

Potency of *Stichopus hermanii* extract as oral candidiasis treatment on epithelial rat tongue

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ABSTRACT

Background: Oral candidiasis is the most prominent oral fungal infection with *Candida albicans* (*C. albicans*) as 75% of etiologic factor. Golden sea cucumbers (*Stichopus hermanii*) have been consumed by Asian community as folk medicine. It has been known to have antifungal and immunomodulator agent thus potential to be explored as treatment in oral candidiasis. **Purpose:** The aim of this study was to examine the potency of *Stichopus hermanii* extract as oral candidiasis treatment. **Method:** The study was an experimental laboratories research with post test only control group design. Thirty male wistar rats were divided into 5 groups i.e negative control, positive control and 3 treatment groups. Oral candidiasis condition were induced by spraying *C. albicans* suspension on dorsal tongue of wistar rats, once in 2 days for 14 days. The treatment groups were given *Stichopus hermanii* extract on the dose of 4.25 ml/kgBW, 8.5 ml/kgBW, 17 ml/kgBW once daily for 14 days. The expression of anti *C. albicans* antibody and TNF- α were examined by immunohistochemistry on epithelial tongue. Data was analyzed by Manova and LSD test. **Result:** Anti *C. albicans* antibody expression were higher in positive control group than in negative control group while TNF- α expression were lower in positive control group than in negative control group ($p < 0.05$). Treatment with *Stichopus hermanii* extract on all doses decreased the expression of anti *C. albicans* antibody and increased the expression of TNF- α ($p < 0.05$). **Conclusion:** *Stichopus hermanii* extract decreased the expression of anti *C. albicans* antibody and increased the expression of TNF- α in epithelial rat tongue.

Keywords: *Stichopus hermanii* extract; oral candidiasis; rat; epithelial tongue

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INTRODUCTION

Oral candidiasis is the most prominent oral fungal infection caused by *Candida albicans* (*C. albicans*), the commensal microflora in human skin, vagina, and intestine. *C. albicans* also cause infections in condition with underlying diseases such as diabetes, prolonged broad spectrum antibiotic administration, steroidal chemotherapy and AIDS.¹ Recently, the incidence of any of candidosis type have been increased, it raised about 50% of oral candidosis cases.^{1,2} The impairment of the immune system expression is strongly correlated with the virulence of *C. albicans* in the oral cavity.³ The adherence property, colonization, enzyme production and interactions with host defences plays their role of the pathogenicity.³

The opportunistic fungus *C. albicans* is a major cause of oral and esophageal infections in immunocompromised patients such as human immunodeficiency virus (HIV)-infected individuals and the elderly. Other predisposing condition as hyposalivation, diabetes mellitus, prolonged use of antibiotics or immunosuppressive drugs, use of dentures, and poor oral hygiene played the role in oral *C. albicans* infection. It seems that oral candidiasis is not life threatening but still it caused significant morbidity and were increasing by the time. Some drugs such as azole antifungal agents has been used to treat this fungal infection. HIV-positive patients received highly active antiretroviral treatment showed significantly fewer episodes of oral candidiasis than those without highly active antiretroviral therapy. However, drug-resistance and side effects are

something to be consider regarding to long-term treatment with antifungal drugs.^{2,4}

Nystatin, ravuconazole, clotrimazole, fluconazole and ketoconazole are antifungal drugs choices commonly used in candidiasis treatment. In recent years, polyenes andazole agents have been used for treating infections caused by *C. albicans*.⁴ Empirically, some natural plant products have also been used, but the recent recurrent infections have revived interest in the products. It has been known that some plant essential oils are having some health benefits as antifungal, antibacterial, anti-inflammatory and antioxidative properties. Anyway, the scientific validation of their use as preventive and therapeutic products still need to be considered before the application in human health. In oral candidiasis, herbal formulations and phytotherapies play the major role.⁵

In the era of globalization and trading, it has been noticed that sea cucumbers such as *Stichopus hermanii* have increasing high commercial value. Sea cucumber have some medicinal benefits regarding to its bioactive compound as triterpene glycosides (saponins), chondroitin sulfates, glycosaminoglycan (GAGs), sulfated polysaccharides, sterols (glycosides and sulfates), phenolics, cerberosides, lectins, peptides, glycoprotein, glycosphingolipids and essential fatty acids. Sea cucumber components and bioactives posses the multiple biological and therapeutic properties of their potential uses for beneficial functional foods and nutraceuticals. Recent research stated that sea cucumber extract have its biomedical properties.⁶ It has been proved that the aqueous and organic extracts from some sea cucumber species have antioxidant activities,^{4,5,8} immunomodulator⁶⁻⁸ while it also been known to have antimicrobial properties on Gram negative, Gram positive bacteria^{6,9,10} and antifungal action.¹¹⁻¹³ The biocompatibility to oral cells have to be assured by identified the cytotoxicity. During the last years, the interest of in vitro systems as an alternative to animal experiments in toxicological research has been increased. The current models of predictive toxicology. have been increased by the using of stem cells and their derivatives as the developing in vitro, human cell assays.^{14,15} Considering to the bioactive compound, *Stichopus hermanii* extract is potentially explored its immunomodulator and antifungal property as the potential candidate therapeutic agent in oral candidiasis.

Sea cucumber extract has been consumed as tonic and traditional medicine, some has been produced as small industry herbal medicine products but the doses of treatment has not been established rather than just once to twice a day consumption. Prior to in vivo study to examine the proper doses of treatment in oral candidiasis., we have been studied the antifungal potency of *Stichopus hermanii* extract to *C. albicans* in vitro and its cytotoxicity to gingival derived-mesenchymal stem cell.¹⁶ Based on empirical regular consumption of sea cucumber extract and the conversion to animal model, three doses of *Stichopus hermanii* extract as therapeutic agent of oral candidiasis were explored in this research.

The aim of this study was to examine the potency of *Stichopus hermanii* extract as oral candidiasis treatment using immunohistochemical technique. 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 oral disease.

MATERIALS AND METHODS

The design of this research was post-test only control group design. The materials investigated in this study is the golden sea cucumber (*Stichopus hermanii*) ethanol extract which tested for its anti-inflammatory potency in vivo in Wistar rats induced by *C. albicans* for oral candidiasis condition.

Therapeutic efficacy studies were performed against *C. albicans*. The culture was stored at -20° C in Sabouraud dextrose broth containing 15% glycerol until use. For inoculation, *C. albicans* was grown on Sabouraud dextrose agar plates at 30°C for 24 h. Fungal colonies were then scraped off the agar, washed three times in phosphate buffered saline (PBS) pH 7 and solution was adjusted to appropriate concentration using heamocytometer.⁴

Stichopus hermanii weight of 100-250 grams were taken ± from Karimun Jawa coastal. Sea cucumbers were cleaned, cut into pieces with a size of 3-10 cm, weighed wet weight after it dried in the solar dryer rack for sample until it looks dry (3-4 days) to reduce the water content. Samples were dried sea cucumber, cut into pieces ± 1 cm, pulverized in a blender. The extraction process was done by the maceration process, by soaking 250 grams of dried sample in 500 mL of solvent refined methanol until all samples submerged and allowed to stand at room temperature for 24 hours. After filtered with a filter paper to separate the filtrate and residue, it was then soaked again with 500 mL of methanol solvent for 24 hours. After filtered with a filter paper to separate the filtrate and residue. Filtrate thus obtained results maceration with 250 gram sample comparison/1000 ml of solvent (1: 4 w/v). Filtrate methanol (polar) conducted homogenization with hexane solvent (non-polar) and 1,000 mL done with separatory funnel partition, then each layer of the filtrate solvent methanol and hexane solvent separated. Methanol filtrate done homogenization back with chloroform solvent (semi-polar) and 1,000 mL done with separatory funnel partition, then each layer of the filtrate solvent methanol and chloroform solvent separated. Each filtrate was then separated from the solvent using a rotary evaporator to obtain the extract.^{11,17}

The whole experimental plan was approved by Faculty of Dentistry, Universitas Hang Tuah ethical committee. Three months-old, male Wistar strain rats weighing approximately 175 g each were used. The rats were housed in 480 x 270 x 200 mm cages. Photoperiods were adjusted to 12/12 h light and darkness cycle daily, and the environmental temperature was constantly maintained

at $21 \pm 1^\circ \text{C}$. Access to food and water was allowed ad libitum to rats.

Sample were 30 male wistar rats, divided into 5 groups i.e negative control, positive control and 3 treatment groups. Oral candidiasis condition were induced in all groups except negative control group, performed by spraying *C. albicans*. Suspension to dorsal tongue of wistar rats, once in 2 days for 14 days. The treatment groups were treated by *Stichopus hermanii* extract using feeding tube, consecutively on the dose of 4.25 ml/kgBW, 8.5 ml/kgBW, 17 ml/kgBW. On the 14th day rats were terminated and the tongue were biopsied and fixed in 10% neutral-buffered formalin and embedded in paraffin. Tissue was sliced (6 to 10 mm) at the cryostat temperature of around -18 to -20°C . Sections were air dried at room temperature for at least 30 minutes, and then immersion-fixed in acetone for 1 to 2 minutes at room temperature. Subsequently, endogenous peroxidase activity was blocked for 10 minutes with 1% H₂O₂ diluted in PBS. Slides were then washed with PBS containing 1% bovine serum albumin (BSA) and background staining was blocked with powdered skim milk (3% in phosphate buffered saline). Sections were incubated overnight at 4°C with anti *C. albicans* antibody and anti-TNF- α (Santa Cruz Biotechnology, USA) and then were washed three times for 5 minutes each in PBS plus 1% BSA and incubated at room temperature for 45 minutes with biotinylated antibody. After being washed three times, in PBS plus 1% BSA, for 5 minutes each, sections were incubated with AB complex (Vector Laboratories, USA) for 45 minutes. Sections were washed again and the reaction was revealed by DAB (Sigma-Aldrich, USA) and finally counterstained with Mayer's hematoxylin. The control slide was not incubated with the primary antibody. Tissue sections from at least one mouse of each group were processed at the same time. Photomicrographs were taken with light microscope (Olympus America Inc., USA) equipped with an automatic camera system with magnification of 400x by two histopathologists, blinded to sample type.^{18,19} Data was analyzed with oneway Anova and LSD test. Data was analyzed by Anova and LSD test at 95% significance level.

RESULTS

The data on Figure 1 were presented as means + standard deviation. Statistical analysis was performed using Anova and LSD test to determine the effect of *Stichopus hermanii* extract as oral candidiasis treatment using immunohistochemical technique. Results of Anova and LSD test showed a significant difference between the control and treatment groups ($p < 0.05$).

The result on Figure 2 showed the highest anti *C. albicans* antibody expression in epithelial tongue of positive control group that inoculated with *C. albicans* compared to negative control group and treatment groups. The expression of anti *C. albicans* antibody were not found in negative control group which were not induced by *C. albicans*. The higher dose of *Stichopus hermanii* extract given, the lower anti *C. albicans* antibody expression resulted. Thus indicates that *Stichopus hermanii* extract could decrease anti *C. albicans* antibody expression with dose of 4.25 ml/kgBW, 8.5 ml/kgBW, 17 ml/kgBW ($p < 0.05$).

The result in Figure 3 showed the lowest TNF- α expression in epithelial tongue of positive control group that inoculated with *C. albicans* compared to negative control group and treatment groups. In treatment groups, the higher dose of *Stichopus hermanii* extract given, the lower TNF- α expression resulted. Thus indicates that *Stichopus hermanii* extract could decrease TNF- α expression with dose of 4.25 ml/kgBW, 8.5 ml/kgBW, 17 ml/kgBW ($p < 0.05$).

DISCUSSION

In vivo studies used Wistar rats that had been commonly used in oral candidiasis experimental because it has several advantages that are relatively easy to manage and have adequate oral cavity size for inoculation and sampling because of tongue is the most area predilection for candida infection.²⁰ Negative control group is a group of normal mice were not inoculated with *C. albicans*. The result showed that the expression of anti *C. albicans* antibody were not found in the negative control group, which is in

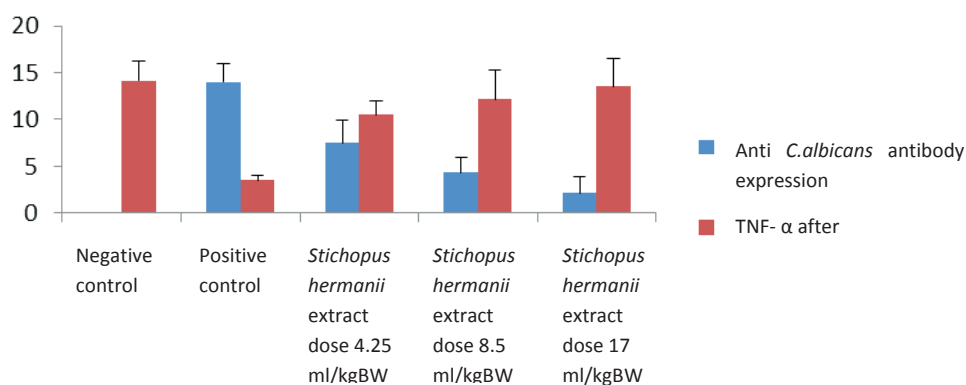


Figure 1. The expression of anti *C. albicans* antibody and TNF- α after addition of *Stichopus hermanii* extract.

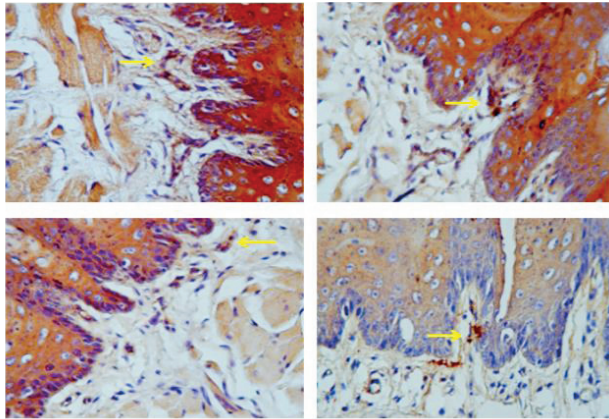


Figure 2. The expression of anti *C. albicans* antibody intongue epithelial. Expression of anti-*C. albicans* antibody in rat tongue epithelial with immunohistochemistry technique (400 magnification). Brown color shows presence of anti *C. albicans* antibody expression (→), A) expression of anti *C. albicans* antibody of positive control group; B) expression of anti *C. albicans* antibody of treatment group 1 (*Stichopus hermanii* 4.25 ml/kgBW); C) expression of anti *C. albicans* antibody of treatment group 2 (*Stichopus hermanii* 8.5 ml/kgBW); D) expression of anti *C. albicans* antibody of treatment group 3 (*Stichopus hermanii* 17 ml/kgBW).

line with the assumption that in the normal animals there is no process of candidiasis. *C. albicans* is not the normal flora of the oral cavity of mice.²¹

The positive control group and the treated group inoculated with *C. albicans* for 14 days. *Stichopus hermanii* extract were given orally with three concentrations, compared with positive and negative control group were given only 1% CMC solution. The procedure adopted in the present study exhibited a progressive increase in the number of colonies after inoculation, which demonstrated that infection was successful on the dorsal side of the tongue. After inoculation of *C. albicans* does not appear to be any significant differences in the clinical manifestations between the normal group and infected group. Inoculated with *C. albicans* for 14 days is not expected to provide significant clinical manifestations appear but the immunohistochemical examination is expected to give an idea about the condition of oral candidiasis by examining the expression of anti *C. albicans* antibody. Based on the presence of the expression of anti *C. albicans* antibody is assumed that there is the presence of *C. albicans* in a certain amount of tongue that indicates a condition of oral candidiasis.

Oropharyngeal candidiasis is the most common infection associated with oral injuries.²² *C. albicans* is part of the normal microbial flora of mucous surfaces, can be present as acquired defects of cell-mediated immunity. The adherence capacity, colonization, enzyme production and interactions with host defences determine its pathogenicity.²⁰ The impairment of the immune system is highly related with the expression of *C. albicans* virulence

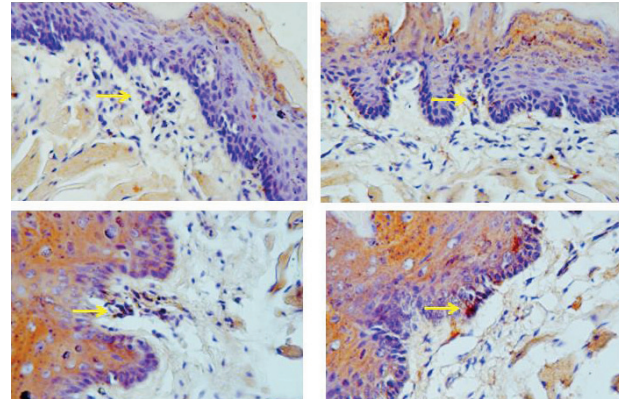


Figure 3. The expression of TNF- α intongue epithelial. Expression of TNF- α in rat tongue epithelial with immunohistochemistry technique (400 magnification). Brown color shows presence of TNF- α expression (→), A) expression of TNF- α of positive control group; B) expression of TNF- α of treatment group 1 (*Stichopus hermanii* 4.25 ml/kgBW); C) expression of TNF- α of treatment group 2 (*Stichopus hermanii* 8.5 ml/kgBW); D) expression of TNF- α of treatment group 3 (*Stichopus hermanii* 17 ml/kgBW).

in the oral cavity. The defense immune mechanisms to fungal infections are various, started from protective mechanisms that were in innate immunity to adaptive immunity mechanisms that are specifically induced during the infection. Skin and mucous membranes are the first-line innate defense as physical barriers, which is complemented by cell membranes, some cellular receptors and humoral factors.²³

Oral epithelial cells has its role on the process of inflammation as protective effort or destructive result, via amplifying signals in infection responses. It considered to play the important role in host defense, including antigen presentation. As response to infection, cytokines/chemokines were produced, one of those are TNF- α which capable to induce expression of other mediators such as prostaglandins. It could amplify the inflammatory response therefore leads lytic enzymes production and stimulates the production of chemokines. It has been studied that cytokines, such as TNF- α played important roles in the control of oral mucosal infection and can be lead to tissue destruction. Based on these findings, the enhancement of TNF- α expression by *C. albicans* infection appears to be involved in the initiation and/or amplification of inflammatory responses to oral candidiasis.^{23,24}

The antifungal drugs of choice to treat oral candidiasis commonly are nystain, ravuconazole, clotrimazole, fluconazole and ketoconazole. In recent years, polyenes and azole agents have been usedfor treating infections caused by *C. albicans*.^{25,26} The existing conventional drugs, however pose several undesirable side effects. A continuous need for new drugs, especially the biocompatible and bio-based drugs is expected to overcome the side effect and resistantance. Natural plant products have also been used

as folk medicine, but the recent recurrent infections have revived interest in the products. In oral candidiasis, herbal formulations and phytotherapies play a major role.²⁷

Sea cucumbers, especially *Stichopus hermanii* have been known to have various health benefits, such as antifungal, antibacterial, anti-inflammatory and antioxidative properties. The lack of scientific validation of their use as preventive and therapeutic products restricts their application in human health. *Stichopus hermanii* extract is known to have anti-fungal properties which contained saponins, alkaloids, and triterpenes that to act as antifungal agent. In earlier laboratory studies using ethanol extract of *Stichopus hermanii*, it was found that the active fraction of the ethanolic extracts were able to control the biofilm formation of *Candida spp.* and more over the extracts were able to kill *C. albicans*. Biofilm formation is one of the pivotal factors in establishing the infections in the host.^{7,11,17}

This study is a continuation of previous studies which have known of the power of its anti fungal agent against *C. albicans in vitro*.²⁸ In the present study we carried out animal experiments to assess the effect of *Stichopus hermanii* extract as a marine medicinal to treat oral candidiasis. In this study *Stichopus hermanii* extract contains triterpene and saponins as an active anti-fungal agent with ethanol extraction method according to research Pranoto *et al.*¹¹ This antifungal compounds soluble in ethanol as a polar solvent that has antifungal and antibacterial activity.⁸ Triterpenes, which comprise a broad chemical group of active principles, are implicated in the mechanisms of action and pharmacological effects of many medicinal plants used in folk medicine against diseases in which the immune system is implicated.²⁹

The results showed that administration of *Stichopus hermanii* extract in all treatment groups decrease anti *C. albicans* antibody expression and increase TNF- α expression. It is estimated that there are other possible mechanisms of saponins in the potential anti-fungal and immunomodulatory agent. *Stichopus hermanii* extract are one of the potential marine animals with multiple biological activities and medicinal value. Therefore, marine echinoderms can be explored as a sustainable natural source for the discovery of novel antifungal agent. Bioactive substances in sea cucumbers, such as triterpene glycosides, enzymes, amyloses, fatty acids, cytotoxins, etc. with potential capabilities to antifungal activities and increase immunity as well as contribute to immunopotentiality. In this study, ethanol extract of *Stichopus hermanii* showed promising antifungal and immunostimulatory activity *in vivo*. It was shown that *Stichopus hermanii* extracts were effective against *C. albicans* at a concentration of 4,25 mg/kg BW until 17 mg/kg BW. The inhibition of fungal growth started from the lowest to the highest tested extract concentrations showed that the amount of extract present related with its antifungal activity. Saponins produced as a form of chemical defense mechanism for sea cucumbers in nature. In addition to alleged as a defense from predators,

also believed to have biological effects, including anti fungal and immune activity.⁶ Saponin is a class of compounds that inhibit or kill microbes by interacting with membrane sterols, contribute as antifungal with membrane sterols that can decrease surface tension of the cell wall of *C. albicans*, so the permeability was increased,²⁹ similar to the mechanism nystatin action. Saponins may also result in apoptosis because it can damage the mitochondrial membrane cell, lowering the transmembrane potential, increase cytosolic calcium and activates apoptosis pathways through calcium.^{30,31}

Its capabilities that triggers macrophages in response to an infection in which this mechanism as antifungal. Prior research showed that sea cucumber protein contained rich of glycine and arginine, especially produced from its body wall. Glycine contributes to enhancing phagocytosis by stimulating production and release of IL-2 and B cell antibody, while arginine can enhance cell immunity by promoting activation and proliferation of T-cell. Sea cucumbers have remarkable function in immune regulation due to these amino acid components.⁶

This research showed that the expression of TNF- α were low in the control positive group, suggesting the reduction of immune activity happened in infection of *C. albicans*. In immuneresponse to *C. albicans*, TNF- α played the role as primary immunity in immune system regulation. Specifically to macrophage, this cytokine increased the activity in killing pathogens, in which this action became an important mediator in inflammation.³² Treatment with *Stichopus hermanii* extract could increase TNF- α activity. Its related to macrophage phagocytosis activity to *C. albicans*. Function of phagocytosis and TNF- α is playing important role at macrophage level which also influence the degradation of adaptive immune response on *C. albicans* on the other side with existence of ethanol extract from *Stichopus hermanii* condition of immunosuppression resulted from *C. albicans* will be improve and repaired. Macrophage as professional phagocyte function to break immunogen as antigen presenting cells (APC) which recognizes microbe through some receptors its to stimulate migration of cell to the place of infection and stimulates the production of substance.

Improvement mechanism of TNF- α by *Stichopus hermanii* to *C. albicans* started by the existence Toll-like receptor (TLR)-2 and TLR-4. It has been highlighted that TLR-2 and TLR-4 are involved in recognition of *Candida* and *Aspergillus*. Fungal wall components zymosan, phospholipomannan and glucuronoxylomannan have been identified as ligands or pathogen associated molecular patterns (PAMPs) for TLR-2, while glucuronoxylomannan and O-linked mannan are ligands for TLR-4.³² Recognition of microorganism by TLR-2 and TLR-4 then activated nuclear factor kappa B (NF- κ B) and activator protein-1 (AP-1) through jun kinase via the mitogen-activated protein kinase (MAPK) pathway. After the release of I- κ B, an increase would occur in the activity of transcription factor NF- κ B which stimulated gene expression that affected the

production of TNF- α and phagocytic activity. Stimulation of gene expression among others affected the production of TNF- α .^{32,33}

It also been stated that *C. albicans* can evade the host defense through TLR2- derived signals. The TLR2-deleted macrophages have been found to have enhanced anti-candidal capabilities, while in-vivo study stated that TLR-2 knock out mice are relatively more resistant to disseminated *C. albicans* infection. Hence the tissue-invasive hyphal forms of *C. albicans*, by means of evading TLR4 in favor of a predominant TLR-2 activation, are able to tilt the balance towards a Th2 response³³ resulted in changes in inflammatory response, assumed to be related with TNF- α production and activation. The capability of *Stichopus hermanii* extract in increasing the expression of TNF- α facilitate the proper immune response in combating *C. albicans* infection.

The decreasing of anti- *C. albicans* antibody expression showed the antifungal property of *Stichopus hermanii* extract and with addition of its property in enhancing the immune response by increasing TNF- α expression will be the advantage of the potential agent for oral candidiasis treatment. It is concluded that ethanol extract from *Stichopus hermanii* has the potency as oral candidiasis treatment by decreasing the expression of anti *C. albicans* antibody and increasing the expression of TNF- α significantly in epithelial tongue of rats inoculated with *C. albicans*.

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