 90% and 100% at 10 µg/mL and 100 µg/mL, respectively. Fraction 7 is considered for being potent as it showed least cell growth comparable to cisplatin at both concentrations tested. Therefore, fraction 7 was selected for further in vitro MTT cytotoxic assay at a wider range of concentrations to evaluate the IC₅₀ value (concentration of the active fractions or cisplatin that inhibits the number of viable cells by 50%).

In vitro MTT cytotoxic assay of fraction 7
Three human cancer cell lines (MCF-7, H-460, and HepG-2) were treated with the fraction 7 at a wider range of concentrations (0.01–1000 µg/mL) and then, cell viability was assessed by MTT assay. The presence of viable cells was visualized by the development of purple color due to the formation of formazan crystals. The IC₅₀ for each cell line was determined using probit analysis with the help of the statistical program SPSS 22. The values of the IC₅₀ are summarized in Table 1.

Cisplatin is one of the most effective synthetic anticancer agents widely used in the treatment of solid tumors. It is generally considered as a cytotoxic drug which kills cancer cells by damaging DNA. However, patients who initially respond to cisplatin therapy often develop resistance to the drug during the course of the treatment [3].

The results in cell growth inhibition by fraction 7 and cisplatin against the cell lines are shown in Fig. 2. A concentration-dependent manner could be observed in all cell lines tested. As the concentration increases, there is also an increase in the cell growth inhibition. Fraction 7 succeeded to inhibit 99% of MCF-7 cells with concentration of 100 µg/mL. These data are of interest, as it suggests that fraction 7 showed potent cytotoxicity on MCF-7 with IC₅₀ 1.925 µg/mL compared with cisplatin, 0.977 µg/mL. As established by the American National Cancer Institute, the IC₅₀ value is standardized <30 µg/mL for anticancer agents, thereby fraction 7 is considered for being potent against MCF-7.

In concern of H-460 and HepG-2 cells, the standard cisplatin showed consistently strong cytotoxic activity, with IC₅₀ values of 1.459 and 1.316 µg/mL, respectively. Contrarily, the percentage inhibitions of fraction 7 averagely decrease from that on MCF-7 cells. In this case, fraction 7 showed less significant cytotoxic activity toward H-460 and HepG-2 cells. It is proved by the IC₅₀ values which were found to be lesser than that on MCF-7 (1.65.322 and 53.011 µg/mL, respectively, for H-460 and HepG-2).

Fraction 7 was then identified for the compounds by LC–MS analysis. The chromatogram separation result of fractions 7 shown in Fig. 3. Identification of the structure was done using literature study of marine natural product compounds, Lit [12], which has a similar structural...
The predicted existing compounds are shown in Table 2.

LC–MS analysis of the fraction 7 reveals that the sponge has components, mostly alkaloids (N-containing heterocycles: Bengamide Q, 4’-N-methyl-5’-hydroxystaurosporine, and clavepictine A). Those isolated compounds have been reported to have a high anticancer activity [13-15]. In regard to mechanism of action, several recent studies reported that alkaloids have the ability to promote apoptosis through inducing DNA damage [16-18]. Other isolated compounds can also be expected to contribute to the anticancer activity possessed by marine sponge Calthropella sp.

Table 2: Compounds identification of fraction 7

<table>
<thead>
<tr>
<th>RT</th>
<th>Measured mass (amu)</th>
<th>Molecular formula of theoretical mass (amu)</th>
<th>Name of compound</th>
<th>Structure of compound</th>
</tr>
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<tbody>
<tr>
<td>4.4</td>
<td>582.4256 [M]+</td>
<td>C_{32}H_{58}N_{2}O_{7}, 582.4244</td>
<td>Bengamide Q</td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>497.2316 [M+H]+</td>
<td>C_{28}H_{29}N_{6}O_{3}, 497.2223</td>
<td>4’-N-methyl-5’-hydroxystaurosporine</td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>348.2991 [M+H]+</td>
<td>C_{21}H_{38}N_{3}O_{3}, 48.2991</td>
<td>Clavepictine A</td>
<td></td>
</tr>
<tr>
<td>9.6</td>
<td>363.3193 [M+H]+</td>
<td>C_{20}H_{38}N_{6}, 363.3145</td>
<td>Biemnic acid</td>
<td></td>
</tr>
<tr>
<td>12.9</td>
<td>540.4622 [M+H]+</td>
<td>C_{32}H_{62}N_{5}O_{5}, 539.4549</td>
<td>New compound</td>
<td></td>
</tr>
<tr>
<td>13.6</td>
<td>501.3893 [M+H]+</td>
<td>C_{33}H_{48}N_{4}, 500.3866</td>
<td>Carteriofenone A</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2: Cytotoxic activity of the active fraction 7 at different concentrations against: (a) MCF-7, (b) H-460 cells, and (c) HepG-2 cells

Fig. 3: Chromatogram liquid chromatography fraction 7 (Eluent 80% MeCN: 20% H_{2}O)
CONCLUSION

EtOAc fraction of Indonesian tidal sponge, Calthropella sp., is considered as a bioactive fraction as it is exhibited an interesting cytotoxic anticancer potential. The active fraction 7 showed moderate to strong cytotoxic activity on MCF-7, H-460, and HepG-2 cell lines (IC\textsubscript{50} 1.925, 165.532, and 53.011 µg/mL, respectively). In addition, fraction 7 promises a strong potent cytotoxicity against MCF-7 cell lines with IC\textsubscript{50} value as low as 1.925 µg/mL compared to cisplatin (IC\textsubscript{50} 0.977 µg/mL). The high-resolution LC–MS revealed that fraction 7 predicted to contain mostly known bioactive compounds and one possibility of a new compound.

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AUTHORS’ CONTRIBUTIONS

First author performed the procedure. Second, third, and fourth author guided through the procedure. All authors discussed and contributed to the final manuscript.

CONFLICTS OF INTEREST

All authors have none to declare.

REFERENCES