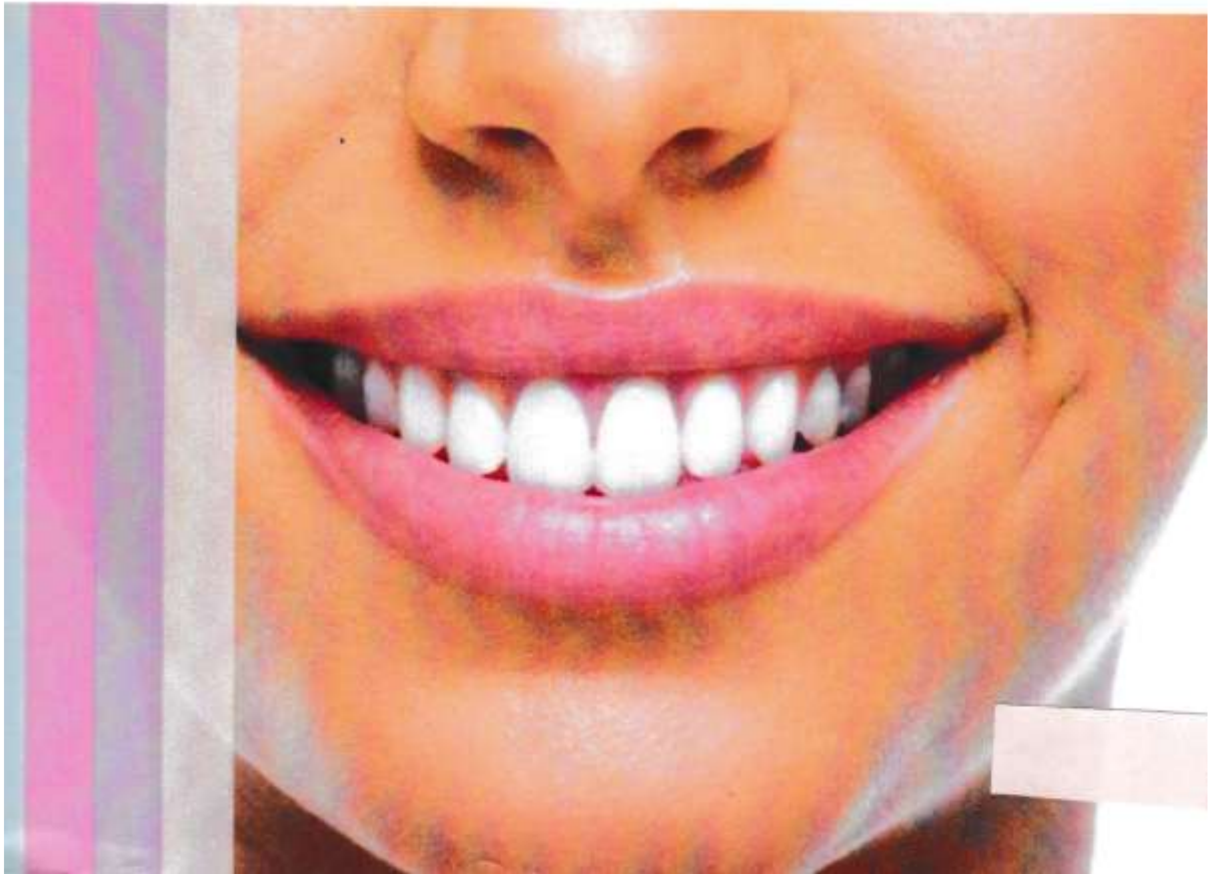
Proceeding



PROCEEDING
PDGI-FDI CONTINUING EDUCATION 2017

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Evaluation Of Antifungal Activity Of Stichopus Hermaniiethanol Extract As Oral Candidiasis Treatment

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ABSTRACT

INTRODUCTION : The presence of Candida species as a human commensal, alarming rates of oral infections have been observed, including oral candidiasis. Currently available antifungal drugs have progressively lost their effectiveness. Stichopus hermanii has been used traditionally as food preservatives and disease remedie, it"s antifungal potency need to be explored. **Objective**: to evaluate the antifungal activity of Stichopus hermanii ethanol extract against oral candidiasis. **Material and Method :** Twenty four wistar rats were randomly divided into four groups, i.e. 1st group (normal rats as control group), 2nd group (oral candida infection group), 3rd group (treatment group received nystatin), 4th group (treatment group received Stichopus hermanii ethanol extract 0.09 mg/gBW). The antifungal activities of Stichopus hermanii ethanol extract were examined by estimating the fungal burden in rat dorsal tongue. The in vitro antifungal activities were assessed by tongue swab sample examined by broth micro-dilution method. The in vivo activity was evaluated by histology assay of tongue biopsy. **Result** : Microbiologically, Stichopus hermanii ethanol extract reduced the number of colony forming units (CFU) sampled from treated rat's oral cavity similar to nystatin, compared to untreated control rats ($p<0.05$). Histologically, Stichopus hermanii ethanol extract and nystatin reduced the biofilm formation on the dorsal tongue epithelium from the oral cavity of rats treated, compared to untreated control rats ($p<0.05$). The histological data were confirmed by the microbiological result, pointing that Stichopus hermanii extract could be considered as antifungal agents. **Conclusion :** Stichopus hermanii extract could be considered as antifungal agents for oral candidiasis.

Keywords: *Stichopus hermanii, experimental oral candidiasis, antifungal activity*

INTRODUCTION

Oral candidiasis has emerged and are being increasingly recognized as important public health problems expanding population of immuno-compromised patients[1]. Oral candidiasis are usually associated with Candida species represent the main opportunistic fungal infections, leading to high morbidity and mortality in the population[2,3]. The antifungal agents are numerous, but antifungal agents are currently due to the high toxicity [4,5]. This is indicating that current antifungal therapy to treat oral candidiasis is still ineffective. Therefore, the discovery of new antifungal agents is still needed. Among the potential sources of new agents, marine biotas have long been investigated because they contain many bioactive compounds that can be used in therapy, because of their low toxicity [6,7]. Sea cucumbers, also known as Stichopus hermanii, are marine biotas, is widely used orally and topically to treat various desease such as wound healing, antibacteria, antifungal, antitumour, antianaphylactic, anti-inflammatory, antinociceptive and antioxidant agent[8]. Thus, the objectives of this study were to evaluate the antifungal activity of Stichopus hermanii ethanol extract against oral candidiasis.

METHOD

Strains and media

The microorganisms used in this study were *Candida albicans* ATCC (American Type Culture Collection) 9002. Sabouraud dextrose agar (SDA) was used for the maintenance and culture of fungal strains while Sabouraud dextrose broth (SDB) was used for the determination of the minimum inhibitory concentrations (MICs). A single colony from SDA was grown in yeast extract-peptone glucose medium (YPG : yeast extract, 2%; bacto peptone, 1%; glucose, 2%) for 18 hours at 30°C in a shaker. The culture was harvested by centrifugation at 2500 g, and then cells were washed three times in Phosphate Buffer Saline (PBS) and adjusted to $3x10^8$ CFU/mL (using a hemocytometer chamber for counting cells). The viability of the inoculum was confirmed by quantitative cultures of serial 10 fold dilutions on SDA plates. The number of viable cells was determined using the drop count method [9].

Antifungal agents

Sticophus hermanii was collected from Karimun Jawa coastal region. Adult sea cucumber was selected to get the best extract result considering to its maximum secondary metabolit contents. The collected samples were cleaned, immersed in water for one night to get rid of salt and parasite then dried in dryer machine. Sticophus hermanii then splitted, the inner abdomen were removed then cleaned and washed, so only the flesh of the body proceed to next process. Each sample 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). Ethanol (polar) filtrate got homogenized with 1000 mL hexane solvent (non polar) then performed partition with separatory funnel the each of the filtrate layer of ethanol and hexane solvent were separated. Ethanol (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[10]. Nystatin was purchased from Bristol-Myers Squibb.

Animals

The animals used for the in vivo experiments were 150-170 g male Wistar rats. Infected rats distributed into four groups of 6 animals each were housed in cages and had access to food and water *ad libitum*. Rats were randomly divided into four groups, i.e. 1st group (normal rats as control group), 2nd group (oral candida infection group), 3rd group (treatment group received nystatin), 4th group (treatment group received Stichopus hermanii ethanol extract 0.09 mg/gBW). All animal experiments were maintained in laboratory animals and were approved by the animal ethical committee of the Hang Tuah University.

Induction of oral infection with Candida albicans in rat

Our experiments were repeated three times, to enhance the infection rate; rats were immunosuppressed with dexamethasone and treated with tetracycline. One week before infection, rats received drinking water with 0.5 mg/L of dexamethasone with tetracycline (0.1%). On the day of infection, dexamethasone was raised to 1 mg/L, while tetracycline was reduced to 0.01% and maintained throughout the experiment. The rats were orally infected three times at 48 h intervals (days-7, -5 and -3) with 0.1mL of saline suspension containing 3.10^8 viable cells of Candida albicans. Oral infection was achieved by means of a cotton swab rolled twice over dorsal tongue. Just before inoculation, the animals were sampled to confirm the absence of Candida albicans in the oral cavity, and 72 h after the last inoculation all groups were sampled in the same manner to check for the presence of the fungi and to quantify the number of CFU in the oral cavity before the beginning of the treatment. Before the start of the treatment, all groups of infected animals were sampled. The oral swabs were all positive for the presence of Candida albicans[11,12].

Antifungal treatment

Stichopus hermanii ethanol extract at the doses of 0.09 mg/gBW were administered orally over 7 days, starting 24 h after infection. Two control groups were used; untreated control received distilled water and a positive control group treated with reference antifungal drug nystatin at 10 mg/kgBW.

Quantification *of viable yeasts in vitro and in vivo*

The quantification of the infection was assessed by microbiological (in vitro) and histopathological techniques (in vivo).

In vitro

Seven days after the treatment, oral samples were collected and cultured to quantify the CFU in the dorsal tongue of the animals of each group. Fungal were collected by rolling a sterile cotton swab over the dorsal tongue, which was then suspended in 1 mL of sterile saline. 25µl samples from this suspension were dropped in duplicate, after serial ten fold dilution on SDA plates containing 0.05% chloramphenicol. All plates were incubated at 30°C for 24 h, and the colonies were counted. The number of viable cells was determined using the drop count method to calculate the log of the CFU/mL[13]. At day 8, i.e. 24 h after the administration of the last dose of antifungal agent or saline, All animals in each group were sacrificed by cervical dislocation and tonge were collected from each rat. The tongues were removed, fixed *in toto* by immersion in Bouin solution for at least 48h. Tongue sections were embedded in paraffin and 5µm thick serial transverse sections were stained with both hematoxylin-eosin stain and Periodic Acid Schiff (PAS), to assess the fungal infection by the thickness of fungal biofilm[11,12].

Statistical analysis

One way analysis of variance was used to analyze the fungal load in all animals between each treatment group and the control group. When there were differences between groups, the means were compared using the Least Significant Difference test at a 5%. Results are expressed as mean ± standard deviation. All data were analyzed using SPSS Statistics 17.0.2.

RESULT

Stichopus hermanii ethanol extract have been used traditionally for generations by humans as food and to treat illness. Many studies have confirmed their role in health maintenance and promotion, but the major challenge is to provide scientific evidence. Therefore, the *in vitro* and *in vivo* antifungal potentials of Stichopus hermanii ethanol extract were investigated. The results of *in vitro* antifungal assay are presented in Table 1 and 2.

Table 1. Antifungal activity of Stichopus hermanii ethanol extract against Candida albicans in vitro

Group	Zone of Inhibition (mm)		
	Mean	Standart Deviation	*ANOVA test*
Control group	0.00	0.0000	
Infection group	6.00	0.0050	
Nystatin treatment group	2.00	0.0012	
Stichopus hermanii treatment group	3.00	0.0015	p = 0.000*

The results indicated that the extracts can inhibit fungal growth but the greatest inhibition zone found in Nystatin treatment group.

Table 2. Antifungal activity of Stichopus hermanii ethanol extract against Candida albicans in vitro

Group	Biofilm thickness (µm)		
	Mean	Standart Deviation	*ANOVA test*
Control group	0.00	0.00	
Infection group	146.50	20.05	
Nystatin treatment group	12.68	0.12	
Stichopus hermanii treatment group	30.10	0.15	p = 0.000*

Based on the thickness of the biofilm formed by Candida albicans, the data showed that a decline in the thickness of the biofilm in the group treated with nystatin and ethanol extract Stichopus hermanii, but the thickness of the biofilm group treated with ethanol extract Stichopus hermanii still larger than the group treated with nystatin.

DISCUSSION

Oral candidiasis is a common infection both in oral and perioral which usually result from excessive development of endogenous Candida albicans. Oral candidiasis may not appear immediately because the Candida albicans is less pathogenic fungi that are predisposing factors that lead to oral candidiasis. Clinical experience indicates that an increase in the biofilm layer related to the severity of an infection disease[14].

Therefore, this study aimed to analyze the effectiveness of the golden sea cucumber (Stichopus Hermanni) in preventing the formation of biofilm on Wistar rats with oral candidiasis. Observation of biofilm formation in Wistar rats that experienced oral candidiasis can be indicated on the tongue. This is because the tongue is one organ in the oral cavity is the most sensitive to changes that occur in the body[15]. The result of this study proves that the ethanol extract of Stichopus hermanii 0.09mg / gBW can prevent biofilm formation of Candida albicans significantly. This proves that Stichopus hermanii has potential as an antifungal. Flavonoids and tannins belonged to phenolic compounds. Phenolic compounds interact with the cell membrane proteins that cause precipitation and terdenaturasinya cell membrane proteins[16]. Damage to the cell membrane causes changes in membrane permeability, resulting in fungal cell membrane lysis[17]. Phenol can damage proteins in the cell membrane so that the membrane of Candida albicans cells into lysis, phenol is able to penetrate the cell nucleus and the fungus Candida albicans fungus can not multiply. Tannins are compounds that are lipophilic so easily bound to the cell wall and cause damage to the cell wall. In addition, the tannins can inhibit the synthesis of chitin which is an essential component of fungal cell walls[18].

Saponins are surfactants which form polar so that it breaks down the fat cell membranes, which in turn cause the permeability of the cell membrane, it resulted in the diffusion process materials or substances needed by the fungus can be disrupted, eventually the cells swell and rupture[19]. Terpenoids, including triterpenoids and steroids are bioactive compounds that function as an antifungal. The compound can inhibit fungal growth, either through the cytoplasmic membrane and interferes with the growth and development of fungal spores[20].

In general, sea cucumbers have compounds that can serve as an antifungal. Based on

prior research it demonstrated that the compound triterpene glycosides in n-butanol fraction of trepang (Actinopyga lecanora) showed antifungal activity against 20 fungal isolates tested[21]. The crude extract and the purified fraction isolated from Holothuria Polii (Mediterranean Sea cucumbers) showed antifungal activity against Aspergillus fumigatus and Trichophyton rubrum[22]. In addition, it was reported that the sea cucumber patagonicoside type A has a good antifungal activity against pathogenic fungi (Cladosporium cucumerinum)[23]. Compounds triterpene glycosides from sea cucumber (Psolus patagonicus) also have potential as a potent antifungal antifungal compared to synthetic effective antifungal product[24]. Based on the potential antifungal as expressed by some research on it is interesting to explore the sea cucumber as a natural source of new antifungal agents to be developed as drugs against infectious diseases.

CONCLUSION

Stichopus hermanii extract could be considered as antifungal agents for oral candidiasis.

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