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Mas Ngur Oyster (*Atactodea striata*) Extract Therapy on Protease Activity and Rat's (*Rattus norvegicus*) Occludin Ileum With Indomethacine-Induced Inflammatory Bowel Disease (IBD)

Celcius Waranmaselembun[1,2*], Eddy Suprayitno[1], Aulanni'Am[4],Andhardoko[1]

[1]Faculty of Fisheries and Marine Science, Brawijaya University, East Java, Indonesia
[2]State Fisheries Polytechnique Tual, Southeast Mallucas, Indonesia
[3]Faculty of Basic Sciences, Brawijaya University-Malang, East Java-Indonesia

Abstract: *Inflammatory Bowel Disease* (IBD) is an inflammatory disease occurring in gastrointestinal tract, particularly colon, that can result from the side effect of antiinflammatory *non-steroid* (AINS) drug usage, such as Indomethacine. "Mas ngur"oysters (*Atactodea striata*) have long been known by people of Kei-Southeast Mallucasas traditional drug, but their use as antiinflammation has never been studied yet. This study was intended to measure the active compound ability of the oyster to reduce the protease activity and occludin increment in rat's (*Rattus norvegicus*) ileum with indomethacine-induced IBD. Test animals were 8-12 weeks old-male rats of 150 - 200 grams. They were separated into 3 groups, healthy, sick (induced with 15 mg/kg BW indomethacine), therapy goups (15 mg/kg BW indomethacine oral induction then treated with mas ngur oyster powder of100, 400, 700 mg/kg BW). Indomethacine induction of 15 mg/kg BWand therapy of mas ngur oyster powder extract were administered orally. The protease activity was measured using a spectophotometer, while the occludin expression was measured using immunohistochemistry. Results showed that the extract therapy gave significantly different effect ($P<0.05$) among treatments with effective dose of 400 mg/kg BW that could reduce the protease activity to 52.03%, andincrease the occludin expression to 989.706%.
Keywords: IBD, Indomethacine, Mas Ngur oyster extract, Protease Activity, Occludin expression.

Introduction

In recent years, attempts to find natural materials beneficial for human health are being icessantly carried out. Most of Indonesians living in rural areas have long and even herediterily employed these natural materials for human health, one of which those in Key islands, Southeast Mallucas,who have used marine oysters called 'mas ngur' as one of the traditional medicines.This oyster holds active compounds, such as alkaloid, steroid, saponin[1], and glutathione S-transferase (GST) enzyme, that filter organic hydroperoxide toxins[2,3].

Alkaloid, steroid,and saponin are potential antiinflammatory bioactive compounds,due to their ability to bind free radicals (ROS,*Reactive Oxygen Species)*as inflammatory trigger inileum, so that ROS cannot be produced excessively in the cell.Fastening of bioactive compounds and ROS could also prevent phosphorilation

and NF-κB activation. The action of alkaloid, steroid and saponinas antiinflammatory agents is to reduce endema as one of the inflammatory indicators in ileum and inhibit exudate formation and vascular permeability increment.

Inflammation is one of the main responses of the immune system to infections or irritations[4,5]. Diseases causing gastroinstestinal inflammation is calledInflammatory Bowel Disease(IBD)[6]. In inflammation treatment, the drug group largely administered is anti inflammatory non steroid, AINS, and one of which is indomethacine[7,8].Some studies, however, found that IBD could result from the side effect of the use of non steroidal anti-inflammatory drugs(NSAIDs), such as indomethacine[9]. The side effect shown by the use of indomethacine can result in inflammation of the gastrointestinal tract, in either human or animals[10]. When indomethacinepresents in the body it will quickly be absorbed by the intestine after oral administration[11].

Indomethacinegiven at the dose of 15mg/kg BWcan activate the macrophages then release Reactive Oxygen Species (ROS). If ROS is produced in excessive amount, it will activate the NF-kB and phosphorilation inhibition (I-kB)occurs. Then,the NF-kB moves to nucleus and expresses pro-inflammatory cytokine**,** such as TNF-α, and ifTNF-α is excessively produced, inflammation will occur. This inflammation will activate neutrophile by releasing protease enzyme causing tissue damages[12,13].Indomethacineinduction can reduce the occludin expression so that the function of small intestine in handling various coming toxins will be disturbed. Based on information above,this study is expected to be a treatment solution ofInflammatory Bowel Disease(IBD)through application of mas ngur oyster extract to reduce the protease activity and to increasethe occludin expression.

Materials and Method

Materials used in this study weredry mas ngur oyster (*Atactodea striata*)extract collected from Ohoililir, Kei Kecil District, Southeast Mallucas Regency, white rats (*Rattus norvegicus*), indomethacine, corn oil, NaCl 0,9%, PFA 10%, PBS-azida, standard solution oftirosin, PBS-Tween, PSMF solution, aquadest, cool absolute ethanol, cool Tris-HCl 20 mM pH 6.8; casein substrate, buffer solution of phosphate pH 7; 100 µL of protease enzyme; 400 µL of 4% (b/v)Tri Chloro Acetic Acid (TCA)solution.

Mas NgurOyster (*Atactodea striata*) Extraction

Extraction process was done by weighing 100 gof mas ngur oyster (*Atactodea striata*) powder, put into an erlenmeyer, added 200 ml of methanol solution, covered with alumunium foil to prevent solution evaporation, and macerated for 24 hours. It was then filtered through filter paper and macerated for 24 hours.

Filtrateobtained was evaporated under the temperature appropriate to the solvent used (± 40^{o}C) until paste-extract was formed. Methanol extract obtained was then washed using methanol solvent as follows[14]: 1). Methanol solvent was added in the methanol extract with 2 : 1, then shaken for 1 hour and left for 24 hours at 4^{o}C;2). If deposition occurred the supernatant was pippetted, and evaporated up to paste formed. The extract was then redissolved in methanol solvent and left for 24 hours at 4^{o}C. If there was still deposition, the supernatant was pippetted and evaporated, then added methanol solvent and left for 24 hours at 4^{o}C. This leaching process was performed until no deposition was found, and therefore, the obtained extract was really free of other components contained in extraction process. The methanol extract of evaporation was scrapped and put into sample bottle, then stored at 4^{o}Cfor further leaching.

Rat grouping

This study used 3-month old-male wistar-strained rats (*Rattus novergicus*) ethically acceptable certified by theCell and Molecular Laboratory of Faculty of Basic Sciences, Brawijaya University, Malang,acclimated and then separated into 3 groups, healthy, sick (orally inducedwithindomethacineof 15 mg/kg BWonce), and treatment group (orally with indomethacineof 15 mg/kg BWonce and then administered mas ngur extracts at the dose of 100, 400, and 700 mg/kg BW, respectively, for 14 successive days).

Indomethacine induction

Indomethacinewas orally given once at the dose of 15mg/kg BWto gain colon with acuteinflammatory bowel disease (IBD)[10].

Mas Ngur oyster (*Atactodea striata*) extract therapy

Mas Ngur oyster (*Atactodea striata*) extract treatment was administered orally at the dose of 100, 400, and 700 mg/kgBW, respectively, for 14 successive days.

Ileumcollection

Ileum was obtained from the test animal by killing through dislocation of the rat's neck part. The rats were then dissected their abdomen for ileum collection. Ileum was taken and washed in 0.9% NaCl and soaked in PBS for 5 minutes, then immersed in PBS-azidafor protease activity analysis.

Protease activity determination

a. Determination of tyrosine maximum wavelength.

As much as 1 ml of 10 µg/mL tyrosine standard solution was measured the absorbance at the wavelength of 230-320 nm using a UV-Vis spectrophotometer and found the maximum absorbance.The highest absorbance at the wavelength range is the maximum wavelength and used to make tyrosine standard curve and as sample absorbance measurement.

b. Tyrosine Standard Curve Preparation.

Ten10 mL volumetric flasks were prepared and each of which was filled with 1 mL, 2 mL, 3 mL, 4 mL, 5 mL, 6 mL, 7 mL, 8 mL, 9 mL, and 10 mL of 20 µg/mL tyrosine standard solution, respectively, to make 2 µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL, 10 µg/mL, 12 µg/mL, 14 µg/mL, 16 µg/mL, 18 µg/mL and 20 µg/mL of tyrosine standard solution, respectively, added aquadest up to the limit line and shaken to homogenous. Each 1mL of 2 µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL, 10 µg/mL, 12 µg/mL, 14 µg/mL, 16 µg/mL, 18 µg/mL and 20 µg/mL of tyrosine standard solution was measured the absorbance at the maximum wavelength obtained, and the tyrosine standard curve was made from the absorbance value. The blank used onlyaquadest.

c. *Crude* Protease Isolation.

Ileum organ in PBS-azida solution was taken 1 g, cut in small piece using a dissecting scisso, and added PBS-Tween solution: One-mL of PMSF (9:1) was added some quartz sand and crushed with cold mortar placed on ice cube. The homogenat was added4 mL of PBS-Tween solution:PMSF (9:1) and moved into polypropylene flask sterilized in an autoclave**,** homogenized for 10 minutes, sonicated with sonicator for 10 minutes and centrifuged for 15 minutes at 6,000 rpm at room temperature. The supernatant was then collected and added cool absolute ethanol at 1:1 ratio and left overnight at 4^{o}C until deposition was formed. It was then centrifuged for 15 minutes at 10,000 rpm, the pellet was taken and dried until the ethanol smell disappeared. The pellet was then added 20 mM of cool Tris-HCl solution of pH 6.8 with volume ratio of 1:1 and homogenized.

d. Protease activity determination of Ileum isolate.

Protease activity measurements were based on tyrosine product formation. These measurements used as much as 200 µL of 500 µg/mL casein substrate inserted into eppendorf flask, added 300 µL of phosphate buffer solution pH 7 and 100 µL of protease enzyme from isolation, and left for 60 minutes at 37^{o}C in an incubator. Then, add 400 µL of 4% (b/v) TCA solution, left for 30 minutes at room temperature, then centrifuged at 4,000 rpm for 10 minutes. The supernatant was taken 200 µL, dilluted 5 times sample volume with phosphate buffer and the absorbance value was measured using a UV-Vis spectrophotometer at the maximum wavelength of tyrosine obtained. Blanksolution used was made using activity determination procedure, but casein solution was replaced with aquadest addition. The protease activity was then measured using the following formula:

$$\text{Protease Activity} = ([\text{Tyrosine formed})/(\text{Mr.Tyrosine}) \times v/pxq \times fp$$

where : v = total volume of sample (mL)
q = incubation time (60 minutes)
fp = dillution factor

p = number of enzymes (mL)

Results and Discussion

Results

Statistical tests using SPSS 21 for windows at P<0.05 showed that mas ngur oyster powder extract treatment on the ileum protease activity of the rat with indomethacine-induced IBDsignificant effect among treatments. Further honest significant difference test indicated significant difference among treatments.

Table 1 shows that in healthy group, mean protease activity is0.0787 ± 0.004990673 µmol/ml.menit. this group was then used as a standardto determinethe increment or reduction of protease activity after mas ngur oyster extract treatment.The sick group induced with indomethacine(e)is significantly different from sick group (a). Also, extract treatments of 100 mg/kg BW(c), 400 mg/kg BW(b), and 700 mg/kg BW(d) had significantly different effect from those of the healthy group (a). Indomethacineinduction (Table 1) of 15 mg/kg BWwas proved to cause the rat's ileum protease enzyme activity increment as nuch as0.1814 ± 0.009820778 µmol/ml.min. (230.41%) of the healthy group with a value of0.0787 ± 0.004990673 µmol/ml.min. After treated with mas ngur oyster extract of 100 mg/kg BW,the protease enzyme activity could be reduced up to67.51%compared with the sick group.The extract treatment of 400 mg/kg BWcould reduce the protease activity up to 52.03%, but that of 700 mg/kg BWcould only reduce the protease activity up to86.29%.

Table 1 Protease activity of rat'sileum in healthy, sick and treatment groups.

Treatment Group	Mean Protease Activity (µmol/ml.min.) ± SD	Protease Activity (%)	
		Increment	Reduction
Healthy group	0.079 ± 0.005^{a}	0	0
Sick group	0.181 ± 0.010^{e}	230.409	-
Therapy 100 mg/kg BW	0.122 ± 0.003^{c}	-	67.513
Therapy 400 mg/kg BW	0.094 ± 0.004^{b}	-	52.030
Therapy 700 mg/kg BW	0.157 ± 0.005^{d}	-	86.294

Note: a,b,c,d,e indicate significant difference among treatment groups (p < 0.05).

Table 2 Occludinexpression of rat'sileum in healthy, sick and treatment groups.

Treatment Group	Mean Occludin Expression (% area) ± SD	Occludin Expression (%)	
		Increment	Reduction
Negative Control	$5/376 \pm 0.056^{a}$	0	0
Positive Control	0.408 ± 0.082^{e}	-	7.589
Therapy of 100 mg/kg BW	2.576 ± 0.059^{c}	631.373	-
Therapy of 400 mg/kg BW	4.038 ± 0.059^{b}	989.706	-
Therapyof 700 mg/kg BW	0.730 ± 0.051^{d}	178.922	-

Note: a,b,c,d,e indicate significant difference among treatment groups (p < 0.05).

Table 2 demonstrates that in sick group, occludin has expression decline of0.408 ± 0.082 % area or7.589 % compared with that of the healthy group, 5.376 ± 0.056 % area. After treated with mas ngur oyster extract, it increases as much as2.576 ± 0.059 % area or631.373 % of the sick group at the dose of 100 mg/kg BW, and 4.038 ± 0.059 % area or989.706 % at the dose of 400 mg/kg BW, and 0.730 ± 0.051 % area or178.922 % at the dose of 700 mg/kg BW, respectively.

Fig.1 shows that indomethacineinduction reduces the occludinexpression indicating that small intestine or ileum damages of the test rat (B) occur, then the treatment of 100 mg/kg BWmas ngur oyster extract makes the damaged tissues start recovering through increased occludin expression (C), and the highest recovery occurs at the extract treatment dose of 400 mg/kg BWwith occludin expression increment of989.706 % (D). The lowest recovery occurs at the extract treatment of 700 mg/kg BW (E).

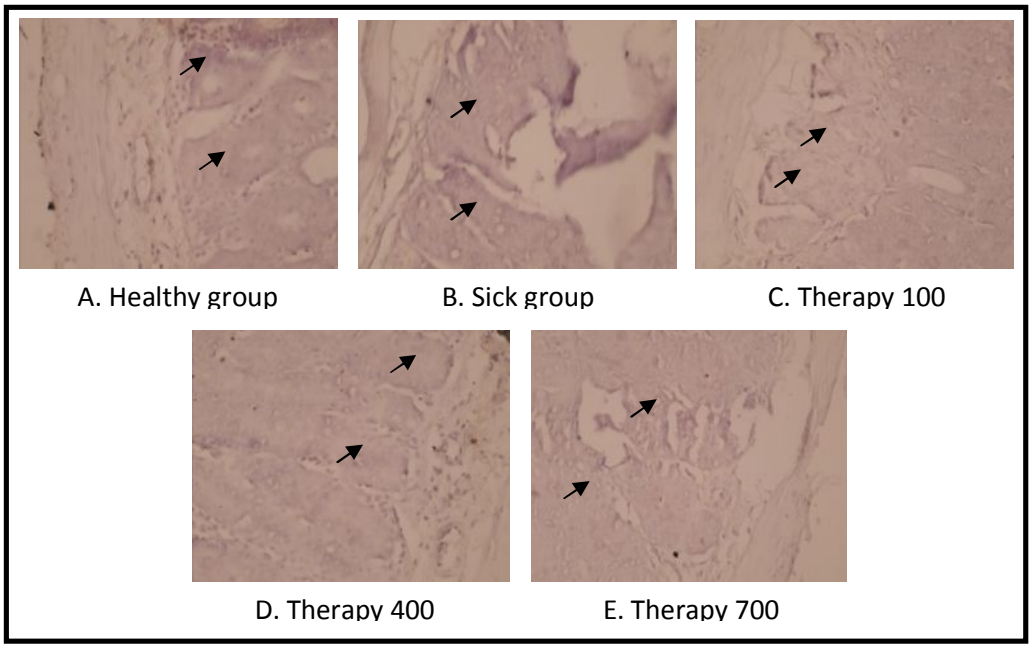

Figure1 Occludinexpression in rat's ileum indicated with brown color of epithelial cell (400x enlargement).
Note:
A. Negative control rat (healthy)
B. Positive control rat (sick) induced with indomethacine
C. Rat of therapy 100 (indomethacine induction + mas ngur oyster extract of 100 mg/kg BW)
D. Rat of therapy 400 (indomethacine induction + ekstrak kerang mas ngur 400 mg/kg BW)
E. Rat of theraphy 700 (indomethacine + mas ngur oyster extract of 700 mg/kg BW)

Discussion

Decline in protease enzyme activity (Table 1) from mas ngur oyster extract could be caused by the bioactive compound contained in the oyster, alkaloid, steroid and saponin. The alkaloid fraction of *C. maculatum* showed significant antiinflammatory activity at the dose of 200 mg/kg since the alkaloid was responsible as analgesics and antiinflammatory agent[15].

Alkaloid can accrelerate lesion healing and increase mucous production of the stomach because of induced materials[16], through bicarbonate output and pH increment, inhibit lesion and alter stomach acid secretion[17], reduce the blood flow of the stomach and react with free radicals[18], and function as antioxidantas well[19].

Sea cucumber steroids, including saponin, free sterol and binded sterol (triterpen glucoside), have benefits for health. Several findings proved that steroid and saponin compounds of sea cucumbers possessed antitoxic, antibacteria**,** antifungi,antitumor andantiinflammatory activities**.** Pharmacological activiies of saponin known are asantiinflammation, antibiotics, antifungi, antivirus, hepatoprotector and antiulceration. Antiinflammatory mechanism of saponin is to inhibit exudate formation and vascular permeability increment[20].

Decline in occludin expression (Table 2 andFig.1) couldresult from ROS formation at the induction of indomethacine that impacted on the destruction of mucosa assembling cells including vili in the small intestine.After mas ngur oyster extract treatment, the occludin expression increased again. It means that the oyster extract has an ability to inhibit ROS formation from indomethacine induction, so that the damaged mucosa constructing cells in the small intestine can recover indicated with the expression increment of the occludin tight junction ileum.

The recovery of small intestine tissues treated with mas ngur oyster extract is also reflected from the presence of more compacted intestinal vili than that in rats of the sick group, meaning that the active

compounds of the mas ngur oyster have ability as free radicals inhibitor that could press ROS formation and repair the damaged tissues through expression increment.

In indomethacine-induced sick group, the occludin expression declines. It could result from enzyme hydrolysis on the protein assembling tight junction by serine protease of neutrophile. This protease will hydrolyze protein tight junction, so that permeability of the enterosit cells, as mucosa producer, increases.Consequently, bacterial invasion in the small intestinal lumen triggers inflammation.

The ability of the protease to hydrolyze ZO-1 protein and occludin occurs due to the availability of substrate broken down by the enzyme.Elastase can break down the arginine and lysine-containing substrate, while trypsine can break down the substrate containing alanine, glysine and serine in ZO-1 and occludin proteins. Beside protease activity, tissue damages of small intestine could also be brought about by excessive ROS production that will react with protein in ileum tissue. ROS can react with the protein of brush border bulders, such as ZO-1 and occludin that can cause tissue damages. This reaction could cause the protein break down ton protein fragments.

Mas ngur oyster extract treatment administered to the sick rats was proved to be able to raise ZO-1 and occludin expressions. It could result from that the bioactive compounds of the oyster, as alkaloid, steroid, and saponin,could inhibit the oxidation of protein and could prevent enzymatically hydrolytic reaction so that the formed ROS could be neutralized.

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