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Protective role of *Sticophus hermanii* ethanol extract supplementation to oxidative stress and oral hyperkeratosis in smoking exposed rats

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Abstract : Objective : Cigarette smoking (CS) is known as one of the most important risk factors for oral leukoplakia. CS contains various oxygen-free radicals which are considered as the main causes of oxidative stress and oral hyperkeratosis. *Stichopus hermanii* (SH) ethanol extract contains antioxidant and antiproliferative agents. The aim of this study was to examine the preventive effect of SH ethanol extract on cigarette smoke-induced oxidative stress and oral hyperkeratosis in wistar rat animal model.

Materials and Methods: Thirty wistar rats were randomly divided into five groups, i.e. sham air (SA) as normal control group, cigarette smoke exposed group (CS) , and three treatment groups each received supplementation with SH 4.25; 8.5; and 17 ml/kgBW.

The exposure to smoking was carried out in a smoking machine, given as a single daily dose for 90 days. SA were exposed to air instead of cigarette smoke. Saliva were collected for lipid peroxidation and catalase activity measurement, while tongue were biopsied for histology assay. Data were anaylized by Anova and LSD.

Result : The mean value of lipid peroxidation were found to be increased and catalase activity were decreased in CS group when compared to the SA group ($p<0.05$). Elevated thickness of epithelial corneum layers were observed in CS group when compared to the SA group. The administration of SH inhibit the increase of lipid peroxidation, the decrease of catalase activity, and the elevation of corneum layer thickness ($p<0.05$).

Conclusion: Supplementation with SH prevented oxidative stress and oral hyperkeratosis in cigarette smoke-induced wistar rat.

Keywords: Stichopus hermanii, saliva, MDA, catalase activity, oral hyperkeratosis.

Introduction

Cigarette smoking is the most widely risk factor in oral leukoplakia[1]. Approximately 80% of oral leukoplakia patients are smokers, when large groups of adults are examined, the smokers are much more likely to have oral leukoplakia (23%) than non-smokers (4%). Oral leukoplakia in smokers is usually seen as a well-defined white plaque (hyperkeratosis) of the oral mucosa. Pipe smokers and heavy cigarette smokers have greater numbers of lesions and larger lesions than other smokers, especially after many years of cigarette or tobacco abuse. Furthermore, 60% of smoke induced oral leukoplakia that dissapear 6-12 months after affected patients stop smoking. Oral leukoplakia has potency transformed into oral cancer. This lesion is most certainly

produced by heavy and frequent contact with smoke. We can find this condition in heavy cigarette smokers (which usually have more than two packs of cigarette per day)[2].

Cigarette smoke contains more than 4,000 identified chemical compounds including 60 known carcinogens[3]. The gaseous components of mainstream smoke (92% of the total smoke) contain from 400 to 500 different gases. These gases include carbon- monoxide, nitrogen oxide, hydrogen cyanide, ozone and formaldehyde. Particulate matter (8% of main stream smoke) contains tar product such as naphthalene, pyrene and nitrosamine[4]. Also particulate matter contains metals such as polonium, cadmium, selenium, mercury, lead and arsenic[5,6].

Cigarette smoke encompasses and creates various reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as superoxide radical, hydrogen peroxide, hydroxyl radical, and peroxynitrite[7]. Highly reactive radicals can damage the cell membrane and also induce DNA fragmentation, tissues damage and alter the cellular antioxidant defense system. Antioxidants, glutathione peroxidase, superoxide dismutase and catalase, can work to dispose free radicals[8]. Peroxidation of membrane lipids generates well characterized, mutagenic lipid peroxidation end products, such as lipid hydroperoxides (LHP) and malondialdehyde (MDA). MDA is a major genotoxic carbonyl compound generated by lipid peroxidation and during arachidonic acid metabolism for the synthesis of prostaglandins. Hence, MDA levels are used to indicate oxidative and cellular damage to tissues due to ROS and free radicals[9].

It has been suggested that free radicals, ROS and RNS in the inhaled CS induce this gradually evolving process, initially expressed by hyperkeratosis, hyperplastic and dysplastic lesions of the mucosa, are then transformed into in situ carcinoma lesions and eventually result in full-blown infiltrating and metastasizing oral squamous cell carcinoma (SCC). Role of free radicals in the pathogenesis of oral SCC is found in a recent study demonstrating that ROS, such as hydroxyl radical, are formed in the human oral cavity during areca quid chewing, and that the activity might cause oxidative DNA damage to the surrounding tissues. In this respect the salivary anticarcinogenic capacity, which has only recently been recognized, may be based on its antioxidant system[10].

However, antioxidant supplement commonly used to prevent oxidative stress. Natural plant products have also been used since ancient times. The lack of scientific validation of their use as preventive and therapeutic products restricts their application in human health. Sea cucumbers such as SH have high commercial value with increasing global production and trade. Therapeutic properties and medicinal benefits of sea cucumbers can be linked to the presence of a wide array of bioactives. Sea cucumbers contain nutrients such as Vitamin A, Vitamin B1 (thiamine), Vitamin B2 (riboflavin), Vitamin B3 (niacin), and minerals, especially calcium, magnesium, iron and zinc. A number of unique biological and pharmacological activities including anti-angiogenic, anticancer, anticoagulant, anti-hypertension, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, antitumor and wound healing have been ascribed to various species of sea cucumbers. 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. The high-value components and bioactives as well as the multiple biological and therapeutic properties of sea cucumbers with regard to exploring their potential uses for functional foods and nutraceuticals. Recent research stated that sea cucumber extract have its biomedical properties. Regarding to its contents, sea cucumber species has been proved to have antioxidant and antiproliferative activities[11]. Considering to the bioactive compound, Sticophus hermanii extract is potentially explored its antioxidant and antiproliferatif property as the potential candidate therapeutic agent.

Materials and methods

Experimental design

The design of this research was post-test only control group design. The materials investigated in this study was SH ethanol extract which were tested for its prevention of oxidative stress and oral hyperkeratosis in Wistar rats induced by chronic cigarette smoke exposure. Male wistar rats (150-200 g, age 12 wk) were procured and housed in animal research centre of the Laboratory of Biochemistry, Faculty of Medicine, Airlangga University, Surabaya, Indonesia. The rats were housed in 480x270x200 mm cages. Light and the

environmental temperature was constantly maintained daily. The rats were fed standard chow diet and water ad libitum. The study was conducted in accordance to Institution guidelines for animal research and Ethical Clearence Committe of Hang Tuah University.

Thirty wistar rats were randomly divided into five groups, i.e. sham air (SA) a normal control group, cigarette smoke (CS) exposed group, and three treatment groups each received supplementation with SH 4.25; 8.5; and 17 ml/kgBW, respectively by gastric intubations every day during 12 weeks together with exposed cigarette. Wistar rats were obtained at 3 month of age (150–200 g) and rested for at least 1 week prior to their first smoke exposure. They were housed in group cages with unlimited access to rat chow and tap water. All rats were weighed weekly to monitor growth rates.

Preparation of SH ethanol extract

SH sea cucumber in the 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 was dried in the solar dryer rack for sample until it looks dry (3-4 days) to reduce the water content. Dried sample of sea cucumber were then cut into pieces ± 1 cm, and 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 it was filtered with a filter paper to separate the filtrate and residue, it was then soaked again with 500 mL of ethanol solvent for 24 hours. After it was 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 ethanol (polar) conducted homogenization with hexane solvent (non-polar) and 1,000 mL done with Separatory funel partition, then each layer of the filtrate solvent ethanol and hexane solvent separated. Each filtrate was then separated from the solvent using a rotary evaporator to obtain the extract. The evaporated extract then placed in the vial and stored in -30^{0}C until the next analysis[12].

Chronic Cigarette Smoke Exposure

An electronically controlled smoking machine was used to expose the animals to cigarette smoke. The smoking machine is composed of an electronic valve, a vacuum pump and a timer to control the sequence of puffs - and fresh air- inlet and exit in the inhalation chamber allowing enough intake of tobacco smoke and preventing oxygen deprivation in chamber. Rats were passively exposed to cigarette smoke once daily in electronic whole-body chambers. Each exposure chambers was large enough to accommodate 10 cages, allowing simultaneous exposure of up to 10 rats. Smoke for each exposure was generated over $\pm$ 1 hour by 30 cigarettes smoked in 6 cycles of 5 cigarettes each. Each cycle of the smoking regimen lasts for 90 sec and consists of three successive steps, operating as follows: cigarette smoke is drawn through the inhalation chamber continuously for 30 sec. An inlet to fresh air is then opened, allowing fresh air to be introduced instead of smoke, which will be washed out of the chamber. The washing out process will also take 30 sec. In the last 30 sec, the vacuum pump will be turned off, and rats will be allowed to breathe fresh air normally. The machine was automated to load, light, and puff each cigarette before ejecting them. Both mainstream and side-stream smoke were collected, mixed with air in a conditioning chamber, and then pumped into the exposure chambers. SA animals were placed in similar chambers for equivalent lengths of time, but exposed to room air only. To control for potential differences in smoke exposure among cage in chambers, each rats was rotated through the 10 possible cage locations in chamber upon successive exposures. The exposure to smoking was carried out as a single daily dose (3 cigarette/rat) for a period of 90 days[13,14].

Food Consumption

Rat chow consumption was measured daily during 12 weeks SA and CS group. Because the rats were housed in cage, the total amount of chow eaten per cage was divided in

half to calculate mean consumption per rat. To account for the obvious differences in body weights of SA vs CS animals, relative food consumption also was calculated by dividing the total weight of the animals in each cage into the weight of food eaten and expressing it as g of food consumed per 100 g of rat weight[15].

Sticophus hermanii (SH) supplementation

The extract was placed in suspension at 2% Na-CMC to administer the SH ethanol extract. An oral dose of SH ethanol extract (administered by gavage) was given to animals in treated groups 1 (4,25 mg/kgBW), group 2 (8,5 mg/kgBW), and group 3 (17 mg/kgBW). Distilled water was given to animals in SA and CS groups. These extraxt were administered orally every other day before CS exposure for 12 weeks experiment.

Saliva Collection

At the end of the experimental duration, rats were fasted overnight and anaesthetised by intramuscular injection of with a combination of ketamine chloride (6 mg/kg BW) and xylazine (0.6 mg/kg BW) then euthanized by cervical decapitation[16]. The secretion of saliva was stimulated with a subcutaneous injection of pilocarpine 2.5 mg/kg (i.m.). During saliva collection, the rats were placed in a restrained position. Their heads of the animal were held down on a table inclined at the angle of 10º and positioned over plastic vessels in a way that prevented contamination by nasal secretions. The drooling saliva was collected for 10 min with micropipette than replace into sterile microtube[17]. Saliva were collected for measurement lipid peroxidation and antioxidant enzymes activity.

Assay MDA Level

The level of oxidative stress was calculated by measurement of salivary MDA level using spectrophotometer by measuring thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation[18]. To 1 mL of sample, 1.5 mL of 0.8% thiobarbituric acid (TBA) was added. Then, 1.5 mL of acetic acid and 0.4 mL of 8.1% sodium dodecyl sulfate were added. Distilled water was added to make the mixture up to 5 mL, and it was then placed in a hot water bath at 95°C for 1 h. The mixture was allowed to cool, and 5 mL of pyridine and n-butanol (15:1, v/v) along with 1.0 mL of distilled water were added. The mixture was vortexed and centrifuged at 4,000 rpm for 10 min. With a spectrophotometer, absorbance of the upper layer was measured at 532 nm against distilled water. When allowed to react with TBA, MDA formed a colored complex that was measured using the spectrophotometer[19].

Assay Catalase Activity

The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at χ 240 nm. One catalase unit is defined as the enzyme concentration required for the decomposition of 1 µmol of H_2O_2 per min at 25^{o}C, as described by Beers and Sizer (1952). The complete reaction system for catalase consisted of 0.1 mM phosphate buffer, pH 7.4 and 10 mM H2O2. The reaction was initiated by the addition of 10 mM H_2O_2 and absorbance was monitored for 2 min at 240 nm with spectrophotometer[20] (Merck KGaA, Darmstadt, Germany).

Histological Slide Preparation

At the end of the experimental period, the rats were killed by decapitation. Tongue tissues were dissected out surgically, rinsed for cleared off blood with physiological saline. For the microscopic analysis, the tongue tissue was fixed in 10% formaldehyde for 24 hours and then the tissues were embedded in paraffin. The embedded tissues were sectioned using microtome. The pieces were sectioned in the sagittal form, divided into four parts and included in paraffin. From each animal, sixteen 7µm cuts of the tongue were obtained and stained with hematoxylin and eosin (H&E) for thickness of corneum layer measurement. Slides were observed and analyzed macroscopically, using a light microscope (Olympus, Shinjuku-ku, Tokyo, Japan) with magnifications of 400x[21].

Histopathological examination

Tongue epithelial lesions of hyperkeratosis or hyperplasia in the oral cavity were diagnosed according to the criteria described by Cawson and Lehner (1968)[22]. The technically best section from each specimen was used to measure corneum layer thickness. Three independent measurements were performed. Corneum layer was measured using a calibrated ocular micrometer and all measurements were adjusted for magnification optics. Total epidermal thickness was calculated by adding corresponding values of corneum layer. One trained pathologist performed all measurements. Thirty randomly selected biopsies were evaluated twice. Two-way

analysis of variance showed no significant difference between the two measurements. Thickness for a given subject was reported as the mean thickness value across observers[23].

Statistical Methods

All data were tested for normality and equal variance before statistical tests were performed. Differences between smoke- and sham-exposed animals were determined by Students t test or the Mann-Whitney Rank Sum test if the data were non-parametric. Differences among the supplementary groups within the smoke- or sham- exposure groups were determined for parametric data by One Way ANOVA with LSD post-hoc testing for pair-wise comparisons or the Holm-Sidak pairwise multiple comparision when a chow-fed control group was included. For non- parametric data, the Kruskal-Wallis ANOVA on Ranks was performed, with post-hoc testing by Dunn's method. All statistical comparisons were made using SPSS, and a p value of <0.05 was considered significant.

Results and Discussion

Lipid Peroxidation

Oxidative stress of chronic cigarette smoke in this study was expressed by lipid peroxidation. The measurement of TBARS is commonly used to monitor lipid peroxidation. MDA is a marker of lipid peroxidation, was used in this study to monitor the degree of modification occurred for lipid in response to chronic cigarette smoke and the protective role of SH ethanol extract.

The result of this study showed salivary MDA level of wistar rat inhaled chronic cigarette smoke once daily for 12 weeks (CS group), showed a highly significant ($p < 0.05$) increase compared to SA group. On the other hand, supplementary SH ethanol extract with chronic cigarette smoke inhalation resulted inhibit the increase of salivary MDA level significantly ($p < 0,05$) compared to CS group (Table 1 and Figure 1).

Table 1. Mean value of salivary MDA level (nmol/mL) on the protective role of SH ethanol extract in wistar rat inhaled chronic cigarette smoke.

Animal Group	Mean	Standart Deviation
SA group	22.86	5.90
CS group	63.28	15.26
CS-SH ethanol extract 4,25 mg/kgBW group	45.81	11.09
CS-SH ethanol extract 8,5 mg/kgBW group	42.04	10.63
CS-SH ethanol extract 17 mg/kgBW group	37.46	8.95

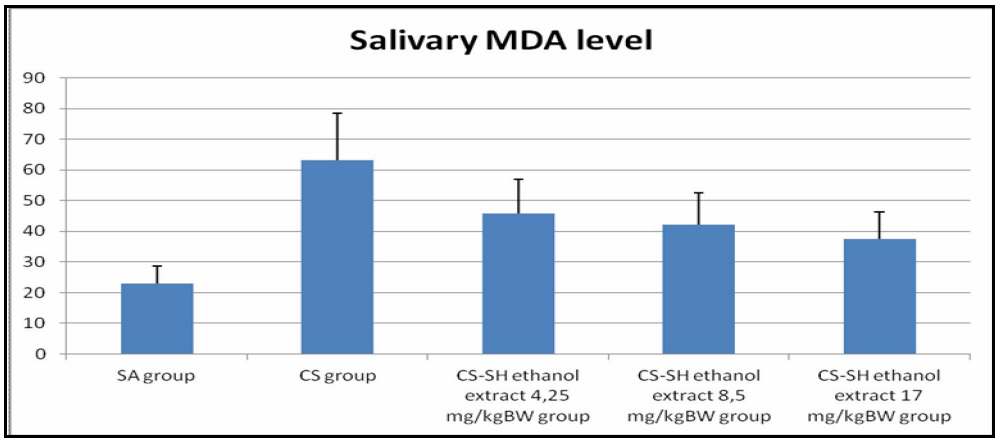

Figure 1. Mean value of salivary MDA level (nmol/mL) on the protective role of SH ethanol extract in wistar rat inhaled chronic cigarette smoke.

Catalase activity

To examine the effects of smoking on salivary antioxidant activity, we measured catalase enzyme activities. Cigarette smoke exposure caused significantly decrease ($p<0,05$) in salivary catalase activity compared to SA exposed rats. On the other hand, supplementary SH ethanol extract with chronic cigarette smoke inhalation resulted inhibit the decrease of salivary catalase activity significantly ($p<0,05$) compared to CS group (Table 2 and figure 2).

Table 2. Mean value of salivary catalase activity (U/mL) on the protective role of SH ethanol extract in wistar rat inhaled chronic cigarette smoke.

Animal Group	Mean	Standart Deviation
SA group	18.19	4.08
CS group	3.55	0.89
CS-SH ethanol extract 4,25 mg/kgBW group	6.43	1.66
CS-SH ethanol extract 8,5 mg/kgBW group	10.38	2.11
CS-SH ethanol extract 17 mg/kgBW group	13.69	2.13

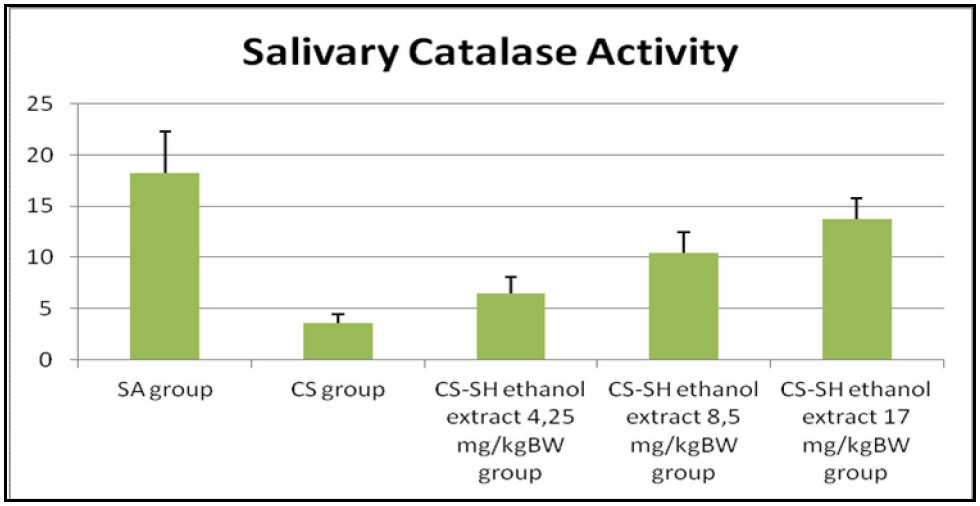

Figure 2. Mean value of salivary catalase activity (U/mL) on the protective role of SH ethanol extract in wistar rat inhaled chronic cigarette smoke.

Oral hyperkeratosis

To examine the protective role of SH ethanol extract inhibit oral hyperkeratosis induced by chronic cigarette smoke, we measured the thickness of tongue corneum layer. A significant difference in thickness of corneum layer was found in sections of rat tongue epithelial (Figure 3). The corneum layer of CS group was clearly thicker than SA group. Layer thicknesses, measured with an eyepiece micrometer, are given in Table 3. The corneum layer of CS group were approximately twice as thick as SA group. On the other hand, supplementary SH ethanol extract with chronic cigarette smoke inhalation resulted inhibit the increase of corneum layer thickness significantly ($p<0,05$) compared to CS group.

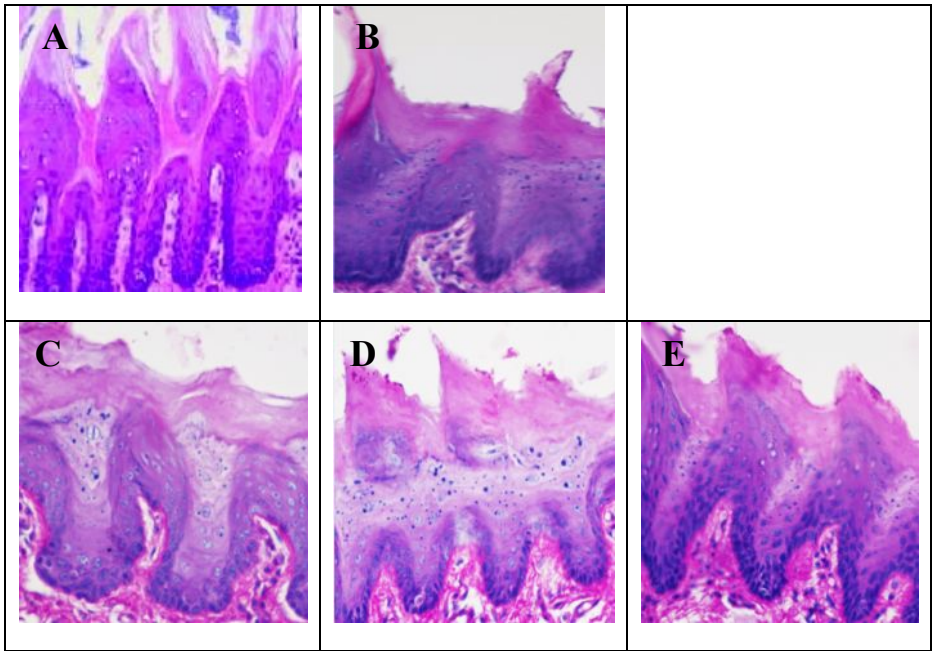

Figure 3. Photomicrographies of rat tongue epithelial. (A) no histopathological change (SA group); (B) epithelial hyperkeratosis in CS group; (C) epithelial hyperkeratosis in CS-SH ethanol extract 4,25 mg/kgBW group; (D) epithelial hyperkeratosis in CS-SH ethanol extract 8,5 mg/kgBW group; (E) epithelial hyperkeratosis in CS-SH ethanol extract 17 mg/kgBW group. (Hematoxylin and Eosin stain; X 400 magnification).

Table 3. Mean value of corneum layer thickness (µm) on the protective role of SH ethanol extract in wistar rat inhaled chronic cigarette smoke.

Animal Group	Mean	Standart Deviation
SA group	336,68	7,06
CS group	449,54	20,27
CS-SH ethanol extract 4,25 mg/kgBW group	419,91	14,53
CS-SH ethanol extract 8,5 mg/kgBW group	392,81	12,29
CS-SH ethanol extract 17 mg/kgBW group	382,26	10,68

Discussion

Habitual smoking is the most significant threat to the world's population today. It is known that cigarette and other tobacco products have extremely harmful effects on human health. Smoking has been reported to be a risk factor for oral disease[24]. CS constitutes thousands chemical compounds from many different classes such as alkenes, nitrosamines, aromatic and heterocyclic hydrocarbons and amines, etc. in both the gaseous form and condensed particle. CS leads to generation of enormous amount of ROS such as hydrogen peroxide, hydroxyl ion, superoxides and peroxyl radical, which in turn causes oxidative stress[25]. Accumulation of ROS, inducing cellular injury, oxidizing cellular macromolecules such as DNA and playing a crucial role in carcinogenesis[26]. Normal cells can be transformed into malignant cells due to oxidative modification. These transformed tumor cells produce high levels of ROS, which in turn increases lipid peroxidation levels. Owing to high cytotoxic properties, lipid peroxidation products such as MDA modulate cell growth by activating signal transduction pathways, therefore acting as tumor promoters and co-carcinogenic agents[27].

In this present study, indirect methods of oxidative-stress evaluation include estimation of secondary lipid peroxidation products, such as MDA. Hence, the extent of lipid peroxidation and free radical mediated damage are assayed by salivary MDA levels[28]. Saliva is the first body fluid to encounter cigarette smoke. The salivary antioxidant system plays a very important role in the carcinogenic capacity of saliva and includes various enzymes and molecules, such as catalase[29].

The present study was specifically meant to evaluate salivary MDA levels and catalase activity to examine the protective role of SH ethanol extract inhibit oxidative stress induced by chronic cigarette smoke. The result of this study showed that the salivary MDA level was significantly increased ($p<0,05$) and catalase activity was significantly decreased ($p<0,05$) in rats with chronic cigarette smoke exposeure compared to normal rats. This indicated that chronic cigarette smoke causes oxidative stress. The use of CS may have increase lipid peroxidation and suppressed the production of antioxidant enzymes. Several studies have reported elevated lipid peroxidation and decreases in antioxidant status in oral cancer who were tobacco users[30].

We found in this study that prophylactic treatment with SH ethanol extract significantly inhibit the increased of salivary MDA level and the decreased of catalase activity in rats with chronic cigarette smoke exposure. The finding suggests the possibility that free radical scavenger from SH ethanol extracr was able to protect rats from lipid peroxidation and reduced oxidative stress and suggests the potential benefit of treatment with SH ethanol extract.

SH is one of the marine organisms that can be explored as a potential source of valuable antioxidants[31]. The antioxidant activity of sea cucumber-derived peptides has been confirmed by Chenghui et al. (2007)[32]. Considerable amounts of phenolics, flavonoids and free radical scavengers have also been determined in SH[33]. These antioxidant compounds include flavonoids and phenolic acids that can inhibit oxidation, scavenge free radicals, and act as reductants[34]. Phenolic compounds also have strong H-donating activity[35].

Smoking is considered a risk factor for developing Oral leukoplakias (OL) that are typically white plaque of the oral mucosa because of hyperkeratosis process[1]. Hyperkeratosis is thickening of the stratum corneum, often associated with the presence of an abnormal quantity of keratin, and also usually accompanied by an increase in the granular layer[36].

The present study was measure the thickness of corneum layer to examine the protective role of SH ethanol extract inhibit oral hyperkeratosis process induced by chronic cigarette smoke. The result of this study showed that the thickness of corneum layer was significantly increased ($p<0,05$) in rats with chronic cigarette smoke exposeure compared to normal rats. This indicated that chronic cigarette smoke causes oral hyperkeratosis.

Smoking induced changes in the apoptosis of human oral keratinocytes, which may be signs of alterations in oral epithelium biology. The altered number of oral epithelial cells with early and late apoptotic features with increased time of smoking indicates interference with the physiological death of oral keratinocytes that a basic process in oral epithelium homeostasis[37]. The previous study found smoke activated caspases, phosphatidylserine externalization, and lost integrity in the plasma membrane significantly increases[38]. The majority of publications which evaluated the influence of tobacco smoke on the intracellular processes of oral keratinocytes, examined only the general features of apoptosis, such as caspase activation, and changes in mitochondria, plasma membrane, chromatin and maturation of keratinocytes[39].

We found in this study that prophylactic treatment with SH ethanol extract significantly inhibit the increased of the corneum layer thickness in rats with chronic cigarette smoke exposure. SH are reported to contain several compounds with antiproliferative properties such as triterpene glycosides. Antiproliferative functionality of sea cucumber extracts might be discribed to the presence of considerable amounts of total phenols and flavonids which are valued as effective antioxidants to protect from oxidative stress[31]. The proliferation inhibition potential was followed by the magnitude of marked apoptosis that is supposed to induce apoptosis by means of mitochondrial and cascade activation pathways[40,41].

Conclusion

In this study we found that chronic cigarette smoke caused oxidative stress and oral hyperkeratosis. Prophylaxis with Sticophus hermanii ethanol extract inhibit oxidative stress and oral hyperkeratosis process.

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