Antifungal effect of *Sticophus hermanii* and *Holothuria atra* extract and its cytotoxicity on gingiva-derived mesenchymal stem cell

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**ABSTRACT**

**Background:** Sea cucumber had been acknowledged to have some medical properties *Sticophus hermanii* and *Holothuria atra* are species of sea cucumber which has been known to have antifungal properties thus potentially explored as therapeutic agent in oral candidiasis. **Purpose:** The aim of this study was to examine the antifungal property *Sticophus hermanii* and *Holothuria atra* extract against Candida albicans and its cytotoxicity to human gingiva-derived mesenchymal stem cell. **Methods:** The study was an experimental laboratories research with post test only control group design. Methanolic extract of *Sticophus hermanii* and *Holothuria atra* in concentrations of 1%, 0.5%; 0.25%; 0.13%, 0.07%; 0.03%, 0.02% and 0.01%; were tested its cytotoxicity on gingiva-derived mesenchymal stem cell. Cell viability were measured by MTT assay. The antifungal property against Candida albicans was tested by disk diffusion method. Data were analyzed by ANOVA followed by LSD. **Results:** Extract of *Sticophus hermanii* showed no cytotoxicity in all concentrations (p>0.05), while *Holothuria atra* showed toxicity in the concentration of 1% and not cytotoxic in the concentrations below (p<0.05). Both sea cucumber extract could inhibit the growth Candida albicans, in vitro, proved by the clear zone around the disc in all concentrations (p<0.05). **Conclusion:** *Stichopus hermanii* and *Holothuria atra* extract had the antifungal effect against Candida albicans. Sea cucumber extract were not cytotoxic to gingiva-derived mesenchymal stem cell in the concentration of *Sticophus hermanii* ≤ 1% and *Holothuria atra* ≤ 0.5%.

**Key words:** *Sticophus hermanii*, *Holothuria atra*, cytotoxicity, gingival, mesenchymal stem cell
INTRODUCTION

Sea cucumbers belong to the phylum *Echinodermata*, meaning that, they are spiny-skinned, under the class *Holothuridea*. Sea cucumbers are important components of the marine ecosystem. Sea cucumbers, informally named as bêche-de-mer, or gamat, have long been used for food and folk medicine in the communities of Asia and Middle East.\(^{1,4}\)

Marine biota is the source of structurally unique natural products that are mainly accumulated in living organisms, not just as food consumption and industrial need but later has been known to have biomedical properties. Therapeutic properties and medicinal benefits of sea cucumbers can be linked to the presence of a wide array of bioactives especially triterpene glycosides (saponins), chondroitin sulfates, glycosaminoglycan (GAGs), sulfated polysaccharides, sterols (glycosides and sulfates), phenolics, cerberosides, lectins, peptides, glycoprotein, glycosphingolipids and essential fatty acids. Nutritionally, sea cucumbers have an impressive profile of valuable nutrients such as vitamin A, vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B3 (niacin), and minerals, especially calcium, magnesium, iron and zinc.\(^{4,7}\)

Generally, most species of sea cucumber share the same bioactive compound mentioned above but in different level contain.\(^{4,7}\) Regarding to its contents, the aqueous and organic extracts from some sea cucumber species has been proved to have antioxidant and antiproliferative activities,\(^{3,4,7}\) immunomodulator,\(^{1,4}\) while the other has been known to have antimicrobial properties on Gram negative, Gram positive bacteria,\(^{3,4,8}\) and antifungal action,\(^{3,9,10,11}\)

Candidiasis is the most common fungal infection in oral cavity caused by *Candida albicans*, which its prevalence raised specially along with the raise prevalence of HIV-AIDS.\(^{12,13}\) A natural source of antifungal agent could become the novel alternative solution in therapy of oral candidiasis.\(^{14}\)

Considering to the bioactive compound, the extract of *Sticophus hermanii* and *Holothuria atra* are potentially explored its antifungal property to *Candida albicans* and as the potential candidateatherapeutic agent in oral candidiasis.\(^{8,14}\) Its cytotoxicity should be well identified to assure the biocompatibility to oral cells.\(^{15}\) During the last years, the interest of in vitro systems as an alternative to animal experiments in toxicological research has been steadily increasing.\(^{16-18}\) Stem cells and their derivatives represent a promising opportunity for developing in vitro, human cell assays that would ultimately replace, enhance, or surpass the current models that are used for predictive toxicology.\(^{19-23}\)

In this paper, two sea cucumber extract *Sticopus hermanii* and *Holothuria atra* were studied its antifungal property to *Candida albicans* and its cytotoxicity to gingiva-derived mesenchymal stem cell. These two species of sea cucumber are found plenty in Karimun Jawa coastal and so far had been explored mostly for food consumption.\(^{12}\) Considering to the bioactive compound, there are some opportunities to explore sea cucumber for medical properties and yield more great value on it specially in oral disease.

The aim of this study was to examine the antifungal property and cytotoxicity of various concentration of *Sticopus hermanii* and *Holothuria atra* extract onhuman gingiva-derived mesenchymal stem cell. The result of this study could be served as preliminary data to be continued in preclinical and clinical research with marine natural products which will probably result in novel therapeutic agents for the treatment of fungal infection in oral disease.

MATERIALS AND METHODS

Two sea cucumber species: *Sticopus hermanii* and *Holothuria atra* were collected from Karimun Jawa coastal region. Adult sea cucumber were selected to get the best extract result considering to its maximum secondary metabolit contents. The collected samples were cleaned from dirt, immersed in water for one night to get rid of salt and parasite then dried in dryer machine. Sea cucumber then splitted, the inner abdomen were removed then cleaned and washed, so only the flesh of the body proceed to next process. Each samples were cut in small piece of 3-10 cm, the wet weight then measured then dried up in solar dryer for 3-4 days to reduce the water content. The dried sea cucumber then cut into smaller pieces of 1 cm, mashed by blender the the weight were measured and ready for the maceration process. Two hundred and fifty (250) gram...
mashed dry sea cucumber sample immersed until soaked in 500 mL methanol solvent for 24 hours at room temperature, then filtered with filter paper to separate filtrate and residue.

Residue then reimmersed in 500 mL methanol solvent for 24 hours, again filtered with filter paper to separate filtrate and residue, resulted in maceration filtrate with the ratio of 250 gram sample / 1000 mL solvent (1:4 w/v). Methanol (polar) filtrate got homogenized with 1000 mL hexane solvent (non polar) then performed partition with separatory funnel the each of the filtrate layer of methanol and hexane solvent were separated. Methanol (polar) filtrate then got re-homogenized with 1000 mL chloroform solvent (semi polar), performed partition with separatory funnel the each of the filtrate layer of methanol and chloroform solvent were separated. Each filtrate were separated by its solvent with rotary evaporator until extract produced. The evaporated extract then placed in the vial and stored in -30°C until the next analysis.

Candida albicans were cultured in Sabouraud dextrose agar, suspension were prepared by inoculating one single loop of fungal colony to Sabouraud broth medium, incubated in 37°C for 24 hours and adjusted its turbidity to standard McFarland 0.5. The samples were divided into 5 groups each consisted of 6 samples i.e: positive control was given nystatin oral solution 100.000 IU, negative control was given DMSO 1%, treatment group were given Sticophus hermanii and Holothuria atra extract separately, each diluted by DMSO 1% with concentration of 20%, 40% and 80%. Antifungal activity test was performed by disk diffusion method on Mueller Hinton agar.

Fungal suspenion of Candida albicans sequal to 0,5Mc Farland was swabbed on to Muller Hinton agar plate. Sterile paper disks were immersed for 15 second into each concentration of extracts for treatment groups, for control negative groups in DMSO 1%, each, and for the positive control group in nystatin oral solution, then put on to Muller Hinton agar, gently pressed for a while and leave, incubated in 37°C for 48 hours. The clear zone around the disk showed inhibition effect to the growth of Candida albicans. Diameter of inhibition zone was measured with digital caliper.

The MTT 3-(4,5-Dimethylthial-2-yl)-2, 5-Diphenyleterazalium Bromide (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) cytotoxicity test is tests for in vitro cytotoxicity, was performed to evaluate the viability of gingiva-derived mesenchymal stem cell after treated with Sticophus hermanii and Holothuria atra extracts which diluted with DMSO 1% into 9 groups concentration of 1%, 0.5%; 0.25%; 0.125%, 0.006%; 0.003%, 0.00015%, 0.00007%; 0.00003%. The gingiva-derived mesenchymal stem cellwere obtained from ITD, Surabaya. After the thawing process, the cell were resuspended with culture medium of α alpha minimum essential medium (MEM) (Gibco, Invitrogen Co, New York, USA), sentrifuged for 5 minute in 1500 rpm, repeated for 3 times then get cultured, passedage every 4 days. In the second passage monolayer was formed and ready to be performed the cytotoxicity test.

The cells were seeded into 96-well microplates (Iwaki, Asahi Glass co, Tokyo, Japan) each containing 200 µlith the density of 5 × 10^4 in α MEM medium incubated at 37°C for 24 hours. Medium then replaced then extract were added as amount of 200 µl and incubated 37°C for 20 hours. Control positive cells were also prepared containing cells in culture medium, assumed to be viable 100%.

Microplates then taken out from the incubator, added the solution of MTT 5 mg/ml in PBS 25 µl for each well, incubated for 4 hour. The medium then discarded, replaced with 200 µl DMSO in each well. Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) to purple formazan. After the exposure, the formazan formations were determined for each treatment concentration by ELISA reader at a wavelength of 595 nm. The relative viability of the treated cells as compared to the control cells were expressed as the % cytoviability, using the following formula:

\[
\text{\% Cell viability} = \left(\frac{A_{\text{sample}}}{A_{\text{control}}} \right) \times 100\% 
\]

Note: A sample is mean value of the measured optical density of the treated cells; A control is mean value of them easured optical density of the control cells.16-18

The data were presented as means ± standard deviation (SD). Statistical analysis was performed using analysis of variance (ANOVA) to determine the effect of sea cucumber extract concentration on the gingiva-derived mesenchymal stem cells cytoviability.

**RESULTS**

Antifungal activity showed by the inhibition zones around the disk were observed in all treatment groups and positive control but not the negative control. All treatment groups in all concentrations showed inhibition zones but the diameter were less than nystatin as positive control. Both Sticophus hermanii and Holothuria atra extracts showed that the increasing concentration tested on Candida albicans resulted in the increasing diameter of inhibition zone as shown in Figure 1.

Further statistical analysis by two way ANOVA test and LSD multiple comparison test at 5% significance level presented in Table 1 showed the significant difference on all concentration of Sticophus hermanii and Holothuria atra extract compared to negative and positive control group (p<0.05).

Result on cytotoxicity test showed that all concentrations of Sticophus hermanii extract were not cytotoxic to gingiva-derived mesenchymal stem, showed by the average of cell viability above 50%, while Holothuria atra extract were not detected.
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Table 1. ANOVA and LSD summary of inhibition zone of Sticophus hermanii and Holothuria atra extracts on serial concentration extracts to Candida albicans compared to control groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Aquaeous</th>
<th>Nystatin</th>
<th>Holothuria atra 20%</th>
<th>Holothuria atra 40%</th>
<th>Holothuria atra 80%</th>
<th>Stichopus hermanii 20%</th>
<th>Stichopus hermanii 40%</th>
<th>Stichopus hermanii 80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquaeous</td>
<td>0.000*</td>
<td>0.032*</td>
<td>0.001*</td>
<td>0.002*</td>
<td>0.011</td>
<td>0.006</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Nystatin</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Holothuria atra 20%</td>
<td></td>
<td></td>
<td>0.238</td>
<td>0.307</td>
<td>0.547</td>
<td>0.53</td>
<td>0.023*</td>
<td></td>
</tr>
<tr>
<td>Holothuria atra 40%</td>
<td></td>
<td></td>
<td>0.872</td>
<td>0.077</td>
<td>0.578</td>
<td>0.263</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holothuria atra 80%</td>
<td></td>
<td></td>
<td>0.107</td>
<td>0.692</td>
<td>0.201</td>
<td>0.023*</td>
<td>0.005*</td>
<td>0.096</td>
</tr>
</tbody>
</table>

Table 2. ANOVA summary of cytotoxicity of Sticophus hermanii and Holothuria atra extracts concentrations on gingiva-derived mesenchymal stem cell

<table>
<thead>
<tr>
<th>Group</th>
<th>F</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sticophus hermanii extract</td>
<td>2.056</td>
<td>0.086</td>
</tr>
<tr>
<td>Holothuria atra extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20.326</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Discussion

Result of antifungal sensitivity test showed inhibition zone around the disk in all treatment group and in control positive group, means that both extract of Sticophus...
hermanii and Holothuria atra has antifungal effect to C. albicans in vitro. The largest diameter of inhibition zone of treatment group was in the concentration of 80 mg/mL but still less than in the control group of nystatin (p<0.05).

Nystatin is a polyene antifungal drug to which many molds and yeasts are sensitive, including Candida spp, used as the positive control for its the common topical antifungal agent therapy on oral candidiasis. Nystatin exerts its antifungal activity by binding to ergosterol found in fungal cell membranes. Binding to ergosterol causes the formation of pores in the membrane. Potassium and other cellular constituents leak from the pores causing cell death.12,13

Sea cucumber extract have been known to have the antifungal property, assumed to be related to its content of alcaloid, saponin and triterpen glycoside.1,4,9,11 Result showed both Sticopus hermanii and Holothuria atra has antifungal activity against C. albicans according to the study stated that generally, most species of sea cucumber share the same bioactive compound mentioned above but in different level contain.4,7 Saponin were identified in the content of sea cucumber extract.1,4,9 It is secondary metabolites of glycosidic nature widely distributed in higher plants and marine invertebrates resulted as the defend mechanism also has the biological properties i.e. the ability to lyse erythrocytes or to foam. It form complexes with cell membrane cholesterol leading in consequence to pore formation and cell permeabilization, alterations in the negatively charged carbohydrate portions on the cell surface.24

Saponin performed its antifungal activity by the interaction with sterol membrane of C. albicans and disrupting the cell wall’s integrity caused the cell death, similar with the mechanism action of nystatin. Sticopus hermanii and Holothuria atra have been extracted by methanolic extract.14 The antibacterial compound of sea cucumber assumed to be polar for it is dissolved in methanol solvent and have been proven to have the antibacterial, antifungal and cytotoxic agent on some studies.1,4,9

Regarding antifungal property of both sea cucumber extract to C. albicans, cytotoxicity test must be performed to examine its biocompatibility prior to explore its potency as treatment in oral candidiasis. Cell culture can be used to screen for toxicity both by estimation of the basal functions of the cell or by tests on specialized cell functions.19,20 Recently, toxicity test have been developed and stem cells were explored regarding to some basic consideration in some advantage in the technique and result. Human stem cells are potentially attractive reagents for predictive toxicology, particularly if they can be shown to be a reliable, large-scale source of differentiated human cells. The use of human cells could increase the correlation between safety studies and clinical trials, an important benefit since conventional animal models of toxicity are not always predictive of human responses. Stem cells that are generated from adult tissues (iPS cells) could allow models to be created from individuals with a diverse range of drug susceptibilities, resistances or disease, which could reduce the rate of adverse effects within patient subpopulations.21

In this research, cytotoxicity test of Sticopus hermanii and Holothuria atra extracts were performed on gingiva-derived mesenchymal stem cell. Gingiva-derived mesenchymal stem cells (GMSCs) were employed on this study considering to its accessibility and potency for further application in oral therapy. GMSC are stem cells from human gingiva, a tissue source easily accessible from the oral cavity, which exhibited clonogenicity, self-renewal, and multipotent differentiation capacities. Most importantly, GMSCs were capable of immunomodulatory functions and able to induce osteogenic, chondrogenic, and adipogenic differentiation.22,25 The cytotoxicity test of sea cucumber extract on GMSC will be a proper predictor regarding to the potency of the cell. Once the extract is not cytotoxic to GMSC the greater possibility of oral application without degrading the potency of stem cell attained.

Cytotoxicity testing includes numerous methods, both qualitative and quantitative. In this study we used indirect test, in which the rate of cell growth (cell number) and the metabolic activity (MTT) have indicated the degree of cytotoxicity of sea cucumber extract. Result showed cytotoxic activity on gingiva-derived mesenchymal stem cell after treated with Holothuria atra extract in concentration of 1%, shown by the cell viability less than 50% (p<0.05).

The cytotoxic effect to gingiva-derived mesenchymal stem cell assumed related to the content of saponin in sea cucumber extract. It has been stated that the bioactive compound of Holothuria atra are mostly triterpene glycoside (saponin). Saponins are secondary metabolites of glycosidic nature widely distributed in higher plants but also found in some animal sources, like e.g. marine invertebrates. Saponins have large structural diversity, but these compounds share some unique biological properties like the ability to lyse erythrocytes or to foam. The latter contributed to naming this group saponins, which is derived from Latin sapo meaning soap. Haemolysis of red blood cells seems to result from saponin ability to form complexes with cell membrane cholesterol leading in consequence to pore formation and cell permeabilization, and also to cause alterations in the negatively charged carbohydrate portions on the cell surface. Surface activity responsible for foaming properties, as well as some other biological functions including haemolytic activity, are attributed to characteristic structural features of saponins and their amphiphilic nature which results from the presence of a hydrophilic sugar moiety and a hydrophobic genin (called sapogenin). It seems that for all saponins both aglycone and sugar part play an important role for cytotoxic activity.

With respect to cytotoxic mechanisms of bothtiterpene and steroid saponins a wide variety of these was reported. Cytotoxic effect of most of the reviewed saponins was due to their ability to stimulate apoptotic process in tumor cells, usually through its intrinsic pathway. Moreover, non apoptotic processes were also involved in saponin cytotoxic
activity, such as cell cycle arrestment, autophagic cell death stimulation, inhibiting of metastasis and cytoskeleton disintegration.23,24

Result showed no cytotoxic activity on gingiva-derived mesenchymal stem cell after treated with all concentration of Sticophus hermanii extract (p>0.05). Regarding to its bioactive component, the saponin content probably have less role than in Holothuria atra extract, but prominently it has been known that Sticophus hermanii mostly contained polyunsaturated fatty acids (PUFA): arachidonic acid (AA C20:4 n-6), eicosapentaenoic acid (EPA C20:5 n-3), docosa hexaenoic acid (DHA C22:6 n-3). It has been stated that fatty acids including arachidonic acid (AA C20:4), eicosapentaenoic acid (EPA C20:5), and docosa hexaenoic acid (DHA C22:6) can play a potential role in tissue repair and wound healing. An appreciable amount of EPA in sea cucumbers might be linked well with the ability of these echinoderms to initiate tissue repair.4 It related to the traditional medicine which believed that direct use of sea cucumber can reduce wound recovery time and help new tissue formation and regeneration in human just as the sea cucumber’s ability to quickly regenerate its own body tissue when damaged.

The conclusion of this research is that Stichopus hermanii and Holothuria atra extract had the antifungal effect against Candida albicans on the concentration of 80 mg/mL. Sea cucumber extract were not cytotoxic to gingiva-derived mesenchymal stem cell in the concentration of Sticophus hermanii ≤1% and Holothuria atra ≤0.5%.

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REFERENCES


