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RESEARCH ARTICLE

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 $AlCl_3$ 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 (IC_{50}) of EDEE was 1031.5 ppm with a total phenolic and flavonoid content of 81.34 ± 0.996mg 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 IC_{50} 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 *Euchema denticulatum* from the East Malaysian coastal region have also been reported (Balasubramaniam *et al.,* 2016). However, the anti-inflammatory potential of *E. dentilculatum* 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^{o}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^{o}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 IC_{50} 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% Na_2CO_3 solution was added and allowed to stand for one hour, 25^{o}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 hexa-hydrate ($AlCl_3.6H_2O$) in ethanol with the same volume. The mixture was shaken vigorously and incubated for ten minutes at 25^{o}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/KEP-FKIKUAJ/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):

$$\boldsymbol{Percentage\ of\ edema}\ (\%) = \frac{\boldsymbol{Vt - Vo}}{\boldsymbol{Vo}}\boldsymbol{x100}$$

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):

$$\boldsymbol{Percentage\ of\ antiinflammatory}\ (\%) = \frac{\boldsymbol{\Delta Vc - \Delta Vt}}{\boldsymbol{\Delta Vc}}\boldsymbol{x100}$$

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 extract 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 ± 58.52g/mL and higher than the positive control, which was 4.557g/mL.

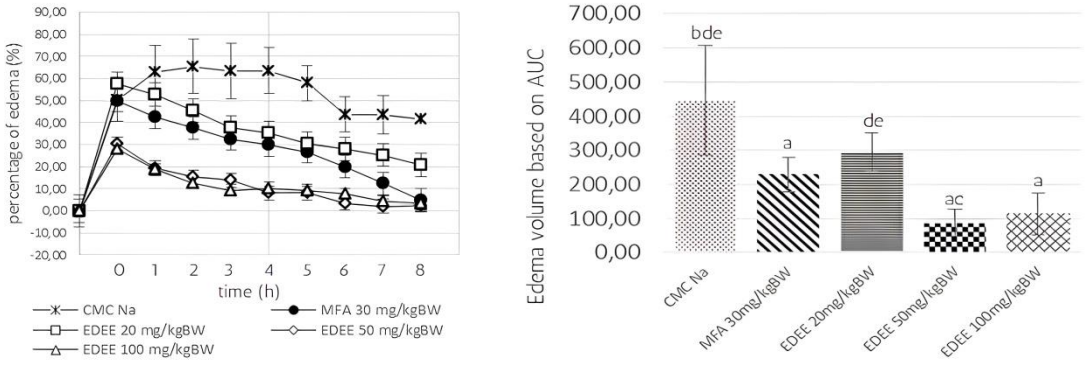
The analysis of the total phenolic content of EDEE was shown as gallic acid equivalent (GAE) and flavonoids as quercetin equivalents (QE). The total phenolic and flavonoid content of EDEE was 93.40 ± 1.068 mg GA/g extract and 5.87 ± 0.118mg QE/g extract, respectively. The carrageenan injection significantly 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.

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.

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

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

Note: MFA = Mefenamic Acid. EDEE = *Euchema denticulatum* ethyl acetate extract. The paw oedema volume (mL) was expressed as mean ± SEM (n=4).
* = significantly different from negative control (p< 0.05)

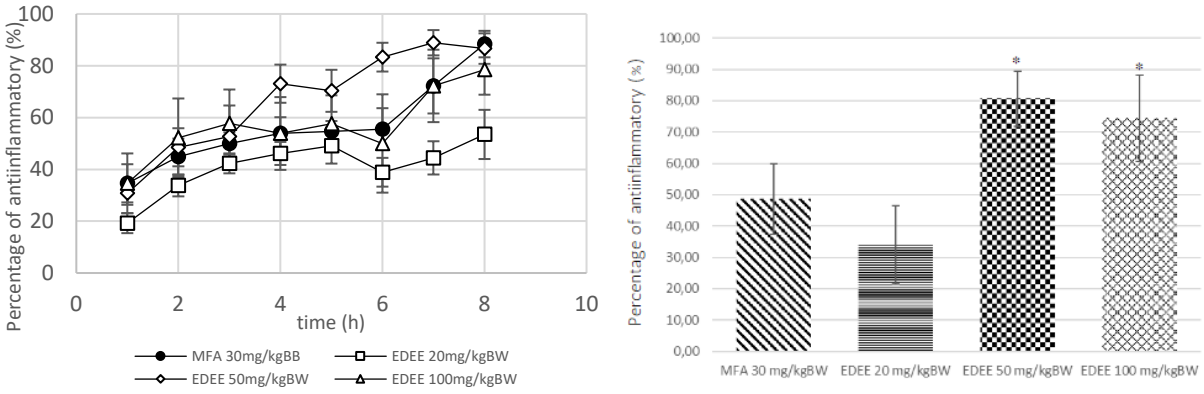


(a) (b)

Data were presented in mean ± SEM (n=4). a = significant difference (p<0.05) compared to negative control. b = significant difference (p<0.05) compared to positive control. c = significant difference (p<0.05) compared to EDEE 20 mg/kg body weight. d = significant difference (p<0.05) compared to EDEE 50 mg/kg body weight. e = significant difference (p<0.05) compared to EDEE 100 mg/kg BW.

Figure 1: (a) Percentage of oedema volume for 8 hours. (b) The AUC values of oedema volume

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).



(a) (b)

Data were presented in mean ± SEM (n=4). * indicates significant difference (p<0.05) compared to positive control

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 ± 58.52 µg/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 µg/mL, 402.80 µg/mL, 384.86 µg/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 µg/mL (Damongilala *et al.,* 2021).

The TPC of EDEE was 93.40 ± 1.068 mg GA/g, which is more excellent than the study by Sofiana and colleagues (Sofiana *et al.,* 2020), which found the TPC of *E. denticulatum* ethanol extract was only 16.47 ± 0.14 mg GA/g extract. Similar results of TPC value from another species of red algae were found by Chan and colleagues in 2015, which stated that the TPC of *Gracilaria changii* ethyl acetate extract was the highest 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 ± 0.118 mg QE/g extract, which was smaller than *E.cotonnii* 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 have 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-, and IL-33) and suppress the activation of the Nf-κB 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 dihydroperoxide and polyunsaturated 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.

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