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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 e*t 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 (Sasongko *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 (Amissi *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 oxo-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 (Sasongko *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 Lumda. 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^{o}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 isooctane, then was shaken well. The isooctane layer was separated with the help of a dropper, then transferred into a tube containing 0.1 g of Na_2SO_4 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 containe 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 acclimatized 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).

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

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 55ºC. 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:

$$\% \text{ analgesic protection} = \frac{(LP_t - LP_n)}{(45 \text{ sec} - LP_n)} \times 100$$

LP_t = Latency period to treatment
LP_n = 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.

$$\text{Percentage inhibition} = \frac{(Vt - V0)}{V0} x\ 100\% \quad \text{..... (a)}$$

$$\text{AUC} = \left(\frac{t_{0-15} x P_{15}}{2}\right) + \left(\left(\frac{(P_{15} + P_{30})}{2}\right) x\ t\right) + \cdots\cdots \left(\left(\frac{(P_{210} + P_{225})}{2}\right) x\ t\right) \quad \text{..... (b)}$$

$$\text{Percentage of anti-inflammation} = \left(\frac{AUC_k - AUC_p}{AUC_k}\right) x\ 100\% \text{..... (c)}$$

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 I. EPA and DHA contents in eel (*Anguilla bicolor bicolor*) oil

Parameters	Results	Unit
Eicosapentaenoic acid (EPA)	843.4	mg/100 g
Docosahexaenoic acid (DHA)	3045.8	mg/100 g

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.

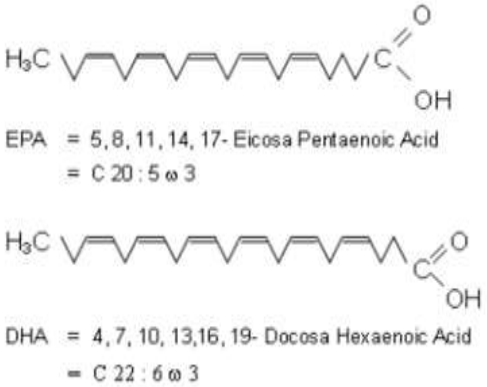

Figure 1. The chemical structures of EPA and DHA (Hadipranoto, 2010)

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,

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

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

*** 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 ($p \geq 0.05$). The administration of eel oil reveals that a higher dose leads to fewer writhing episodes and, in other words, higher protection against writhing ($p \leq 0.05$). 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.

Table III. The cumulative data of jumps in each group of mice

Groups	Average of jumps	% analgesic protection	Post hoc test
Negative control (Aquadest)	15.82±1.49		
Positive control (Tramadol)	6.28±1.58	60.30%	.012*
Eel oil 2400 mg/kg b.w.	15.77±2.14	0.32%	.911
Eel oil 4800 mg/kg b.w.	8.43±1.83	46.77%	.045*
Eel oil 9600 mg/kg b.w.	6.97±0.46	55.94%	.021*

*** significant difference with negative control**

Table IV. The cumulative data of the latency period in each group of mice

Groups	Average of latency period	% analgesic protection	Post hoc test
Negative control (Aquadest)	8.7±1.45		
Positive control (Tramadol)	19.18±2.83	28.87%	.009*
Eel oil 2400 mg/kg b.w.	9.9±7.85	3.3%	.016*
Eel oil 4800 mg/kg b.w.	16.03±4.25	26.61%	.009*
Eel oil 9600 mg/kg b.w.	20.63±2.79	32.86%	.009*

*** significant difference with negative control**

Tables II and III present the central analgesic activity showed during the hot plate method. The negative control has the most number of jumps and the shortest latency period because aquadest is pure water that does not contain any ingredients (Linandarwati, 2010) and, thereby, exhibits no activity in reducing central pain. Tramadol was chosen as a positive control because it belongs to the opioid group and is a drug of choice in the group of analgesics that work centrally. Furthermore, the positive control has the smallest average number of jumps and the most extended latency period compared with the negative control, which means that the test method is valid and that tramadol is effective in treating central pain.

At a higher dose, the administration of eel oil induces a fewer number of jumps and more extended latency period. The percentage of analgesic protection based on jumps and latency period becomes more significant as the dose of eel oil increases, i.e., from 2400 mg to 4800 mg then 9600 mg/kg b.w. According to Winarti and Wantiyah (2011), a high percentage of analgesic protection implies that the test animal can withstand the pain caused by thermal exposure to its feet. When administered at the doses of 4800 and 9600 mg/kg b.w., the eel oil showed no significant difference in the percentage of analgesic protection compared with the positive control.

Eel oil contains omega-3 (EPA and DHA), which is known to have many benefits. Also, some studies have affirmed that omega-3 in fish oil can reduce pain. DHA can release β-endorphin that stimulates μ receptor—that is, receptors associated with the occurrence of pain, especially central pain. If the receptor is stimulated, there will be no release of neurotransmitters in the body (Laino, 2017). In other words, the body does not experience any pain.

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.

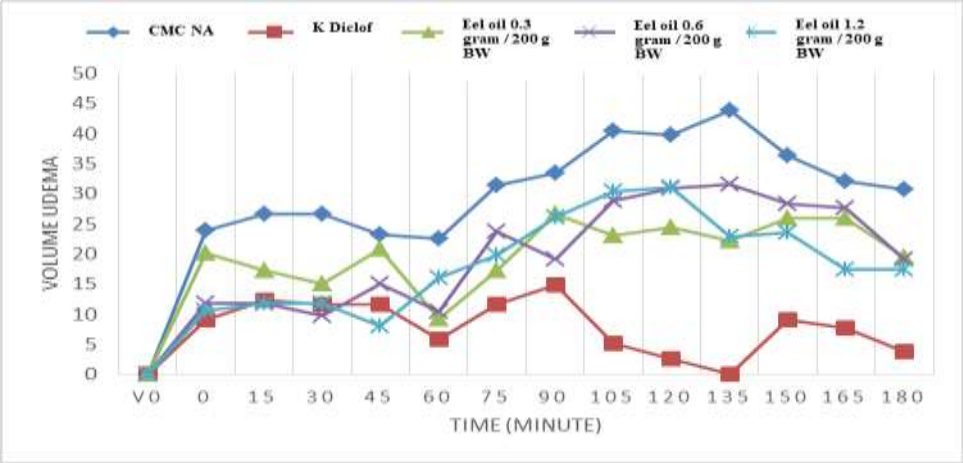

Figure 2. Percentage of the volume of edema in rat's footpad in each treatment group

Table V. Percentage of anti-inflammatory inhibition in all group

Groups	Percentage of anti-inflammatory (%)
Negative control (CMC-Na)	0
Positive control	73.43
Eel oil 1500 mg/kg b.w.	34.35
Eel oil 3000 mg/kg b.w.	35.13
Eel oil 6000 mg/kg b.w.	40.28

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 ($p \leq 0.05$) 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 ($p \geq 0.05$). 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 $p < 0.05$ 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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