Anti-stress Activity of *Sargassum polycystum* Extracts Using a Cold Restraint Stress Model

Radipta Lailatussifa, Amir Husni*, and Agung Endro Nugroho¹

Department of Fisheries Faculty of Agriculture Universitas Gadjah Mada, Jalan Flora Gedung A4 Bulaksumur, Yogyakarta 55281, Indonesia

¹Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy Universitas Gadjah Mada, Sekip Utara, Yogyakarta 55281, Indonesia

Received August 12, 2015
Revised December 22, 2015
Accepted December 23, 2015
Published online April 30, 2016

*Corresponding Author
Tel: +62-274-551218
Fax: +62-274-551218
E-mail: a-husni@gadjahmada.edu

pISSN 1226-7708
eISSN 2092-6456

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Abstract Anti-stress effects of polyphenol extracts of *Sargassum polycystum* were evaluated. Polyphenol extracts of *S. polycystum* and diazepam were compared for anti-stress activities using a cold restraint animal stress model. *S. polycystum* extracts were administered orally at dosages of 150 and 450 mg/kg. Diazepam, an anti-stress agent, was used as a standard drug at 0.18 mg/kg p.o. Both dosages of *S. polycystum* extracts showed good anti-stress effects. Due to cold restraint stress there was an imbalance in levels of biochemical parameters, including glucose, triglycerides, cholesterol, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate amino transferase (AST), which were near normalized following administration of *S. polycystum* extracts. Polyphenol extracts of *S. polycystum* at oral dosages of 150 and 450 mg/kg exerted anti-stress effects.

Keywords: anti-stress, *Sargassum polycystum* extract, polyphenol, marine algae, gastric ulcer

Introduction

Stress is a phenomenon experienced by individuals that has become an integral part of modern human life (1). Stress is a biological response that can cause anxiety in an organism (2). Stress is thought to be involved in the a etiopathogenesis of hypertension, peptic ulcer, diabetes, immunosuppression, reproductive dysfunction, and behavioral disorders such as anxiety due to involvement of the central nervous system (CNS), endocrine, and metabolic systems (3). Human diseases derived from stress can be caused by free radicals (4) as free radicals can cause damage to tissues in the body (5).

Marine algae are organisms that have anti-oxidant, anti-inflammatory, and anticancer potentials (6). *Sargassum* sp. is a brown algae found in southern beach areas of Gunungkidul, Yogyakarta, Indonesia. Effects of *Sargassum* sp. treatments both *in vivo* and *in vitro* have indicated anticancer, anti-inflammatory, antibacterial, and antiviral pharmacological activities due to terpenoids, phlorotannin, and fucoidan (7). Phenolic components are effective antioxidants in brown algae (8). Bioactive compounds in *Sargassum* sp. have antioxidant, antitumor, antifungal, antiviral, antihypertensive, and antidiabetic potentials (9,10). Thus, marine algae can lower oxidative stress levels associated with antioxidant effects.

There have been many pre-clinical models for induction of stress in animals, including restraint stress (11). Cold restraint stress has been used for evaluation of anti-stress effects of *Caesalpinia bonduc*

seed extracts (12) and *Eugenia caryophyllis* bud extracts (13). This study was conducted to determine activities of polyphenol compounds of *S. polycystum* for influencing glucose, triglyceride, cholesterol, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate amino transferase (AST) levels, and the incidence of gastric ulcers as important effects against oxidative stress.

Materials and Methods

Materials The main material used in this study was the brown seaweed *S. polycystum* obtained from coastal Gunungkidul Yogyakarta, Indonesia in March of 2014. The chemical used for extraction of polyphenols was methanol (Merck KGaA, Darmstadt, Germany). All other chemicals used in this study were of analytical grade.

Seaweed extraction Extraction from seaweed was used to obtain extracts containing polyphenols. Polyphenol extraction was conducted as described by Kang *et al.* (14) with modification. Seaweed was washed thoroughly and dried at room temperature ($\pm 26^{\circ}\text{C}$) for 3–4 days prior to extraction. Dried seaweed was then cut into small pieces of ± 0.5 cm using scissors. A total of 40 g of seaweed pieces was put into an Erlenmeyer flask and covered with aluminum foil for maceration using 600 mL of 50% methanol for 48 h at room temperature. Resulting extracts were filtered using a coarse filter paper, then centrifuged (Centrifuge 5810 R; Eppendorf, Hamburg,

Germany) at 1,118×g for 10 min at 20°C. Methanol was evaporated using a rotary evaporator (RV 10 basic; IKA, Staufen im Breisgau, Germany) at 40°C, then extracts were freeze-dried (Virtis; SP Scientific, Gardiner, NY, USA) and stored at –20°C (GC-124GGFP; LG Electronics Inc., Seoul, Korea). Extracts were subsequently compared with diazepam for anti-stress activities.

Animals Albino Wistar rats of both sexes weighing 150–200 g were obtained from Integrated Research and Testing Laboratory Universitas Gadjah Mada, Yogyakarta, Indonesia. Rats were housed under standard conditions of 24±1°C at a relative humidity of 65±10% with a 12 h light and 12 h dark cycle and fed with a standard pellet diet with water *ad libitum*. All experimental protocols involving animal studies were approved after careful scrutiny by the Institutional Animal Ethics Committee (IAEC) of Integrated Research and Testing Laboratory Universitas Gadjah Mada.

Experimental method A cold restraint stress model was used for evaluation of anti-stress activities. Animals were divided into 5 groups of 5 animals each. Un-stressed group Group 1 rats did not receive any stress or treatment. Negative control Group 2 rats did not receive any treatment. Group 3 rats received standard diazepam at 0.18 mg/kg p.o. Groups 4 and 5 rats received 150 mg/kg p.o. and 450 mg/kg p.o. *S. polycystum* extracts, respectively. Stress was induced in all rats, except the un-stressed group, by subjecting animals to cold restraint stress with immobilization at 7°C for 10 min daily in a cylindrical cage over a period of 14 days. All 5 rats of each treatment group were placed in a 90 cm×50 cm×70 cm (L×W×H) tub with a water height of 30 cm. Stress was applied once daily for 14 consecutive days. Body weights of rats were measured every day. On day 14, rats were anaesthetized with anesthetic ether and dissected immediately after application of stress and blood was collected in microtubes. Blood was centrifuged (Centrifuge 5810 R; Eppendorf) at 1,610×g for 20 min at 4°C to provide serum that was collected and stored at –20 °C (GC-124GGFP; LG Electronics Inc.) prior to testing. Blood serum levels were measured to determine the profile of biochemical parameters, including glucose, triglycerides, cholesterol, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate amino transferase (AST). Each animal stomach was opened along the greater curvature and qualitatively examined macroscopically for gastric ulcers. Gastric tissue was stored in a 10% formalin solution before testing for gastric ulceration. Ulceration was analyzed using the method described by Gregory *et al.* (15).

Statistical analysis Anti-stress effects were reported as means± standard deviation (SD) and were assessed using an analysis of variance (ANOVA), and differences among samples were determined based on Duncan's multiple range test using the Statistical Analysis System (SAS release 6.12) program. A level of $p < 0.05$ was used as a criterion for statistical significance.

Fig. 1. Effects of polyphenol extracts from *Sargassum polycystum* and diazepam on restraint stress-induced body weight changes in rats.

Results and Discussion

Rat body weight Body weight changes before and after cold stress treatments are shown in Fig. 1. Normal group rats did not exhibit significant ($p \geq 0.05$) weight differences between before and after treatment. Rats stressed without treatment showed a significant ($p < 0.05$) decrease in body weight after administration of stress. Diazepam-treated rats showed relatively minor body weight changes after stress. Lima *et al.* (16) reported that diazepam influenced the central nervous system as a sedative and as a pain reliever.

Body weights of group 5 rats receiving *S. polycystum* extracts at 450 mg/kg p.o. after application of stress were increased above the level before stress. Therefore, the best treatment for prevention of weight loss after stress was administration of extracts at 450 mg/kg. Stress due to cold temperature can result in weight loss that can be adaptively overcome with long term anti-stress drug delivery (17). Firdaus *et al.* (18) mentioned that *Sargassum echinocarpum* extracts contain an anti-stress oxidative ingredient that is able to increase the uptake of glucose based on regulation of the cellular metabolism by adenosine monophosphate-activated protein kinase (AMPK) that is activated by algae phlorotannin contents.

Glucose level Rat blood glucose levels are shown in Table 1. Rats given extracts containing polyphenols from *S. polycystum* at a dose of 450 mg/kg showed a significant ($p < 0.05$) decrease in blood glucose levels to near levels of un-stressed rats, in agreement with a report of Firdaus *et al.* (18) in which methanol extracts of the brown algae *S. echinocarpum* at a dosage of 450 mg/kg body weight lowered blood glucose levels of diabetic mellitus rats to below 200 mg/dL. *S. echinocarpum* extracts inhibited α -glucosidase activity and showed insulin mimic activities. Lamela *et al.* (19) also reported that *Sargassum* sp. extracts exerted hypoglycemic properties in normal and diabetic rabbits. Borriello *et al.* (20) suggested that activation of

Table 1. Effect of polyphenol extracts from *Sargassum polycystum* and diazepam on restraint stress-induced biochemical changes in rats

Treatment groups	Glucose (mg/dL)	Triglycerides (mg/dL)	Cholesterol (mg/dL)	Alkaline phosphatase (U/L)	Alanine amino-transferase (U/L)	Aspartate amino-transferase (U/L)
Control	93.38±15.95 ^{a1)}	73.44±7.86 ^c	43.60±6.50 ^a	160.98±19.81 ^a	26.26±1.87 ^a	55.66±3.89 ^a
Cold restraint stress	181.20±28.69 ^c	26.56±6.69 ^a	79.32±9.90 ^c	304.02±13.57 ^c	54.10±5.02 ^c	101.58±6.40 ^d
Diazepam 0.18 mg/kg p.o	124.48±15.68 ^b	49.94±7.93 ^b	50.66±1.68 ^{a,b}	237.87±27.42 ^b	29.64±4.17 ^a	63.92±5.58 ^b
Polyphenol 150 mg/kg p.o	135.38±11.54 ^b	43.88±5.61 ^b	57.44±5.40 ^b	247.80±14.95 ^b	38.92±4.19 ^b	76.74±3.98 ^c
Polyphenol 450 mg/kg p.o	118.9±4.60 ^b	66.82±7.88 ^c	46.36±2.71 ^a	185.09±14.82 ^a	26.50±3.57 ^a	53.72±4.78 ^a

¹⁾Values with different letters in the same column indicate significant difference ($p < 0.05$)

AMPK by polyphenols can increase the Akt activity causing glucose transporter 4 to immediately translocate to the cell membrane. The glucose transporter is useful for up-take of glucose in the blood and muscles.

Triglyceride levels Rat triglyceride levels showed a significant ($p < 0.05$) difference between cold stress group rats and rats of all other groups (Table 1). Rat triglyceride levels in the diazepam 0.18 mg/kg group were not significantly ($p \geq 0.05$) different from *S. polycystum* extracts at 150 mg/kg group rats, but were significantly ($p < 0.05$) different from un-stressed rats and from *S. polycystum* extracts at 450 mg/kg group rats. Triglyceride levels of un-stressed rats were not significantly ($p \geq 0.05$) different from *S. polycystum* extracts at 450 mg/kg group rats. The best triglyceride level was produced by *S. polycystum* extracts at 450 mg/kg.

Lower triglyceride levels were observed in cold stress treatment group rats than in negative control group rats. Neelima *et al.* (21) reported that stressing Wistar rats via swimming in a negative control group without any medication increased triglyceride levels that decreased with treatment of *Embllica officinalis* fruit extracts at 400 mg/kg, approaching normal control levels. However, Singh *et al.* (13) reported that induction of stress using a voice in negative control group Wistar rats without a drug reduced triglyceride levels, but levels increased after treatment with Zeetress extracts at a dosage of 14 mg/kg, and treatment with clove extracts at 100 and 200 mg/kg, compared with normal control group rats. Decreased triglyceride levels in stressed rats caused stimulation of the hypothalamo-pituitary axis (HPA) and the sympathetic nervous system as a result of release of catecholamines and glucocorticoids (22). In addition, triglyceride serves as a rapid source of energy under stressful conditions (13). Thus, administration of an adaptogen drug can increase triglyceride levels to levels equal to triglyceride levels of rats in normal control groups.

Total cholesterol Total cholesterol blood serum levels of rats showed no significant ($p \geq 0.05$) difference between un-stressed group rats and rats that received *S. polycystum* extracts 450 mg/kg and diazepam at 0.18 mg/kg (Table 1). Total cholesterol levels diazepam 0.18 mg/kg group rats were also not significantly ($p \geq 0.05$) different from *S. polycystum* extracts at 150 mg/kg group rats. Total cholesterol levels of cold stress group rats were significantly ($p < 0.05$) different from

rats of all other groups. The best total cholesterol level was shown by *S. polycystum* extracts at a dosage of 450 mg/kg group rats.

A decrease in cholesterol levels after rats were administered an adaptogen of either diazepam or *S. polycystum* extracts for treatment of cold stress followed the report of Neelima *et al.* (21) in which extracts of the fruit of *E. officinalis* and the *Withania somnifera* standard drug reduced levels of serum cholesterol in rats. Singh *et al.* (13) also reported that Zeetress extracts lowered cholesterol levels in Wistar rats subjected to voice stress treatments because administration of a drug adaptogen can inhibit stimulation of the sympathetic nervous system so that mobilization of fat does not happen and synthesis of cholesterol is reduced.

Alkaline phosphatase (ALP) Blood serum levels of ALP Wistar rats showed no significant ($p \geq 0.05$) differences between un-stressed and *S. polycystum* extracts at 450 mg/kg group rats (Table 1). On the other hand, levels of ALP in diazepam 0.18 mg/kg group rats were not significantly ($p \geq 0.05$) different from *S. polycystum* extracts at 150 mg/kg group rats. Levels of ALP in negative control group rats that were stressed without medication were significantly different from all other group rats. On day 14, good ALP serum levels were best demonstrated by *S. polycystum* extracts at 450 mg/kg group rats. Decreased ALP levels for treatment of Wistar rats given cold stress adaptogen drugs followed the methods of Singh *et al.* (13) in which clove extracts at dosages of 100 and 200 mg/kg reduced ALP levels to 393.95 and 379.95 U/L, respectively, which was better than the value for control group normal rats of 415.67 U/L due to decreased levels of anti-oxidants in extracts that can inhibit stimulation of the hypothalamo-pituitary axis (HPA) and the sympathetic nervous system (22). Zaidi *et al.* (23) also reported decreased ALP levels in immobilization stress-induced rats treated with garlic extracts.

Alanine aminotransferase (ALT) Wistar rat serum ALT levels on day 14 showed no significant ($p \geq 0.05$) differences between un-stressed rats and *S. polycystum* extracts at 450 mg/kg and diazepam 0.18 mg/kg group rats (Table 1). Serum ALT levels in negative control group rats with stress but without medication were significantly ($p < 0.05$) different from all other group rats. Thus, administration of *S. polycystum* extracts 450 mg/kg was effective for reduction of serum ALT levels and prevention of oxidative stress at levels similar to the standard drug treatment of diazepam at 0.18 mg/kg.

Fig. 2. Effects of polyphenol extracts from *Sargassum polycystum* and diazepam on restraint stress-induced gastric ulcers in Wistar rats. +++=very severe; ++=moderate severe; +=low severe; -=no severe

Decreased ALT levels in Wistar rats administered adaptogen *S. polycystum* extracts and diazepam drugs followed reports of Neelima *et al.* (21) and Singh *et al.* (13). Neelima *et al.* (21) reported that ALT levels of Wistar rats treated with stress increased, compared with untreated group rats, but ALT levels decreased in Wistar rats treated with *Emblica officinalis* extracts at different concentrations. Singh *et al.* (13) also reported that ALT levels of Wistar rats treated with stress but without treatment increased, but ALT levels decreased in Wistar rats treated with clove extracts at 100 and 200 mg/kg, and with Zeetress extracts at 14 mg/kg. Wistar rats exhibited decreased ALT levels due to *S. polycystum* extracts containing polyphenolic compounds that acted as antioxidants for prevention of ROS formation. Stimulation of the hypothalamo-pituitary axis (HPA) and the sympathetic nervous system was, thus, affected.

Aspartate aminotransferase (AST) Wistar rat AST serum levels on day 14 showed no significant ($p \geq 0.05$) differences between un-

stressed group rats and *S. polycystum* extracts at 450 mg/kg group rats (Table 1). Wistar rat AST serum levels of these two groups were significantly ($p < 0.05$) different from all other group rats. Likewise, rat AST serum levels of negative control group rats with stress but without medication differed from all other group rats. Rat AST levels for diazepam at 0.18 mg/kg were significantly ($p < 0.05$) different, compared with *S. polycystum* extracts at 150 mg/kg group rats. The best treatment was indicated in rats treated with *S. polycystum* extracts at 450 mg/kg, characterized by improvements in rat AST levels to near AST levels in healthy rats of the normal group. Thus, *S. polycystum* extracts at 450 mg/kg administered orally can prevent and inhibit oxidative stress.

A decrease in AST levels of rats administered drug adaptogens was also reported by Neelima *et al.* (21) and Singh *et al.* (13). Wistar rat AST levels increased in stressed but untreated rats, but decreased in rats administered drug adaptogens of *E. officinalis* extracts (21). Singh *et al.* (13) also mentioned that Wistar rats treated with voice

stress exhibited increased AST levels in untreated rats, but decreased in Wistar rats administered drug adaptogens, such as clove extracts at 100 and 200 mg/kg, and Zeetress extracts at 14 mg/kg. Decreased AST levels in Wistar rats due *S. polycystum* extracts containing polyphenolic compounds that act as antioxidants for prevention of ROS formation can affect stimulation of the hypothalamo-pituitary axis (HPA) and the sympathetic nervous system.

Gastric ulcers Qualitative analysis showed that un-stressed Wistar rats exhibited little erosion of the mucosal lining of the stomach based on no change in the pathology of the gastric mucosal wall (Fig. 2). Thus, the un-stressed rat stomach was characterized by an absence of severe ulceration. The rat restraint stressed group showed very severe (+++) gastric ulceration, characterized by an occurrence of severe hyperplastic, severe inflammation, and irregular erosion of the outer wall of the gastric mucosa, the epithelium of the stomach. Thus, stress applied to rats can lead to gastrointestinal damage characterized an increased incidence of gastric ulcers in the outer mucosa of the stomach epithelium in rats.

Diazepam administered rats showed ulceration of low severity (+), characterized by slight erosion of the epithelial lining of the outer wall of the gastric mucosa. Erosion of the gastric mucosa can be prevented by drugs administered to minimize the occurrence of oxidative stress, suggesting that administration of diazepam at 0.18 mg/kg orally before application of cold stress in Wistar rats is effective for prevention of ulceration of the rat gastric mucosa. *S. polycystum* extracts at 150 mg/kg group rats showed ulcers of intermediate severity (++), characterized by severe erosion of the gastric mucosal wall. Thus, administration of ethanol extracts of *S. polycystum* at a dosage of 150 mg/kg orally given before application of stress in Wistar rats was not effective for prevention of ulcers of the rat gastric mucosa. *S. polycystum* extracts at 450 mg/kg group rats showed mostly no ulcers, characterized by an absence of pathological changes in the mucosal lining of the stomach. Therefore, the gastric mucosal wall was not prone to erosion. Thus, administration of ethanol extracts of *S. polycystum* at a dosage of 450 mg/kg orally given before application of stress in Wistar rats was effective for prevention of ulcers of the rat gastric mucosa, and similar to conditions of the gastric mucosa of normal healthy control group rats. *S. polycystum* extracts at 450 mg/kg were most effective for prevention of gastric ulcers in Wistar rats, and for maintenance of a healthy gastric mucosal layer in normal healthy control rats.

Ethanol extracts of *S. polycystum* at 450 mg/kg inhibited oxidative stress via maintenance of the gastric mucosa layer, similar to conditions in control group rats. Key factors of a gastric mucosal defense are anti-oxidants contained in extracts that increase secretion of bicarbonate and prostaglandins (24) and maintain the amount of nitric oxide (NO) via activation of endothelial and neuronal nitric oxide synthase (nnos). NO can dilate blood vessels, improving blood flow and stimulating angiogenesis in the gastric ulcer healing process. NO also stimulates proliferation of gastric mucosal cells and

the granulation (sealing) network in ulcer formation (25). In addition, anti-oxidants also improve cyclooxygenase-1 (COX-1) and COX-2 levels which is vital to defense of the gastric mucosal layer (26), in agreement with Amaral *et al.* (24) where anti-oxidants from *Rosmarinus officinalis* ethanol extracts at concentrations of 500 and 1,000 mg/kg prevented formation of gastric ulcers induced using 70% ethanol. Morsy *et al.* (27) reported that application of cold stress resulted in formation of acute gastric ulcers. Cold stress triggered increased secretion of gastric acid, increased pepsin activity, and lead to a decrease in mucosal protection induced by a decrease in mucus secretion, mucosal blood flow, and NO and prostaglandins levels. Morsy *et al.* (28) reported that anti-oxidants derived from nebulivol during cold stress induction healed ulcers in the stomach and increase the thickness of the gastric mucosa to the same thickness as in normal control group mice. In addition, Singh *et al.* (13) reported that the anti-oxidant effect of clove (*Eugenia caryophyllus*) extracts at a concentration of 100 mg/kg with application of cold stress could lower the ulceration index in the stomach.

Acknowledgment This research was supported by a research grant from Flagship Research Grant Universitas Gadjah Mada 2013, number LPPM-UGM/1406/LIT/2013.

Disclosure The authors declare no conflict of interest.

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