Apoptosis induction on human breast cancer T47D cell line by extracts of Ancorina sp. [version 1; referees: awaiting peer review]

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Abstract

Background: Breast cancer is the second leading cause of death in women. Alternative medicine with high efficacy is needed for breast cancer treatments, for example induction of apoptosis using natural products. It has been found that many natural apoptosis-inducing compounds are isolated from marine sponge. The objective of this study is to analyze the ability of extracts of the sponge Ancorina sp. to induce apoptosis on human breast cancer T47D cell line and find out its mechanism.

Methods: T47D cells were treated with crude extracts of methanol, dichloromethane:methanol (1:1) and dichloromethane Ancorina sp. for 24 h, and doxorubicin was used as a positive control. Methods used for this study were MTT assay to examine cell viability and determine IC$_{50}$ of the three extracts, while the percentage of apoptosis and caspase-3 were investigated by flow cytometry.

Results: IC$_{50}$ values of methanol, dichloromethane:methanol (1:1), and dichloromethane extract were 84.25, 121.45, and 99.85μg/mL respectively. The percentages of apoptotic cells after treatment with methanol, dichloromethane:methanol (1:1), and dichloromethane extracts were 88.68, 27.54 and 53.63% respectively, whereas the percentage of caspase-3 was 77.87, 12.66 and 12.97%, respectively.

Conclusions: These results revealed that all extracts of Ancorina sp. have strong or moderate cytotoxicity and have the ability to induce apoptosis on T47D human breast cancer cell line. However, methanol crude extract has high efficacy to induce apoptosis through caspase-3 activation compared to the other extracts. Hence methanol extract warrants further investigation as a natural medicine for human breast cancer.

Keywords
Ancorina sp., cytotoxicity, apoptosis, caspase-3, breast cancer
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**Introduction**

Breast cancer is the second leading cause of death in women after cervical cancer. In 2016 breast cancer cases have occurred in 40 per 100,000 women in Indonesia\(^1\). Medical treatment for breast cancer is currently widely applied\(^2\). However, medical treatment can cause side effects, namely the death of healthy cells surrounding cancer cells\(^1\). Alternative methods of breast cancer treatment with reduced side effects are needed, such as treatments using natural anticancer agents\(^3\).

There are many cancer treatment methods such as antiangiogenesis therapy\(^4\), cell cycle inhibitors\(^5\), and photodynamic therapy\(^6\). Induction of apoptosis is the most common approach in cancer therapy because apoptosis has specific abilities to kill certain cells\(^7\). One characteristic of cancer cell is loss of ability for apoptosis\(^8\). The ability of apoptosis to kill abnormal cells can prevent the occurrence of cancer growth\(^9\). Induction of apoptosis occurs through three apoptotic-signaling pathways: extrinsic, intrinsic and perforin/granzyme pathways. Apoptosis path activation is marked by the activation of caspases. Caspase is found in normal cells as an inactive zymogen (procaspase). Active caspase activates other caspases, forming the ‘caspase cascade’. Activation of caspase 8 and 9 will cause activation of caspase-3 as a downstream effector, which induces apoptosis\(^10\).

Previous studies found many natural apoptosis-inducing compounds isolated from marine sponge that can be developed as natural medicine\(^11\). Fraction of *Negombata magnifica* sponge is able to induce apoptosis in hepatocellular carcinoma\(^12\). Sponge extract of *Haliclona* sp. able to increase the percentage of apoptosis in A549 lung cancer cells\(^13\). Organic compounds have been successfully isolated from the sponge *Condrosia reniformes*, *Tethya rubra*, *Tethya ignis*, *Mycale angulosa* and *Dysidea avara* as a drug therapy for Chagas disease using acetone solvents\(^14\). Terpenoids have been successfully isolated from sponge *Ircina* sp. and *Spongia* sp. using ethanol solvent\(^15\). Anticancer compounds have been successfully isolated from *Petrosia* sp., *Jaspis* sp. and heterogeneous *Pericharax* using dichloromethane:methanol (1:1)\(^16\). Some studies also mention that sponge bioactive compounds, antiviral, antimicrobial, antifungal, and anticancer compounds, have been successfully isolated with methanol\(^17,18\), ethanol\(^19\), dichloromethane and combination of dichloromethane:methanol (1:1)\(^20,21\).

The objective of this study is to determine the cytotoxicity of *Ancorina* sp. extract in breast cancer T47D cells and measure extract-induced apoptosis through activation of caspase-3. In this study we use three solvents: methanol (polar), dichloromethane (non-polar) and mixture of both solvents to determine the most effective solvent. Furthermore this study used T47D cells as a model for breast cancer cells because T47D cells are able to express caspase-3, which is an effector of apoptotic induction\(^22\).

**Methods**

**Sample preparation and determination**

*Ancorina* sp. were collected from Wedi Ombo Beach, Gunungkidul, Yogyakarta, Indonesia. Samples were washed to remove debris and residual salt. Samples were transferred to the laboratory in methanol, dichloromethane and dichloromethane:methanol (1:1) under cool condition.

**Extraction**

Fresh samples were crushed in a blender in methanol, dichloromethane and dichloromethane methanol (1:1) then macerated for 24 hours. The samples were filtered and the residue was re-extracted for two times. The total filtrate was then evaporated to obtain crude extract paste.

**Cell line culture**

We used T47D cells obtained from Integrated Laboratory of Research and Testing, Universitas Gadjah Mada (LPPT UGM).

The cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2% penicillin streptomycin and 0.5% Fungizone. Cells were harvested and re-suspended in 0.25% Trypsin-EDTA. Cells were cultured in 96-well microplates (1 × 10\(^4\) cells/well) in 100 μL RPMI and incubated at 37°C with 5% CO\(_2\) overnight.

Doxorubicin at 5 μg/mL was used as the positive control whereas T47D cells cultured in medium was used as the negative control and cells cultured in 0.5% DMSO in medium was used as the solvent blank.

**Cytotoxicity assay**

Cytotoxicity was assessed using the MTT assay. After the cells were incubated for 24 h with the serial dilution from 15.68 to 250 μg/mL, 0.5% MTT solution was added and the cells were incubated for 4 h followed by addition of stopper reagent (10% SDS in 0.1 N HCl). The optical density (OD) was measured at 550 nm using Microplate Reader BIO-RAD 680XR. MTT data was then analyzed by Probit to determine the IC\(_{50}\) value. IC\(_{50}\) of each extract is used for FACS experiment.

**Apoptosis and caspase-3 assay**

Briefly, T47D cells were seeded in 6-well microplates in 3×10\(^4\) μL RPMI. In total, 1×10\(^5\) cells were treated by IC\(_{50}\) concentrations of three extracts or doxorubicin for 24 h. Cells were stained by Annexin V-PI Biolegend for apoptosis test and by BD Cytofix / Cytoperm™ for caspase-3 activation test. The sample was measured using flow cytometer BD FACSCalibur™. Flowcytometry output by BD FACS Calibur™ was shown in four quadrants. The first quadrant contains normal living cells population that respond negatively to Annexin V-FITC and propidium iodide (PI). Second quadrant contains early apoptotic cells populations that
respond positively to Annexin V-FITC. Third quadrant contains the late apoptotic cells population which responds positively to Annexin V-FITC and Propidium Iodide (PI). Whereas in the fourth quadrant contains a population of necrotic cells that respond negatively to Annexin V-FITC and respond positively to PI².

Data analysis
The IC₅₀ value was determined by Probit analysis. IC50 value and percentage of apoptosis are further analyzed by one-way ANOVA and Tukey’s test at 5% significance level using IBM SPSS Statistic 23.0 program. P < 0.05 indicated statistical significance.

Results
Cytotoxicity
The cell viability of T47D cells after methanol, dichloromethane and dichloromethane: methanol (1:1) extracts treatment are presented in Figure 1. The concentration of extracts reduced the viability of investigated cells by 50% (IC₅₀), which has been reported in Table 1.

All Ancorina sp. extracts inhibited the proliferation of cancer cells in a dose dependent manner. The higher concentration of extract caused the lower percentage of T47D cell viability. All extracts were cytotoxic to T47D cells. IC₅₀ value of methanol was significantly different to dichloromethane: methanol but wasn’t significantly different to dichloromethane.

Apoptosis and caspase-3 activation assay
We analyzed cell death qualitatively by examining cell morphological change and quantitively by flow cytometry using Annexin-V after 24 h incubation of extracts.

Cell morphology after treatment can be seen in Figure 2. Morphology of T47D cell showed that methanol extract caused most cells population to undergo death (approximately more than 70%), while dichloromethane extract resulted in almost half cell population deaths. The combination of methanol and dichloromethane (1:1) extract causes fewer cell deaths (<50%).

The control (DMSO) cell did not cause cell death but after doxorubicin and extract treatment most cells undergo death. This data supports the cytotoxicity assay that the Ancorina sp. extracts successfully induced cell death.

Detection of apoptosis marker after treatment by extracts can be seen in Figure 3.

All Ancorina sp. extracts increase the percentage of apoptotic cells compared to control cells (Figure 3). The highest percentage of apoptosis was obtained in the methanol group (88.68%), which was even higher than doxorubicin as a positive control (75.74%) (Table 2).

The three extracts showed the same pattern with doxorubicin, i.e. a high percentage of apoptotic cell while the percentage of necrotic cells is low (Table 2).

We further investigated the apoptotic mechanism by examining the percentage of caspase-3. Detection of caspase-3 can be seen in Figure 4, while percentage of caspase-3 activation and correlation between percentage of apoptosis and caspase-3 activation can be seen in Table 3 and Figure 5, respectively.

The highest percentage of caspase-3 was detected with methanol extract, which almost equaled doxorubicin, while the value of the other extracts was lower (Table 3).

The three extracts have a positive correlation between percentage of apoptosis and caspase-3. Although dichloromethane showed lower percentage of apoptosis and caspase-3, but they still have strong cytotoxicity (99.85 μg/mL), which shows potency as natural anticancer agents.
Table 1. IC<sub>50</sub> values of Ancorina sp. extracts.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; value (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>84.25 ± 9.52</td>
</tr>
<tr>
<td>Dichloromethane:methanol (1:1)</td>
<td>121.45 ± 10.11</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>99.85 ± 11.79</td>
</tr>
</tbody>
</table>

Note: different letter showed the significant difference at the 0.05 level.

Figure 2. Cell morphology of breast cancer T47D cells after treatment with extracts of Ancorina sp. Control (A), Methanol (B), Dichloromethane:Methanol (C), Dichloromethane (D), Doxorubicin (E) and DMSO (F). Arrow shows dead cells.
Table 2. Percentage of T47D cell population after treatment of crude extracts of Ancorina sp.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Applied concentration (IC₅₀ value)</th>
<th>Normal</th>
<th>Early + Late apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>84.25</td>
<td>6.94 ± 0.21</td>
<td>88.68 ± 0.47</td>
<td>4.38 ± 0.25</td>
</tr>
<tr>
<td>Dichloromethane: Methanol (1:1)</td>
<td>121.45</td>
<td>65.51 ± 2.79</td>
<td>27.54 ± 0.93</td>
<td>7.37 ± 1.65</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>99.85</td>
<td>39.29 ± 1.60</td>
<td>53.63 ± 1.42</td>
<td>7.60 ± 0.91</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>5</td>
<td>20.65 ± 2.09</td>
<td>75.74 ± 1.58</td>
<td>3.67 ± 0.44</td>
</tr>
<tr>
<td>Negative control</td>
<td>-</td>
<td>92.93 ± 0.01</td>
<td>1.84 ± 0.15</td>
<td>5.34 ± 0.21</td>
</tr>
</tbody>
</table>

Note: different letter showed the significant difference at the 0.05 level. Statistical analysis is focused to percentage of apoptosis among group.
Table 3. Percentage of caspase-3 activation after treatment by crude extracts of *Ancorina* sp.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Applied concentrations</th>
<th>Caspase-3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>84.25</td>
<td>77.87 ± 5.81</td>
</tr>
<tr>
<td>Dichloromethane : Methanol (1:1)</td>
<td>121.45</td>
<td>12.66 ± 3.30</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>99.85</td>
<td>12.97 ± 2.11</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>5</td>
<td>91.53 ± 4.09</td>
</tr>
<tr>
<td>Negative control</td>
<td>-</td>
<td>1.54 ± 0.00</td>
</tr>
</tbody>
</table>

Figure 4. Detection of caspase-3 activation in breast cancer T47D cells after treatment with extracts of *Ancorina* sp. Negative control (A), Doxorubicin (B), Methanol (C), Dichloromethane:Methanol (D) and Dichloromethane (E).
Discussion

Sponges are highly diverse in Indonesia. In particular, encrusting sponges abundantly live in Gunung Kidul, Yogyakarta. Marine sponges produce some secondary metabolites, which can be used as antiviral[^2], antimicrobial[^2][^23], antifungal[^2], and anticancer drugs[^17][^24][^29]. The cell adhesion and immune system in sponge allow the different forms of the body plan[^30]. When encrusting sponges grow together, sponges can survive by producing chemicals to kill fast dividing cells from the neighboring sponges. This ability of the chemicals can be used for chemotherapy since the basis of chemotherapy treatments is to disturb cancer cell growth[^31].

Sponge Ancorina sp. is a member of family Ancorinidae, which contains bioactive compounds such as ancorinoside BD, penazetidine A (Penares sollasi), ecionines A & B (Ecionemia sp.) and Iso malabaricane triterpenes (Rhabdastrella globostellata)[^32]. Ancorinoside is a MT1-matrix metalloproteinase inhibitor in the development and metastasis of tumor cells[^33], whereas Penazetidine A strongly inhibits PKC-β1 activity in tumor cells with IC\textsubscript{50} value 0.3 μg / mL[^34].

Ecionines A (biemnadin) and B (meridine) are anticancer compounds for many cancer cells, including bladder cancer cells[^35]. Further, Iso malabaricane triterpenes were also found to be anticancer after testing on three types of cancer cells, namely L5178Y (mouse lymphoma), HeLa (human cervical carcinoma), and PC-12 (pheochromocytoma in mice)[^36]. Ancorina sp. is a source of bioactive compounds such as ancorinoside A Mg salt, ancorinolates AC, bis-ancorinolate B, ancorinazole, indolo [3,2-a] carbazole, and (+) - 7-bromotrypargine[^37][^38]. These previous data show the high potency of Ancorinidae to be used as natural anticancer agents; hence this study is focused on the potency of Ancorina sp. as an anticancer agent and its mechanism, which is possibly through apoptosis induction.

Cytotoxicity is categorized into three levels by IC\textsubscript{50} extract values. Very strong cytotoxicity has IC\textsubscript{50} less than 10 μg / mL, strong cytotoxicity has IC\textsubscript{50} values between 10 -100 μg/mL, and moderate cytotoxicity has IC\textsubscript{50} values between 100 - 500 μg/mL[^39]. According to these ranges, IC\textsubscript{50} of the methanol and dichloromethane extracts in the present study had strong cytotoxic ability, while dichloromethane:methanol (1:1) extract belonged to moderate cytotoxicity. Ancorina sp. extracts have greater value of IC\textsubscript{50} compared with the study[^34], which mentioned penazetidine A, a bioactive compound of marine sponge and highly inhibits PKC-β1 activity in tumor cells with lower IC\textsubscript{50} of 0.3 μg/mL. This difference is due to the non-fractionated extract of our sponge, so that unsorted bioactive compounds possibly affect the cytotoxicity ability of extracts[^34].

Bioactive compounds from natural products depend on solvents. Based on the polarity of solvents, in the present study, Ancorina sp. extracts with polar solvent (methanol) lead to a higher apoptosis than non-polar (dichloromethane) or combination. These results are supported by a previous study that showed some compounds of Ancorinidae, such as ancorinoside BD, penazetidine A, ecionines A and B and isomalabaricane triterpenes, are polar compounds that have anti-tumor and anti-cancer characteristics[^32]. Interestingly, some studies in sponge also show same phenomenon such as cytotoxic compounds from sponge Dactylospongia elegans and Pachychalina alcaloidifera has been isolated using polar solvent methanol[^14][^15]. Terpenoids from sponge Iricina sp. and Spongia sp. have been isolated using polar solvent ethanol[^14][^15][^17]. Bioactive compounds of sponge, both antiviral, antimicrobial, antifungal, and anticancer compounds have been successfully isolated by polar solvent as methanol[^16][^21] and ethanol[^22]. Considering all extracts in this study have low necrosis values (Table 2), they are safe to be used as medicine. Therefore, further studies are needed to find out the specific compounds of Ancorina sp. extracts.

Figure 5. Correlation between percentage of apoptosis and caspase 3 activation in breast cancer T47D cells after treatment with extracts of Ancorina sp. Doxorubicin (Dox), Methanol (M), Dichloromethane:Methanol (1:1) (D:M), Dichloromethane (D), and negative control (control).
Apoptosis can be triggered by extrinsic stimulation through death receptors on cell surfaces, such as TNFα (Tumor Necrosis Factor-α), Fas receptor (CD95 / AP01) and TRAIL (TNF related to ligand-inducing apoptosis) or by intrinsic stimulation through mitochondrial signaling pathways. In these two main pathways, activation of cysteine aspartyl proteases or caspase can produce mitochondrial permeabilization membrane, chromatin condensation and DNA fragmentation. These events stimulate the cells that undergo apoptosis and lead to a distinctive cell morphology, such as the appearance of pyknosis, chromatin condensation, nucleus fragmentation, and apoptotic body formation, but organelles are still intact. This can be seen in the present study in Figure 2.

Apoptotic pathways commonly occur by the activation of caspase-3, which is the effector of intrinsic, extrinsic and perforin pathways. Caspase-3 is a key protease that is activated during the early stages of apoptosis. Caspase-3 is proteolytically active, cuts and activates other caspases, as well as relevant targets such as targets in the cytoplasm (D4-GDI and Bcl-23) and nucleus (poly (ADP-ribose) polymerase; PARP1).

In the present study, the highest percentage of caspase-3 was detected in methanol extract, which almost equal to doxorubicin, while the other extracts was lower (Table 3). Doxorubicin as a commercial drug in chemotherapy revealed a high percentage of apoptotic cells and caspase-3 activation. Among Ancorina sp. treatment groups, methanolic extract showed the highest percentage of both apoptosis and caspase-3. Interestingly, the methanolic extract showed a higher percentage than doxorubicin, and revealed its great potency to be used as a cancer medicine (Table 3).

The three extracts in this study have a positive trend between percentage of apoptosis and caspase-3 activation. Although dichloromethane showed a lower percentage of apoptosis and caspase-3, they had strong cytotoxicity (99.85 μg/mL) which shows potential as natural anticancer agents. It is possible that anticancer mechanism of dichloromethane and mixture of dichloromethane and methanol (1:1) excludes caspase-3 activation as effector caspases. Another pathway, such as caspase-6 or 7, can also induce apoptosis in T47D breast cancer cells. More investigation is needed to elucidate the anticancer mechanism of these extracts.

Conclusions
All extracts of Ancorina sp. have strong or moderate cytotoxicity and have the ability to induce apoptosis in T47D human breast cancer cell line.

Data availability
Underlying data

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

Grant information
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References


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