Anti-inflammatory activity of Eucheuma denticulatum from Warambadi coast: In-vivo study model of carrageenan-induced paw oedema

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Abstract

**Background:** Chronic inflammatory diseases have been declared the leading cause of mortality in the world, with more than 50% of deaths associated with inflammatory cases. Based on previous studies, red algae contained antioxidants such as phenolics and flavonoids, which are correlated to their anti-inflammatory activity, including *Eucheuma denticulatum*. **Objective:** This study aims to analyse antioxidants and anti-inflammatory activity as well as the phenolic and flavonoid content of red algae *E. denticulatum* ethyl acetate extract (EDEE). **Method:** EDEE was macerated in ethyl acetate and the antioxidant activity was performed using the (2,2-diphenyl-1-picryl-hydrazyl-hydrate) DPPH method. Total phenolic and flavonoid content was quantified using the Folin-Ciocalteu and AlCl₃ methods. The anti-inflammatory activity was analysed by measuring the percentage of anti-inflammatory activity in carrageenan-induced mice paw edema. **Result:** The results showed that the Inhibition concentration 50 (IC₅₀) of EDEE was 1031.5 ppm with a total phenolic and flavonoid content of 81.34 ± 0.996 mg gallic acid equivalents (GAE/g) and 5.64 ± 0.12 mg quercetin equivalents (QE/g) extract, respectively. In vivo study showed that EDEE of 20, 50, and 100mg/kg body weight (BW) provided the percentage of anti-inflammatory activity of 53.49%, 86.63%, and 78.49% respectively, which is significantly different (p<0.05) compared to the negative control. **Conclusion:** EDEE 50mg/kg body weight had the highest anti-inflammatory activity due to the presence of phenolic compounds.

Introduction

Indonesia is known as the largest archipelagic country with high biological marine organisms such as red algae (Rhodophyta). The bioactive compounds that have been identified in the organisms include polysaccharides, lipids, polyphenols, steroids, glycosides, flavonoids, tannins, saponins, alkaloids, triterpenoids, anthraquinones, and cardiac glycosides. Currently, chronic inflammatory diseases are known as the leading cause of mortality worldwide, where more than 50% of deaths are often associated with inflammatory cases. During the pandemic, the leading cause of death in COVID-19 patients was a cytokine storm as an uncontrolled inflammatory response. The accumulation of free radicals in tissues can cause oxidative damage to biomolecules and cell membranes, leading to inflammation (Dutta *et al.*, 2020). Antioxidants inhibit oxidative reactions by scavenging reactive free radical compounds to produce stable non-reactive ones. The properties of antioxidants in plants are usually correlated with the presence of phenolic and flavonoid content that can counteract free radicals. Several phenolic compounds in red algae are flavonoids, xanthones, coumarins, carotenoids, phenolic acids, tannins, and anthocyanin (Hmani *et al.*, 2021).

Chan and colleagues conducted a study concerning the antioxidant activity of red algae *Gracilaria changii* by using ethanol, acetone, methanol, and ethyl acetate solvents (Chan *et al.*, 2015). He reported that ethyl acetate was the best solvent, with an IC₅₀ value of 0,51 ± 0,09 mg/mL. Nabil-Adam and Shreadah also...
stated that the red algae Galaxaura oblongata in ethyl acetate fraction had significant antioxidant and anti-inflammatory activity (Nabil-Adam & Shreadah, 2021). Therefore, ethyl acetate should be used to extract bioactive substances that possess fundamental antioxidant activity optimally.

Anti-inflammatory studies of red algae have been carried out on several species, including Sarcodia ceylanica, Gracilaria changii, Sphaerococcus coronopifolius and Eucheuma cotonii (Shu et al., 2013; Abu Bakar et al., 2015; Shih et al., 2017; Salhi et al., 2018). In vitro, anti-inflammatory studies of Eucheuma denticulatum from the East Malaysian coastal region have also been reported (Balasubramanian et al., 2016). However, the anti-inflammatory potential of E. denticulatum from Indonesia must be explored.

Methods
Sample collection and determination
The samples were collected in December 2020 from the Warambadi coast, East Nusa Tenggara, Indonesia, and determined at the Oceanographic research centre of the National Research and innovation agency.

Sample preparation and extraction
The sample was washed thoroughly with fresh water to remove all impurities, such as sand particles, salt, and epiphytes. It was then dried up at room temperature. The dried sample was transported to the Unika Atma Jaya laboratory. It was dried again using an oven at 45°C for 8 hours. Lastly, the samples were pulverised, and the powder was macerated three times with ethyl acetate (1:4 w/v) for 24 hours. The filtrates were concentrated with a rotary evaporator at 40°C.

Antioxidant activity assay
EDEE was dissolved in methanol of 800-1600µg/mL, and 2mL diluted solutions were added with 2mL DPPH solution. The mixtures were vortexed and incubated for 60 minutes, and the absorbance was measured at 516nm using a UV–Vis spectrophotometer. Ascorbic acid of 4-16µg/mL was used as the positive control, and the IC₅₀ value was determined using the linear regression equation.

Total phenolic content (TPC)
The EDEE was dissolved in dimethylsulfoxide (DMSO) (0.1 mg/mL), mixed with 2 mL of Folin-Ciocalteu, and incubated for three minutes (Kim et al., 2003). Subsequently, 2mL of 10% Na₂CO₃ solution was added and allowed to stand for one hour, 25°C. The absorbance was measured at 764 nm using a UV-Vis spectrophotometer, and the total flavonoid content (TFC) value was expressed as mg GA/g extract using the gallic acid standard calibration equation.

Total flavonoid content (TFC)
The EDEE was dissolved in DMSO (0.1 mg/mL) and was added into a solution of 2% aluminium chloride hexahydrate (AlCl₃·6H₂O) in ethanol with the same volume. The mixture was shaken vigorously and incubated for ten minutes at 25°C (Kim et al., 2003), and the absorbance was measured at 436nm using a UV-Vis spectrophotometer. The TFC value was expressed in mg QE/g extract using the quercetin standard calibration equation.

Anti-inflammatory assay
The experimental animal study had been approved by the ethics committee of the School of Medicine and Health Science of Atma Jaya Catholic University with the number: 09/02/FKIKUAI/2022. Male Deutsch denken yoken mice 8-12 weeks old, weighing 20-35 g, were obtained from the faculty of Animal husbandry, IPB University, Indonesia. They were grouped into experimental and control mice (n=4) in each group. The sodium carboxymethyl cellulose (CMC) 1% and mefenamic acid (MFA) 30mg/kg body weight were used as the negative and positive control, respectively. The experimental group was administered with EDEE of 20, 50, and 100 mg/kg body weight. After one hour of oral administration, the mice’s paw was induced subcutaneously with carrageenan 1% (100 µL). Paw oedema volume was measured every hour consecutively for eight hours using a plethysmometer. The percentage of paw oedema volume was calculated using the expression below (Azab et al., 2017):

\[
\text{Percentage of edema} \% = \frac{V_t - V_o}{V_o} \times 100
\]

Where Vo is the paw volume before carrageenan injection (mL), and Vt is the paw volume at t-hour after carrageenan injection (mL). The percentage of anti-inflammatory activity was calculated using the formula (Jayasuriya et al., 2020):

\[
\text{Percentage of anti-inflammatory} \% = \frac{\Delta V_c - \Delta V_t}{\Delta V_c} \times 100
\]

Where ΔVc is the difference in paw volume in the negative control group, and ΔVt is the difference in paw volume in the treatment group.
**Statistical analysis**

The results were analysed among the experimental group of animals, and statistical significance (*p*<0.05) between controls and treated groups was evaluated using multivariate analysis of variance (ANOVA) followed by Tukey’s multiple range test. All experiments were carried out in four replicates. All data are presented as mean ± standard error minimum.

**Results**

The maceration result of 700g sample powder obtained the crude extract of 2.2g of EDEE with a yield of 0.314%. The antioxidant activity testing result was expressed in IC$_{50}$ value (inhibition concentration), which states the concentration of the extract that can reduce 50% activity of DPPH radical. The IC$_{50}$ value of EDEE was 1031.5 ± 58.52 g/mL and higher than the positive control, which was 4.557 g/mL.

The carrageenan injection significantly induced oedema in mice’s paws and was suppressed by MFA. This also occurred in the EDEE of 20, 50, and 100 mg/kg BW groups, where there was a significant decrease in oedema volume (*p*<0.05) compared to negative control at the 5th hour, as shown in Table I and Figure 1a.

![Figure 1](image)

**Table I: The mice’s paw oedema volume after carrageenan induction for eight hours**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>CMC Na</th>
<th>MFA 30 mg/kg BW</th>
<th>EDEE 20 mg/kg BW</th>
<th>EDEE 50 mg/kg BW</th>
<th>EDEE 100 mg/kg BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>0.158 ± 0.01</td>
<td>0.150 ± 0.00</td>
<td>0.158 ± 0.01</td>
<td>0.313 ± 0.02</td>
<td>0.260 ± 0.01</td>
</tr>
<tr>
<td>1 h</td>
<td>0.170 ± 0.01</td>
<td>0.143 ± 0.00</td>
<td>0.153 ± 0.00</td>
<td>0.288 ± 0.02*</td>
<td>0.240 ± 0.01*</td>
</tr>
<tr>
<td>2 h</td>
<td>0.173 ± 0.01</td>
<td>0.138 ± 0.00</td>
<td>0.145 ± 0.00</td>
<td>0.278 ± 0.02*</td>
<td>0.230 ± 0.01*</td>
</tr>
<tr>
<td>3 h</td>
<td>0.170 ± 0.01</td>
<td>0.133 ± 0.00*</td>
<td>0.138 ± 0.00</td>
<td>0.273 ± 0.02*</td>
<td>0.225 ± 0.01*</td>
</tr>
<tr>
<td>4 h</td>
<td>0.170 ± 0.01</td>
<td>0.130 ± 0.00*</td>
<td>0.135 ± 0.00</td>
<td>0.260 ± 0.02*</td>
<td>0.228 ± 0.01*</td>
</tr>
<tr>
<td>5 h</td>
<td>0.164 ± 0.01</td>
<td>0.127 ± 0.00*</td>
<td>0.130 ± 0.00*</td>
<td>0.260 ± 0.02*</td>
<td>0.223 ± 0.01*</td>
</tr>
<tr>
<td>6 h</td>
<td>0.150 ± 0.01</td>
<td>0.120 ± 0.00*</td>
<td>0.128 ± 0.00</td>
<td>0.250 ± 0.02*</td>
<td>0.220 ± 0.01*</td>
</tr>
<tr>
<td>7 h</td>
<td>0.150 ± 0.01</td>
<td>0.113 ± 0.00*</td>
<td>0.125 ± 0.00</td>
<td>0.248 ± 0.03*</td>
<td>0.210 ± 0.01*</td>
</tr>
<tr>
<td>8 h</td>
<td>0.148 ± 0.01</td>
<td>0.105 ± 0.00*</td>
<td>0.120 ± 0.00*</td>
<td>0.248 ± 0.03*</td>
<td>0.207 ± 0.01*</td>
</tr>
</tbody>
</table>

Note: MFA = Mefenamic Acid. EDEE = Eucheuma denticulatum ethyl acetate extract. The paw oedema volume (mL) was expressed as mean ± SEM (n=4).

* = significantly different from negative control (*p*<0.05)

From Figure 1, the mice’s paw oedema volume was calculated as the area under the curve (AUC), where it was negative control (446.30) > EDEE 20mg/kg BW (294.5) > positive control (229.25) > EDEE 100 mg/kg BW (114.175) > EDEE 50 mg/kg BW (86.6). Meanwhile, the reduction in AUC values shows the oedema inhibitory activity.
In Figure 2, it was discovered that the anti-inflammatory properties of MFA were not significantly different from the EDEE 20mg/kg body weight group ($p=0.338$), but varied from the EDEE 50 mg/kg body weight ($p=0.011$) and 100 mg/kg body weight ($p=0.040$). The anti-inflammatory properties of EDEE 20 mg/kg body weight were significantly different from the EDEE 50 mg/kg body weight ($p=0.001$) and EDEE 100 mg/kg body weight ($p=0.002$) groups. However, there was no variation between EDEE of 50 mg/kg body weight with 100 mg/kg body weight ($p=0.878$).

**Figure 2:** (a) Percentage of anti-inflammatory for 8 hours. (b) The AUC values of anti-inflammatory percentage

### Discussion

The IC$_{50}$ of EDEE $(1031.5 \pm 58.52 \, \mu g/mL)$ in this study was different from three previous studies that reported IC$_{50}$ value from ethyl acetate extract of red algae *Eucheuma spinosum* of $430.50 \, \mu g/mL, 402.80 \, \mu g/mL, 384.86 \, \mu g/mL$ respectively (Putri et al., 2019; Inayah & Masruri, 2021; Damongilala et al., 2021). These differences can be influenced by excessive drying time and temperature, reproductive phase, harvest time, and environmental conditions (Holdt & Kraan, 2011; Lumbessy et al., 2020). Similar studies by Novianty and Purbosari, which identified the effect of harvesting age on the phenolic content of the red algae *Eucheuma sp* stated that it had the highest phenolic content at 35 days of harvest (Novianty, 2019; Purbosari et al., 2020). Damongilala and others also stated that the antioxidant activity of *E.denticulatum* ethyl acetate extract was produced by the 3.3-methoxyphenyl-propanal compound with IC$_{50}$ value of $87.97 \, \mu g/mL$ (Damongilala et al., 2021).

The TPC of EDEE was 93.40 ± 1.068 mg GA/g, which is more excellent compared to other solvents such as ethanol, methanol, acetone, and water. Meanwhile, it was also affected by the maceration method, which can produce higher polyphenol content (Chin et al., 2013; Chan et al., 2015). The duration and high temperature during the drying process, species, sunlight, climate, harvest age, and geography also influence the phenolic content (O’Sullivan et al., 2011).

The TFC of the EDEE was $5.87 \pm 0.118 \, mg \, QE/g$ extract, which was smaller than *E.cottonii* ethyl acetate extract of $35.13mg \, QE/g$ and *Gracilaria changii* of $200.87mg \, QE/g$ (Chan et al., 2015; Yanuarti et al., 2017). This is influenced by several factors, such as species, drying method, solubility, genetics, climate, and environmental conditions (Malo et al., 2018). According to a previous study by Ling and colleagues, the methanol extract of the red algae *Kappaphycus alvarezii* had varying TFC values due to the different drying methods (Ling et al., 2015). Similarly, it was also reported that higher drying temperatures produced a lower TFC value because of the decomposition of polyphenolic compounds (Syafarina, Taufiqurrahman, & Edyson, 2019).

Carrageenan injection caused oedema, which was characterised by a biphasic mode of action. In the first phase, between one and two hours, inflammation is associated with the production of inflammatory mediators such as histamine, serotonin, and
bradykinin, while the prostaglandins, leukotrienes, and free radicals were produced in the second phase (three - six hours). Furthermore, nitric oxide (NO) free radicals are also formed and diffuse into smooth muscle blood vessels, as well as activate guanylate cyclase. This causes an increase in cGMP levels in intracellular and vascular permeability, leading to the exudation of plasma proteins and fluids into the tissue, which becomes oedema (Jayasuriya et al., 2020).

Figures 1(a) and 1(b) show that MFA has anti-inflammatory activity by significantly reducing the percentage of oedema at three to eight hours (p<0.05). MFA is an NSAID drug that works by inhibiting cyclooxygenase (COX). Moreover, COX is an essential enzyme responsible for converting arachidonic acid into prostaglandins by stimulating proinflammatory cytokines (TNF-α, IL-1β, IL-6, IFN-γ) (Ghlichloo & Gerriets, 2022). The EDEE 20mg/kg body weight also had the same potency as MFA. However, the EDEE of 50 and 100mg/kg body weight were more potent in reducing paw oedema volume between one to eight hours. Its activity can be due to the inhibition of COX synthesis; however, further investigation is required.

This study found that EDEE from the Warambadi coast had a relatively high phenolic content. The anti-inflammatory activity of phenolic and flavonoid compounds has been studied through several mechanisms, including regulation of cellular activity inside cells responsible for inflammation and modulation of the inhibition of enzyme activity involved in the metabolism of arachidonic acid (phospholipase A2, COX) and arginine (NOS). At the molecular level, the mechanism of anti-inflammatory activity of polyphenolic compounds was related to the inhibition of enzymes associated with proinflammatory properties and through the activation of mitogen-activated protein kinases (MAPK), Protein kinase C (PKC), and erythroid factor 2-related to nuclear factor (Hussain et al., 2016). In addition, phenolic compounds were also known to be able to inhibit the production of cytokines (IL-1β, TNF-α, and IL-33) and suppress the activation of the Nf-kB signalling pathway so that transcription of genes that play a role in the inflammatory response can be suppressed (Di Marzio et al., 2016). Other constituents contained in red algae, such as 2,5 dimethyl-hexane-2,5 dihydropсорoxide and polysunsaturated fatty acids (PUFA) were also known to have anti-inflammatory activity. Thus, based on the current result study, it can be said that the high polyphenol content of EDEE had a potential role in its anti-inflammatory activity.

**Conclusion**

EDEE 50mg/kg body weight had the highest anti-inflammatory activity. However, further investigation of its bioactive compound, safety, and specific mechanisms is recommended.

**Acknowledgement**

This article was presented at the 2022 Annual Scientific Conference of the Indonesian Pharmacist Association.

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Pharmacy Education 23(2) 216 - 222


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