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# NF-κB AND TNF-α EXPRESSIONS AND HISTOLOGICAL IMAGE OF WHITE RAT'S, RATTUS NORVEGICUS, ILEUM WITH INDOMETHACINE-INDUCED IBD (INFLAMMATORY BOWEL DESEASE) AFTER MAS NGUR OYSTER (ATACTODEA STRIATA) EXTRACT THERAPY

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**ABSTRACT**

*Inflammatory disease in gastric tract called Inflammatory Bowel Disease (IBD), particularly colon, in general, results from the use of non-steroid anti-inflammatory drug, such as indomethacine. The use of natural material, such as mas ngur oyster (Atactodea striata) as traditional medicine has been long known by people in Kei Islands–Southeast Mallucas. This study is aimed at measuring the ability of active compounds of this oyster extract in reducing NF-κB and TNF-α expression and showing the histological image of indomethacine-induced rat ileum with IBD after treatment with mas ngur oyster extract. It used 8-12 week old male rats (Rattus norvegicus) of 150 - 200 g BW. The rats were divided into 3 groups, healthy group, sick group (induced with 15 mg/kg BW of indomethacine), and treatment group (orally induced with indomethacine at a dose of 15 mg/kg BW then treated with mas ngur oyster extract at the dose of 100, 400, 700 mg/kg BW). Indomethacine induction at the dose of 15 mg/kg BW and mas ngur oyster extract therapy were orally administered. NF-κB and TNF-α expressions were measured using immunohistochemicals, and the histological image used Hematoksilin-Eosin staining. Results showed that extract therapy gave significant effect (P<0.05) at the effective dose of 400 mg/kg BW that could reduce 86.421% of NF-κB expression and 60.972% of TNF-α expression in the rat ileum and result in tissue recovery of the IBD rat's ileum after the therapy*

**KEYWORDS** *colon, active compound, NF-κB and TNF-α expression, histological image*

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## INTRODUCTION

Atactodea striata is a species of marine mollusk (bivalve) living in the intertidal that has long been identified by people of Kei islands, southeast Mallucas, as "mas ngur". Their growth is influenced by surrounding environmental conditions, such as temperature, water condition, depth, and food availability. This species has calcium-containing soft body cover. The body is usually held in the shell and not seen from outside. In favorable conditions, the body is spread out the shell, and foot firstly appears that is used to crawl or swim. The shell is thin, hard, and yellowish white colored, approximately 1 – 3.5 cm long (Sunarto, 2001). These animals have largely been used by the local people as traditional medicine of liver disease. The bioactivity of its secondary metabolite as anti-cancer is in the form of alkaloid, steroid, and saponin compounds. Previous findings indicated that these bioactive compounds are also potential as anti-inflammatory drugs.

Inflammation is an immune system response to irritations or infections (Erlina & Indah, 2017; Safrina et al., 2018), and if it occurs in gastric tract from viral or pathogenic bacterial infection, it is called Inflammatory Bowel Disease (IBD) (Krzystek-Korpacka et al., 2009). Most inflammatory medications have relied upon non steroidal anti-inflammatory non steroid (NSAP) drugs, and one of which is indomethacine (Hasanah et al., 2011; Wilmana & Gan, 2017). Nevertheless, some previous studies have demonstatrated that IBD could also result from the side effect of this drug group, such as indomethacine (Podolsky, 2002). The side effect can cause inflammation in the gastric tract, both in human or animals. Indomethacine administered at the dose of 15mg/kg BW will be able to increase the activity and the productivity of Reactive Oxygen Species (ROS) (Bures et al., 2011). It is quickly absorbed by the intestine after oral administration (Tanaka et al., 2001).

Indomethacine induction will raise ROS activity and productivity that then activate kappa B (I-κB) inhibitor and necrotic factor of kappa B (NF-κB) which is expression regulator of inflammatory gene and immune genes. The activated NF-κB will then induce the macrophages and neutrophyles, and initiate the formation of tumor necrotic factor-α (TNF-α). If this TNF-α presents in excessive numbers, the neurophyle will be activated and change the protease enzyme activity (Sharony et al., 2013). Thus, NF- κB and TNF-α expressions are one of the indicators of inflammation. The process of ileum inflammation can also observed through histopathology since when indomethacine is induced the inflammation mediators, such as histamine and others, will be released, indicated with endema as an indication of ileum inflammation, and it can be seen through histological image.

IBD therapy must be safe and use natural material with no effect of worsening the inflammatory condition of the ileum (Lanas & Scarpignato, 2016; Laudanno et al., 2016). One of the natural materials holding active compounds (alkaloid, steroid, and saponin) potential as anti-inflammation in IBD case is mas ngur oyster A. striata. This study is expected to be able to use mas ngur oyster (Atactodea striata) extract as one of the IBD drugs through its effect on NF-κB

activity increment and TNF-α level reduction and tissue repair of rat's ileum shown in its histological images.

## RESEARCH METHOD

This study used dry extract of mas ngur oyster (A. striata) extract collected from Ohoililir, Kei Kecil District, Southeast Maluccas, white rat (R. norvegicus), indomethacine, corn oil, 0.9% NaCl, 10% PFA, PBS-azida, tyrosin standard solution, PBS-Tween, PSMF solution, aquadest, cool absolute ethanol, cool pH-6.8 20mM Tris-HCl; casein substrate, pH 7-phosphate buffer solution; 400 µL of 4% (b/v) Tri Chloro Acetic Acid (TCA); PFA, xylol, paraffin, hematoxylin-eosin stain, and alcohol.

Test animals were 8-12 week old male wistar-strained (R. norvegicus) with weight range of 150 - 200 g obtained from Cell and Molecular Laboratory, Faculty of Basic Sciences, Brawijaya University, Malang, meeting ethical certificate, acclimated, and separated into 3 groups, healthy, sick (orally administered with 15 mg of indomethacine/kg BW once), and therapy (oral administration with 15 mg of indomethacine/kg BW once and continued with administration of 100, 400, 700 mg of dry mas gur oyster extract/kg BW for 14 successive days).

A. striata extract processing. Extraction employed a modified multilevel extraction method (Harborne, 2017) (Noviana et al., 2013). Fifty grams of mas ngur A. striata powder were put into an erlenmeyer, added with 100 ml of hexane solvent, and covered with alumunium foil agar to prevent the solvent to evaporate, macerated for 24 hours, and then filtered through filter paper. The residue was added with 100 ml of ethyl acetate, covered with aluminium foil, and macerated for 24 hours. Each filtrate produced was evaporated at suitable temperature for the used solvent (4°C) up to pasta-like extract was produced. Those extracts were coded as hexane extract, ethyl acetate extract, and methanol extract, respectively. Each extract was then washed using the same solvent (hexane, ethyl acetate, and methanol, respectively) as follows: 1) Hexane solvent was added to hexane extract, ethyl acetate to ethyl acetate extract, and methanol to methanol extract with solvent : extract ratio of 2 : 1. The mixture was then shaken for one hour and left for 24 hours at 4°C; 2) when precipitate occurs the upper layer was pippetted and then evaporated until the pasta was formed. The extract was dissolved using the same type of solvent and left for 24 hours at 4°C. This washing process was repeatedly done up to absence of precipitate indicating that the extract obtained is fully free of other unnecessary components. Each extract of evaporation was then scratted and put into sample bottle and stored at 4°C.

Ileum Collection. Test animals were firstly killed through head dislocation on the neck part. Their abdomens were then dissected for ileum collection. It was then washed in 0.9% NaCl and dipped in PBS for 5 min., then in PBS-azida solution for MDA measurement and histopathological preparat preparation with HE staining.

NF-κB and TNF-α Expression with Immunohistochemistry method. Praparat slide of the ileum was washed using pH 7 PBS and dropped 3% H2O2 for 20 min. It was then rewashed 3 times in pH 7 PBS for 5 min. and blocked using 5% FBS (Fetal Bovine Serum) for one hour, then rewashed 3 times for 5 min. in pH 7.4 PBS.

The preparat was then incubated for 24 hours using NF-κB primer antibody (for NF-κB measurement) and TNF-α primer (for TNF-α measurement) at 4oC and washed for 5 min. in pH 7.4 PBS 3 times, then incubated at room temperature for 1 hour using anti rabbit secondary antibody (Santa Cruz). The preparat was rewashed 3 times for 5 min. in pH 7.4 PBS and shedded with Strep Avidin-horse radin peroxidase (SA-HRP) then incubated for 40 min. It was washed again 3 times in pH 7.4 PBS for 5 min. and shedded with Diamino Benzidin (DAB) then incubated for 10 min., and washed 3 times in pH 7.4 PBS for 5 min. Counterstaining was done using Meyer Hemotoxylen for 10 min. The preparat was then washed in running water, rinsed with aquadest and dried. It was mounted with entellan and covered with cover glass.

Hematoxylin-Eosin Staining. Hematoxylin-Eosin staining was firstly accomplished by inserting the ileum preparat into absolute xylol two times for 5 min. The next step was deparaffination, in which the preparat was dipped into 1-3-levelled xylol 1-3 [xylol : absolute ethanol (3:1, 1:1, 1:3)], each of which for 5 min. The preparat was then rehydrated into leveled ethanol from absolute, 95%, 90%, 80% and 70% ethanol, respectively, for 5 min., then dipped in aquadest for 5 min. It was then stained with hematoxylin for 10 min. until the best outcome was gained, washed in running water for 30 min., rinsed with aquadest and inserted into eosin stain for 5 min. The preparat was macerated in aquadest to remove the excessive eosin. The next step was dehydration, in which the preparat was inserted in the levelled ethanol, from 80%, 90% and 95% to absolute ethanol. The preparat was then cleared by putting it into xylol for 5 min., wind-dried, and mounted with entellan, then covered with cover glass.

## RESULT AND DISCUSSION

Table 1 demonstrates that NF-κB expression of the rat's ileum increases with an average area of 5.376 ± 0.056 or 1,317.647 % compared with the healthy rats with an average area of 0.408 ± 0.082 %. It could result from that in normal condition, the NF-κB in the cytoplasm binds with IκBα, and IκBβ prevents its insertion into the cell nucleus. Nevertheless, when ROS stimulates NF-κB, the NF-κB will be released from IκB because IκB is phosphorilated by kinase so that degradation by proteasome occurs**.** It will result in NF-κB activation and go into the cell nucleus, then NF-κB will bind to κB side at the gene promotor side and carry out a series of transcriptions to express protein inflammation, such as TNF-α.

**Table 1 NF-κB expression in negative, positive control, therapized rat's ileum.**

| Treatment Group | Mean NF-κB expression (% area) ± SD | NF-κB Expression (%) | |
|---|---|---|---|
| | | Increment | Decline |
| Healthy Control | 0.408 ± 0.082[a] | 0 | 0 |
| Sick Control | 5.376 ± 0.056[e] | 1,317.647 | - |
| Therapy-100 mg/kg BW | 2.576 ± 0.059[c] | - | 52.083 |
| Therapy- 400 mg/kg BW | 0.730 ± 0.051[b] | - | 86.421 |
| Therapy- 700 mg/kg BW | 4.038 ± 0.059[d] | - | 24.888 |

*Note*:a,b,c,d,e indicate significant difference among treatment groups at $p < 0.05$.

Although the therapy of *A. striata* extract, as a whole, has been able to reduce NF-κB expression of the rat's ileum, it has not reached the normal condition like in negative control yet. This expression decline could result from the ability of the active compounds, such as alkaloid, steroid, saponin, and flavonoid, in ROS activity and productivity inhibition, so that NF-κB activation does not occur as a result of bond termination between NF-κB and IκB through phosphorylation.

Based on Table 1, it appears that *mas ngur* oyster extract therapy could have reduced NF-κB expression of sick rat's ileum (positive control) as much as 2.576 ± 0.059% area or 52.083% at dose of 100 mg/kg BW, 0.730 ± 0.051% area or 86.421% at dose of 400 mg/kg BW, and 4.038 ± 0.059% area or 24.888% at dose of 700 mg/kg BW. Statistical analysis showed that the indomethacine induced in the treatment group gave significant effect on NF-κB expression increment in the rat's ileum, and the treatment groups gave also significantly different effect on NF-κB expression ($p<0.05$).

Mas ngur oyster extract therapy could entirely have reduced NF-κB expression in the rat's ileum even though the decline has not reached normal condition as shown in the negative control. This expression decline could result from the active compounds of the oyster, such as alkaloid, steroid, saponin, and flavonoid, that are capable of inhibiting ROS activity and productivity so that NF-κB activation dos not occur as a result of bond severance between NF-κB and IκB through phosphorilation. Immunohistochemical analysis on the test rat's ileum clearly revealed that indomethacine induction increased NF-κB expression, then drastically reduced after theraphy with *mas ngur* oyster extract (Figure 1).

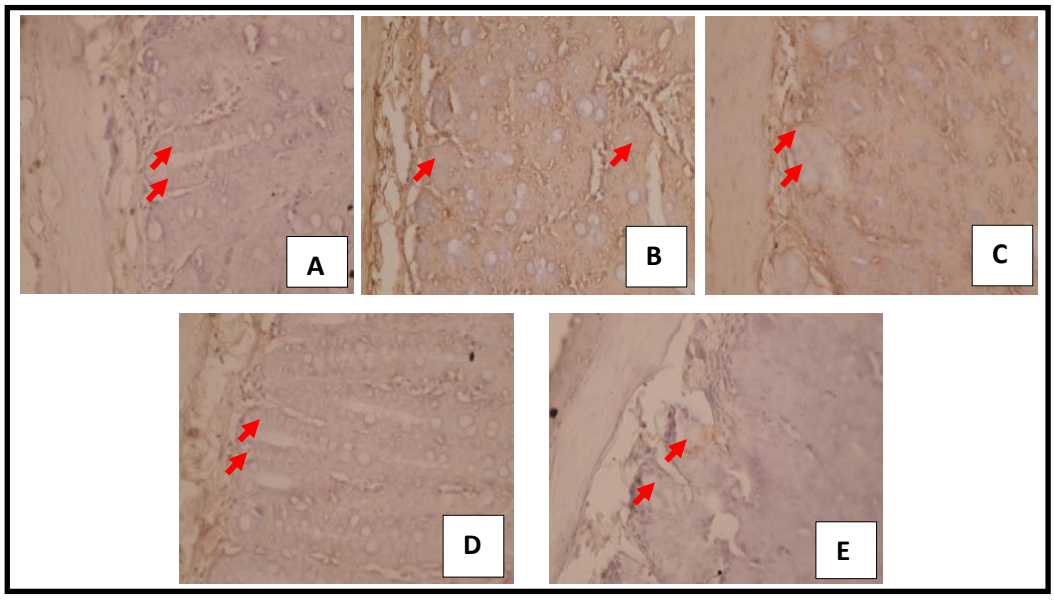


**Figure 1 NF-κB expression in rat's ileum indicated with brown color of the epithelial cell (400x enlargement). A = healthy rat control, B = indomethacine-induced sick rat control, C = therapy-100 rats (induced with indomethacine + 100 mg/kg BW of mas ngur oyster extract), D = therapy-400 rat (induced with indomethacine + 400 mg/kg BW of mas ngur oyster**

**extract), E = therapy-700 rat (induced with indomethacine + 700 mg/kg BW of mas ngur oyster extract).**

Figure 1 demonstrates that indimethacine induction raises NF-κB expression and damages the tissues of the test rat's ileum (B), and then *mas ngur* oyster extract therapy of 100 mg/kg BW repairs the damaged tissues (C), with the highest recovery at the dose of 400 mg/kg BW (D) and the lowest at the dose of 700 mg/kg BW (E). Improvement in small intestinal tissues therapized with *mas ngur* oyster extract is indicated by the presence of more compacted intestinal vili tissues in the positive control (sick). It reflects that the active compound of *mas ngur* oyster has an ability as free radical scavenger that could press ROS formation, and repair the damaged tissues through NF-κB expression reduction.

Increase in ROS activity and productivity impacts on NF-κB activation and then initiates TNF-α formation, and will activate the protease enzyme if presenting in excessive numbers. Therefore, TNF-α is one of the parameters needed to analyze its expression and through immunohistochemical analysis as given in Table 2.

**Table 2 TNF-α expression in negative control, positive control, and therapized group**

| Treatment Group | Mean Expression of TNF-α (% area) ± SD | TNF-α Expression (%) | |
|---|---|---|---|
| | | Increment | Decline |
| Healthy control | $0.400 \pm 0.011^{a}$ | 0 | 0 |
| Sick control | $2.470 \pm 0.061^{e}$ | 617.500 | - |
| Therapy of 100 mg/kg BW | $1.558 \pm 0.072^{c}$ | - | 36.923 |
| Therapy of 400 mg/kg BW | $0.964 \pm 0.061^{b}$ | - | 60.972 |
| Therapy of 700 mg/kg BW | $2.034 \pm 0.079^{d}$ | - | 17.652 |

*Note*:a,b,c,d,e indicate significant difference among treatment groups at $p < 0.05$.

It is apparent that the rats induced with indomethacine have activity increment and TNF-α expression of 2.470 ± 0.061% of the area or 617.500 % of the negative control rats, an area of 0.400 ± 0.011 %. After the rats had been in inflamed condition from indomethacine induction, and therapized with *mas ngur* oyster extract, the decline of TNF-α activity and expression occurred, 1.558 ± 0.072% of the area or 36.923% of the sick condition at the dose of 100 mg/kg BW, 0.964 ± 0.061% of the area or 60.972% of the sick condition at the dose of 400 mg/kg BW, and 2.034 ± 0.079% of the area or 17.652% of the sick condition at the dose of 700 mg/kg BW. Statistical analysis showed that indomethacine induced in the treatment groups gave significant effect on TNF-α expression increment of the ileum, and the expressions of TNF-α are significantly different among the treatment groups ($p<0.05$). Immunohistological analysis revealed that indomethacine induction initiated TNF-α formation, but then drastically reduced the expression after treated with mas ngur oyster extract (Figure 2).

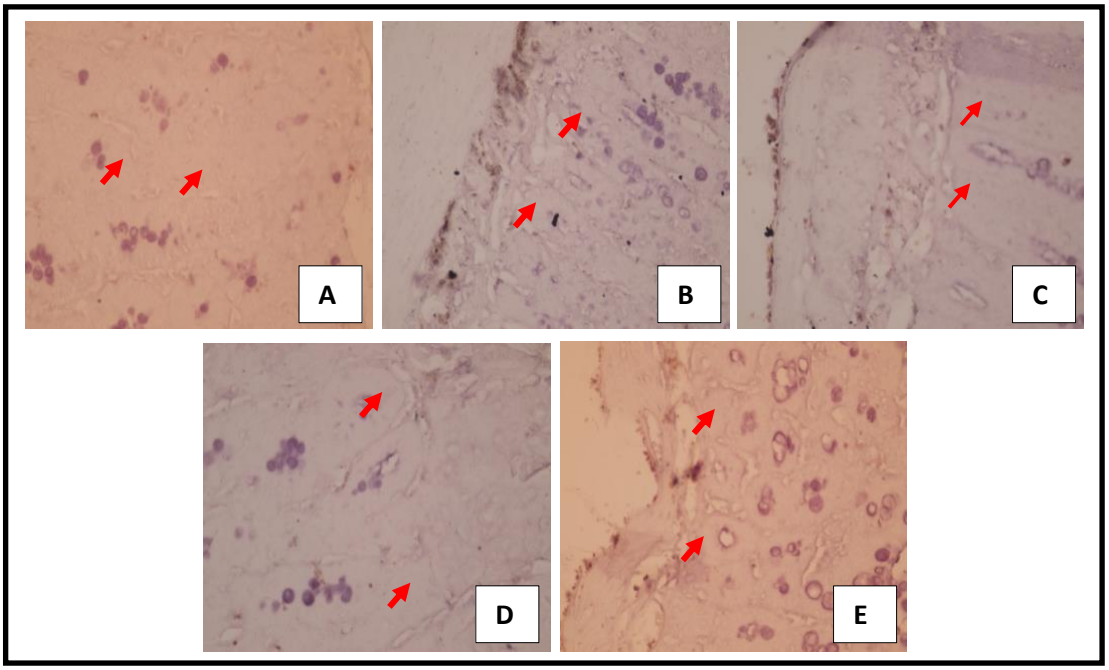


**Figure 2 TNF-α expression of the rat's ileum is shown with brown color of the epithelial cell (400 x enlargement). A = healthy rat control, B = indomethacine-induced sick rat control, C = therapy-100 rats (induced with indomethacine + 100 mg/kg BW of mas ngur oyster extract), D = therapy-400 rat (induced with indomethacine + 400 mg/kg BW of mas ngur oyster extract), E = therapy-700 rat (induced with indomethacine + 700 mg/kg BW of mas ngur oyster extract).**

Figure 2 reveals that indomethacine induction raises TNF-α and damages the ileum tissue of the test rat (B), but the tissue recovers after mas ngur oyster extract treatment (C), with the highest improvement at the dose of 400 mg extract/kg BW (D) and the the lowest at the dose of 700 mg extract/kg BW (E). The recovery of the small intestine treated with mas ngur oyster extract is demonstrated by more compacted intestinal villi than those in positive control (sick) rats, meaning that the active compounds of mas ngur oyster have worked as free radical scavenger that could inhibit ROS formation and repair the damaged tissues by reducing the TNF-α expression.

The damage and recovery level of an organ could be detected through one of the measured parameters, the organ histology (Wresdiyati et al., 2013). The histological conditions of negative (healthy) control, positive (sick) control, and therapized groups are given in Figure 3.

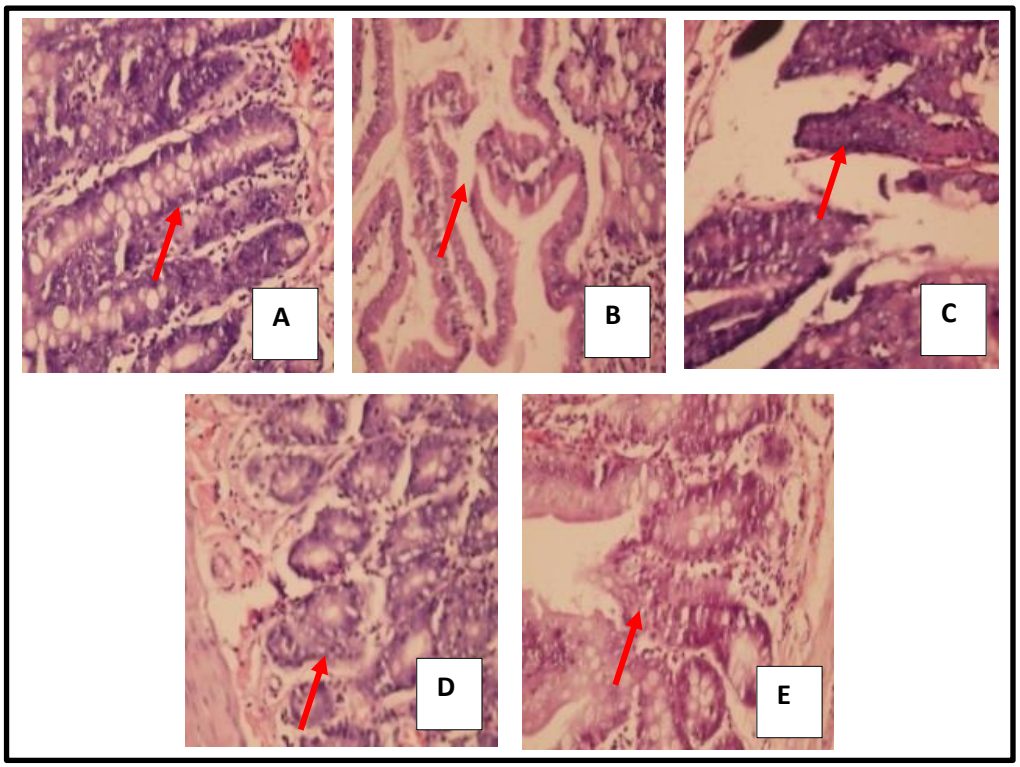


**Figure 3 Histological image of rat's ileum. 400x enlargement. A = healthy rat control, B = indomethacine-induced sick rat control, C = therapy-100 rats (induced with indomethacine + 100 mg/kg BW of mas ngur oyster extract), D = therapy-400 rat (induced with indomethacine + 400 mg/kg BW of mas ngur oyster extract), E = therapy-700 rat (induced with indomethacine + 700 mg/kg BW of mas ngur oyster extract).**

Figure 3 shows that villi of the ileum (A) still look good and have more compacted matrix, but those induced with indomethacine (B) look damaged. It could result from that indomethacine induction will give immune response that eases pathogenic bacterial invasion into the small intestine. This invasion will eventually activate the macrophages through cytokine secretion, such as TNF-α and ROS (Silva et al., 2008). It also shows that the sick rats from indomethacine induction, then therapized with mas ngur oyster extract get small intestinal tissue repair at the dose of 100 mg/kg BW (C) with the highest (aproaching to normal) at the dose of 400 (D), and the lowest at the dose of 700 (E). This improvement is reflected from more compacted intestinal villi than that in the sick group. This improvement indicates that the bioactive compounds of *mas ngur* oyster extract are capable of repairing the damaged tissue since it could reduce ROS formation as the cause of tissue damages from indomethacine induction, in which through cell regeneration mechanism, new absorbing epithelial cells (entrocyte cell) will appear to replace the damaged cells, and the small intestinal ileum tissue can be repaired.

## CONCLUSION

NF-κB and TNF-α expression of IBD rats reduce as much as 86.421% and 60.972% after therapy with mas ngur oyster extract at the best dose of 400 mg/kg BW. There was also tissue repair of the IBD rat's ileum after the therapy at the same dose. Further study needs to focus on pure isolate of *A. striata*. In addition, population study of *A. striata* should be considered in order to promote sustainable utilization and conservation.

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