In-Vivo Analgesic And Anti-Inflammatory Effects Of Eel (Anguilla bicolor bicolor) Oil

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ABSTRACT

Analgesic and anti-inflammatory tests of eel (Anguilla bicolor bicolor) oil on animal models have been performed. Previous studies have proven that oral administration of EPA and DHA exhibits analgesic and anti-inflammatory effects. Gas Chromatography analysis shows that eel contains EPA and DHA. In this research, the analgesic activity was evaluated with the acetic acid-induced writhing test and hot plate test, while the anti-inflammatory properties were identified using carrageenan-induced inflammation. In the writhing test, 25 male Swiss Webster mice were divided into five groups. Group I was given 0.5% CMC-Na as a negative control, group II was given 65 mg/kg b.w. of Acetosal as a positive control, group III-V was given eel oil at different doses, namely 2400, 4800, and 9600 mg/kg b.w. of the mouse. For the hot plate test, 6.5 mg/kg b.w. of tramadol acted as the positive control. Similar to the analgesic effect analysis, the anti-inflammatory test also divided 25 male Wistar rats into five groups. Group I as a negative control was given 0.5% CMC-Na, group II was given 9 mg/kg b.w. of diclofenac potassium as a positive control, and group III-V were given eel oil at different doses, namely 1500, 3000, and 6000 mg/kg b.w. of the rat. The results of the acetic acid-induced writhing test and hot plate test showed that when compared with the positive and negative controls, eel oil had a potential analgesic activity with a significance value of p< 0.05. The analgesic effects were noticeable at doses of 2400, 4800 and 9600 mg/kg b.w. in the writhing test and at 4800 and 9600 mg/kg b.w. in the hot plate test. The anti-inflammatory test showed that eel oil was efficacious when administered at the doses of 1500, 3000, and 6000 mg/kg b.w. with percentage inhibition of 34.35%, 35.132%, and 40.28%, respectively.

Keywords: Anguilla bicolor bicolor, analgesic, anti-inflammation, EPA, DHA

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INTRODUCTION
Pain and inflammation are the most common physical conditions in many people that can be indicated as a symptom of a disease. Pain is defined as an unpleasant physical sensation that differs in type, intensity, duration, and location (Lambert, 2014). Inflammation is an essential immune response that enables survival during infection or injury and maintains tissue homeostasis under a variety of noxious conditions (Medzhitov, 2010). Pain and inflammation with mild to moderate intensity can effectively be treated using a particular drug class, viz., Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) (Swieboda et al., 2013). However, more intense pain requires non-dependent analgesics, such as tramadol (Mullican et al., 2001). In the U.S., around 43 million adults (19.0%) take aspirin at least three times per week for more than three months (i.e., regular users), and more than 29 million adults (12.1%) are regular users of NSAIDs (Zhou et al., 2014).

NSAIDs are commonly used for arthritis pain worldwide, although long-term use may have side effects (Conaghan, 2012). Long-term use of NSAIDs is associated with adverse events involving the gastrointestinal system (Castellsague et al., 2012; Sostres et al., 2010) and the cardiovascular system (Atzeni et al., 2010; Olsen et al., 2011). Tramadol is an opioid analgesic drug used in treating moderate to severe pain (Rafati et al., 2012). The use of tramadol can cause many side effects, such as nausea, vomiting, dizziness, itching, shortness of breath, dry mouth, sweating, and psychological dependence.

The marine product is the new drug discovery for health. The role of natural products in drug discovery has undergone many changes in the past 30 years, with a noticeable decline in participation by the major pharmaceutical companies by the mid-1990s (Molinski et al., 2009). Eel (Anguilla bicolor bicolor) is one of marine biodiversity and generally consumed fish in many countries, especially Japan, China, Germany, and France (Sasonoko et al., 2017). Eel oil is reported to contain fatty acids like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Baeza et al., 2014; Kusharto et al., 2014). EPA and DHA are part of Omega-3 polyunsaturated fatty acids (Amisi et al., 2016). Many studies report that EPA and DHA can reduce inflammation (Camuesco et al., 2005; James et al., 2000; Mori and Beilin, 2004; Simopoulos, 2002) and pain (Goldberg and Katz, 2007). The anti-inflammatory nature of DHA- and DPA-derived EFOX (electrophilic o xo-derivatives) by showing that they can act as peroxisome proliferator-activated receptor gamma (PPAR gamma) agonists and inhibit pro-inflammatory cytokines and nitric oxide production, all within the biological concentration ranges (Groeger et al., 2010).

This paper discusses the analgesic and anti-inflammatory effects of eel (Anguilla bicolor bicolor) oil. In the literature, there has never been a study performing analgesic and anti-inflammatory test on eel oil. The analgesic testing used writhing and hot plate test (Sasonoko et al., 2016; Fan et al., 2014), while the anti-inflammatory analysis used carrageenan to induce inflammation (Shalini et al., 2015).

MATERIALS AND METHODS
Chemicals
Acetosal (Merck), potassium diclofenac (Hexpharm), tramadol (Kimia Farma) were purchased from Apotek Sebelas Maret Surakarta. The carrageenan was bought from Sigma Lunda. The Distilled water, CMC 0.5%, acetic acid glacial (Merck), NaCl 0.9%, NaOH, Na2SO4, and analytical grade methanol were obtained from the Pharmaceutical and Pharmacology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret.
Animals
The experiment used Wistar rat (weighing 150-200 gram) for the anti-inflammation test and Swiss Webster mice (weighing 20-30 gram) for the analgesic test. The test animals were locally purchased from Mouse for Labs, Mojosongo, Boyolali, Jawa Tengah.

Eel for material test
Eels (Anguilla bicolor bicolor) aged 6-8 months and weighing 100-200 g were purchased from the UNAGI business development unit, Universitas Sebelas Maret, Surakarta.

Oil extraction
The reflux extraction technique was performed to extract oil from fresh eels. Eels were cut into small pieces and then refluxed using distilled water under a controlled temperature (70-80°C) for ± 5 hours. The oil phase was then centrifuged at 3000 rpm and separated using a filter paper (Sasongko et al., 2017).

Identification of EPA and DHA
The oil of eel extract was tested for its EPA and DHA content, which was determined by gas chromatography. A total of 30-40 mg of eel oil was placed in a closed tube, added with 1 mL of NaOH 0.5 N in methanol, and heated in a water bath for 20 minutes. Then, the oil was added with 2 mL of 20% BF3 and reheated for 20 minutes. After the oil had cooled down, it was added with 2 mL of saturated NaCl and 1 mL of isoctane, then was shaken well. The isoctane layer was separated with the help of a dropper, then transferred into a tube containing 0.1 g of Na2SO4 and left for 15 minutes. The liquid phase was separated and subsequently injected into the gas chromatogram. To determine the retention time of EPA and DHA, gas of fatty acid ester from the Fatty Acid Methyl Ester (FAME) standard, which contains EPA and DHA as the standard, was injected first into the chromatogram. The presence of EPA and DHA in the samples can be viewed by equalizing the retention time of the EPA and DHA standard (Panagan et al., 2011).

Preparation of test animals
The rats and white mice were aclimatized for one week and given sufficient food and drink. Before the test, they were subjected to fasting for ± 11 hours, without food but still provided with drinking water. This procedure aimed to reduce the effects of bias during the study. All research protocols that involved test animals had been approved by the ethics committee of the Faculty of Medicine, Universitas Sebelas Maret (No. 441/V/HREC/2017).

Analgesic test
Acetic Acid-Induced Writhing Test
The analgesic test was performed by giving the test material orally. After 5 minutes, an intraperitoneal administration of acetic acid 1% was conducted. Pain is characterized by the onset of writhing or stretching, i.e., when the abdomen of the mice touches the floor of the testing area and both front and hind legs are pulled back (Sasongko et al., 2016). The mice were divided into five groups randomly before the experiment. Group I was given 0.5% CMC-Na as a negative control, group II was given 65 mg/kg b.w. of Acetosal as a positive control, group III-V was given eel oil at the doses of 2400, 4800, and 9600 mg/kg b.w. of the mouse. The number of writhing episodes in 30 minutes was counted for each group. When compared with the negative control, a smaller number of writhing episodes signifies an analgesic activity in the test animal (Goenarwo et al., 2011).
% writhing protection = 100 − [(E/C) x 100%]

E = cumulative total of writhing episodes in the test animals after intervention
C = cumulative total of writhing episodes in the negative control

**Hot plate test**

The analgesic activity was also evaluated using the hot plate method. The mice were placed on a hot plate maintained at 55°C. Jumping from the plate and latency period (the first jump after exposure to heat) are considered as a response to pain stimulus (Mondal et al., 2014). Reaction time for each group was recorded at 15, 30, 45, 60, 90 and 120 minutes after drug administration. To avoid any severe tissue damage, a cutoff point of 45 seconds was considered (Fan et al., 2014). The mice were divided into five groups. Group I was given 0.5% CMC-Na as a negative control, group II was given 6.5 mg/kg b.w. of tramadol as a positive control, and group III, IV and V were given eel oil at different doses, namely 2400 mg, 4800 mg, and 9600 mg/kg b.w. The number of jumps and latency period in each group were calculated and then averaged. Afterward, the analgesic intervention groups were compared with the negative control. The percentage (%) of analgesic protection was calculated using the two formula below:

**Analgesic protection based on the latency period:**

\[
\text{% analgesic protection} = \frac{(\text{LP}_t - \text{LP}_n)}{(45 \text{ sec} - \text{LP}_n)} \times 100
\]

\(\text{LP}_t = \text{Latency period to treatment}\)
\(\text{LP}_n = \text{Latency period for negative control}\)

**Analgesic protection based on the number of jumps:**

\[
\text{% analgesic protection} = 100\% - [(E/C) \times 100]
\]

E = cumulative total of jumps in the test animals after treatment
C = cumulative total of jumps in the negative control

**Anti-inflammation test**

The anti-inflammatory test in this study relied on the formation of artificial edema. The rat’s legs were identified with markers and measured in volume using a plethysmometer. After the test rats were given the drug and allowed for 30-minute rest, 0.1 mL of 1% carrageenan was injected. The needle was inserted in the same direction as the rat's leg. The volume of edema was measured every 15 minutes from Minute 0 to 180. The rats were divided into five groups randomly before the experiment. Group I was given 0.5% CMC-Na as a negative control, group II was given 9 mg/kg b.w. of diclofenac potassium as a positive control, and group III, IV and V were given eel oil at the doses of 1500, 3000, and 6000 g/kg b.w., respectively. The data of edema volume before and during the treatment were used to calculate the percentage of increase in the edema volume at \(t\) time, as shown in the following equations.
Percentage inhibition = \( \frac{(V_t - V_0)}{V_0} \times 100\% \) ........................ (a)

\[
AUC = \left( \frac{t_{0-15} P_{15}}{2} \right) + \left( \frac{(P_{15} + P_{20})}{2} \times t \right) + \left( \frac{(P_{20} + P_{25})}{2} \times t \right)
\]

Percentage of anti-inflammation = \( \left( \frac{A_{BC_k} - A_{ABC_k}}{A_{ABC_k}} \right) \times 100\% \) ........................ (c)

Statistical analysis
The data were analyzed statistically with the Shapiro-Wilk test for normality and the test for homogeneity of variance. The normally and homogenously distributed data were analyzed with One-way Analysis of Variance (ANOVA). To identify the differences among the treatment groups, the research employed LSD and Bonferroni post hoc test. As for the data that were not distributed normally and homogenously, they were subjected to the Kruskal-Wallis test, followed with the Mann-Whitney U test.

RESULTS AND DISCUSSION
Extraction yield and EPA and DHA content
The extraction of eel (Anguilla bicolor bicolor) with a gross weight of 1 kg yielded 5.65% w/w. This yield is almost the same as the one produced in the previous research, i.e., 5.33% w/w with a specific gravity of 0.8575 g/mL (Sasongko et al., 2017). The results of the EPA and DHA analysis of the eel oil extract were shown in Table I.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eicosapentaenoic acid (EPA)</td>
<td>843.4</td>
<td>mg/100 g</td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA)</td>
<td>3045.8</td>
<td>mg/100 g</td>
</tr>
</tbody>
</table>

The EPA and DHA contents in eel were used as active compounds in the analgesic and anti-inflammatory testing. A previous study found that fresh Indonesian eel is composed of 1.15% EPA and 5.16% DHA (Kusharto et al., 2014). Fish oil is rich in omega-3, which is an unsaturated fatty acid containing two fatty acids, namely Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) (Almatsier, 2002). Figure 1 shows the chemical structures of EPA and DHA.

![Figure 1. The chemical structures of EPA and DHA (Hadipranoto, 2010)](image)

The analgesic effect of eel (Anguilla bicolor bicolor) oil
Acetic acid-induced writhing test
The test animals received 1% acetic acid to induce pain. This chemical causes severe irritation on the mucous membrane of the abdominal cavity. This pain is manifested in the extension of legs,

In-Vivo Analgesic ...(Sasongko et al.,)
arching of the back, and contraction of the abdomen—causing it to touch the floor of the treatment site) (Sasongko et al., 2016). The data generated from the test include the number of writhing episodes. The average of the writhing episodes in each group is presented in Table II.

Table II. The cumulative number of writhing episodes in each group of mice after stretch induction by 0.5% acetic acid

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average of writhing episodes</th>
<th>% writhing protection</th>
<th>Post hoc test (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>191.6±17.3</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>41.2±8.01</td>
<td>78.5%</td>
<td>.009*</td>
</tr>
<tr>
<td>Eel oil 2400 mg/kg b.w.</td>
<td>167.4±10.09</td>
<td>12.63%</td>
<td>.016*</td>
</tr>
<tr>
<td>Eel oil 4800 mg/kg b.w.</td>
<td>114.6±13.47</td>
<td>40.19%</td>
<td>.047*</td>
</tr>
<tr>
<td>Eel oil 09600 mg/kg b.w.</td>
<td>38.2±4.09</td>
<td>80.06%</td>
<td>.009*</td>
</tr>
</tbody>
</table>

* significant difference with the negative control (p<0.05)

Table II shows that the negative control—given 0.5% CMC-Na—causes the highest number of writhing episodes. In this case, 0.5% CMC-Na is a placebo that has no analgesic activity, resulting in the highest writhing episodes among the test animals. Because the administration of Acetosal, as a positive control, induced a smaller number of writhing episodes than the negative control, the test method is concluded as valid. Acetosal is a non-steroid anti-inflammatory drug (NSAID) that inhibits the activity of the enzyme called cyclooxygenase (COX), which leads to the formation of prostaglandins (PGs)—i.e., a precursor of inflammation, swelling, pain, and fever (Vane and Botting, 2003). There are two structurally distinct forms of the cyclooxygenase enzyme (COX-1 and COX-2). COX-1 is a constitutive member of normal cells, while COX-2 is induced in inflammatory cells. Inhibition of COX-2 activity represents the most likely mechanism of NSAID-mediated analgesia (Cashman, 1996). As seen in Table II, the cumulative writhing episodes in groups receiving eel oil at the doses of 2400, 4800, and 9600 mg/kg b.w. are averagely 167.4 ± 10.09, 114.6±13.47, and 38.2±4.09. When administered at the dose of 9600 mg/kg b.w., the number of writhing episodes is not significantly different from the positive control (p≥0.05). The administration of eel oil reveals that a higher dose leads to fewer writhing episodes and, in other words, higher protection against writhing (p≤0.05). Eel oil (Anguilla bicolor bicolor) is used as an analgesic in this research because it has EPA and DHA content. EPA and DHA are included in the essential fatty acids that are released due to the presence of wound on the cell membranes, which then competitively inhibit the formation of pro-inflammatory interleukins (IL-1β, IL-6, and IL-12), tumor necrosis factor alpha (TNF α), and prostaglandin. Pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, can lead to pathological pain (Zhang and An, 2007). EPA has a long-chain structure like AA (Arachidonic Acid); therefore, it can be a competitor that replaces arachidonic acid in the metabolic processes of cyclooxygenase and lipoxygenase. EPA is also a substrate of COX, LPO, and cytochrome P450 enzymes that produce eicosanoids. Mediators generated by EPA have different structures, such as PGE3 (EPA) and PGE2 (AA) as well as LTB5 (EPA) and LTB4 (AA) (Calder, 2013).

Hot plate test

Hot plate test is a widely used model for centrally acting analgesics through their action at the spinal cord level. Pain caused by heat exposure is very closely related to the ability of high temperature to damage the tissue on mice. Tissue damage will occur at > 45ºC, and, as a result, the sensation of heat will turn into pain (Puspitasari et al., 2003). Reaction to thermal pain such as jumping in mice is considered to be a supraspinally integrated response (Fan et al., 2014). An average number of jumps and the latency period generated from each group can be seen in Tables III and IV.
The anti-inflammatory effect of eel (Anguilla bicolor bicolor) oil

An anti-inflammatory test aims to determine the association of a decrease in the volume of the food pad (edema) in rats with the administration of eel oil. In this study, the edema was induced by intraplantar injection of carrageenan. Carrageenan can release prostaglandins that trigger inflammation in rat's legs. Inflammation is characterized by redness, swelling, elevated temperature, pain, and loss of function (Okuse, 2007). The percentage of inhibition against edema and the percentage of anti-inflammation in all groups of rats are shown in Figure 2 and Table V.

In-Vivo Analgesic ... (Sasongko et al.)
Based on Figure 2 and Table V, the positive control (potassium diclofenac) appears to experience a decrease in the volume of edema when compared with the negative control. CMC-Na as the negative control has no anti-inflammatory effect (no inflammatory response), indicating that the test method is valid. Diclofenac is a proven and commonly prescribed nonsteroidal anti-inflammatory drug (NSAID) that has analgesic, anti-inflammatory, and antipyretic properties, and is useful in treating a variety of acute and chronic pain and inflammatory conditions. Diclofenac exerts its action via inhibition of prostaglandin synthesis by preventing the actions of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) with equipotency (Gan, 2010). The administration of eel oil at the doses of 1500, 3000, and 6000 mg/kg b.w. produced anti-inflammatory effects with the percentages of 34.35%, 35.13%, and 40.28%, respectively. The dose of 6000 mg/kg b.w. yields the highest anti-inflammatory response, and the statistical test results showed that it is significantly different (p ≤ 0.05) from the effects produced by the other two doses. However, the doses of 1500 and 3000 mg/kg b.w. stimulate anti-inflammatory reactions that do not differ significantly (p ≥ 0.05). This finding is explained by Rees et al. (2006), which state that fish oils containing EPA and DHA give a significant effect at a dose of 1350 mg/kg b.w. Another study proves that at a dose of 1.2 grams, EPA and DHA can substitute the effects of NSAIDs (Maroon and Bost, 2006). Many studies have also confirmed that EPA and DHA have anti-inflammatory effects (Camuesco et al., 2005; Simopoulos, 2002). EPA and DHA induce the down-regulation of pro-inflammatory cytokines, which are associated with the etiology of metabolic syndrome, NF-κB transcriptional activity, and upstream cytoplasmic signaling events. Immune
responses are dynamic, and the present study suggests a nutrient-sensitive window of LPS activation at which EPA and DHA are strongly anti-inflammatory (Mullen et al., 2010).

CONCLUSION
The results showed that eel oil had a potential analgesic activity with a significance value of p<0.05 when compared with the positive and negative controls. In the acetic acid-induced writhing test, the analgesic effect is noticeable when eel oil is administered at the doses of 2400, 4800 and 9600 mg/kg b.w. Meanwhile, in the hot plate test, eel oil is efficacious as pain relief when given at the doses of 4800 and 9600 mg/kg b.w. Based on the anti-inflammatory analysis, the eel oil at the doses of 1500, 3000, and 6000 mg/kg b.w. can inhibit inflammation by 34.35, 35.132, and 40.28%, respectively.

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