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Anti-inflammatory and antioxidant properties of *Sargassum polycystum* ethyl acetate extract from Indonesia

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ABSTRACT

Introduction: Inflammatory diseases, including asthma, osteoarthritis, rheumatoid arthritis, and cancer persist with relatively high incidence, which highlights the need to explore natural alternatives such as the bioactive substances of brown algae (*Sargassum polycystum*). Certain substances, such as phenolics and flavonoids, have been demonstrated to have an association with antioxidant activity because of their capacity to fend off free radicals, which cause inflammation. The purpose of this study was to look into the inflammatory inhibition and antioxidant activity of an ethyl acetate extract of *S. polycystum* from Indonesia.

Methods: After maceration in ethyl acetate (1:4 w/v) for 24 hours, the total phenolic and flavonoid content of *S. polycystum* extract was measured using the colorimetric method. The antioxidant activity was tested using 1,1-diphenyl-2-picrylhydrazyl (DPPH), and the models of paw edema induced by carrageenan were used to assess inflammatory inhibition activity.

Results: The findings revealed that *S. polycystum* had a weak antioxidant with a half-maximal inhibitory concentration (IC_{50}) value of 605.24 ± 26.53 g/mL. Furthermore, it had 23.509 ± 0.109 mg gallic acid equivalents/gram (GAE/g) and 15.186 ± 0.046 mg quercetin equivalents/gram (QE/g) of total phenolic and flavonoid compounds, respectively. At 200, 400, and 800 mg/kg BW doses, *S. polycystum* had inflammatory inhibition percentages of 53.80 ± 4.68%, 62.35 ± 4.05%, and 60.90 ± 2.88%, respectively.

Conclusion: *Sargassum polycystum* ethyl acetate extract inhibited inflammation. However, further study is needed to examine the safety, identification, separation of the relevant substances involved, and the precise mechanism of action.

Implication for health policy/practice/research/medical education:
This research provides scientific proof that *Sargassum polycystum* from Indonesia has the potential as a natural anti-inflammatory agent, which can be developed into a dosage form for clinical uses.

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Introduction

The prevalence of chronic inflammatory diseases continues to increase and threaten human health. Chronic inflammation has been linked to advancing the progression of various diseases, including neurodegenerative disease, cardiovascular disease, autoimmune disease, cancer, and asthma. The accumulation of free radicals in cells is a contributing factor to inflammation, which leads to oxidative damage to biomolecules and cells (1,2). Meanwhile, antioxidants can interact with reactive free radical compounds to produce non-reactive free radical compounds that are relatively stable (1). The phenolic and flavonoid content in plants generally correlates with antioxidant activity. Phenolic and flavonoid compounds are known to have inflammatory inhibition activity, which is attributable to their chemical structure. The presence of unsaturated carbon rings can stabilize free radical species in the body via a resonance mechanism (3-5). Brown algae contain phenolic compounds, including flavonoids, phlorotannins, fucoxanthin, cinnamic acid, and benzoic acid (4,6). Several studies have been declared on various biological activities of brown algae species like

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anticancer, antibacterial, antidepressant, antidiabetic, and wound healing (7-11). Previous studies have also reported the inflammatory inhibition of hexane, chloroform, and ethanol extracts of *Sargassum swartzii* (6,12). In addition, there have been several studies suggesting that the ethyl acetate fraction can exhibit the highest antioxidant activity because of its ability to extract polyphenol and carotenoid compounds better than other polar solvents such as methanol, ethanol, water, and acetone. Currently, there paper demonstrating the inflammatory inhibition activity of *S. polycystum* from Indonesia utilizing the ethyl acetate fraction. Therefore, we performed this study to develop it as a natural therapeutic agent for public health benefits.

Materials and Methods

Tools

In this study, the tools used were a set of laboratory glassware, analytical balance (Mettler Toledo), oven (Memmert), orbital shaker (Yih Der TS520-D), rotary evaporator (Heidolph – Hei Vap), hotplate (Heidolph), vortex (Vortex Mixer Heidolph EU 0416), sonicator (Branson M1800-E), UV-Vis spectrophotometer (Shimadzu A11635480009ML), and plethysmometer (PANLAB Pletismometer 2866919).

Chemicals and reagents

The chemicals and reagents that were used consisted of ethyl acetate (Merck, German), methanol (Merck, German), ethanol (Merck, German), Whatman filter paper, DPPH (Sigma-Aldrich, USA), DMSO (Merck, German), ascorbic acid (Kanto Chemical Co., Japan), $AlCl_3.6H_2O$, quercetin (Sigma-Aldrich, USA), Folin's reagent Ciocalteu (Merck, German), sodium carbonate (SmartLab, Indonesia), gallic acid (Merck, German), kappa carrageenan (CV.Karagen Indonesia), and mefenamic acid (Ponstan®, Pfizer).

Preparing the extraction of *Sargassum polycystum*

Sargassum polycystum was collected in December 2020 from Ria Beach, Dompu Regency, West Nusa Tenggara, Indonesia. The taxonomical identification was performed at the Oceanographic Research Center of the National Research and Innovation Agency and a herbarium specimen was deposited there with the number: B-7523/IPK.2/IF.07/XI. *S. polycystum* was thoroughly washed with fresh water to get rid of impurities such as epiphytes, sand particles, and salt. It was then dried at room temperature and transported to the Unika Atma Jaya Laboratory to dry at 45°C for 8 hours using an oven. Finally, the sample was pulverized using a grinder and macerated with ethyl acetate (1:4 w/v) for 24 hours. The filtrate was evaporated with a rotary evaporator at 40°C.

Total phenolic content (TPC)

The ethyl acetate extract solution of *S. polycystum* (0.4 mL) was dissolved in DMSO (1 mg/mL), and 2 mL of Folin-Ciocalteu (10% v/v) was added in distilled water and incubated for 5 minutes. Then, 2 mL of Na_2CO_3 solution (10% w/v) was added and left for 1 hour at room temperature (13,14). The absorbance was analyzed at 764 nm with a spectrophotometer UV-Vis. The TPC was expressed as mg GAE/mg extract.

Total flavonoid content (TFC)

The concentrated ethyl acetate extract solution of *S. polycystum* was dissolved in DMSO (1 mg/mL), and $AlCl_3.6H_2O$ (2% w/v) was added in the same volume. The mixture was mixed and incubated for 10 minutes at room temperature (13,15). The absorbance was analyzed at 436 nm with a UV-Vis spectrophotometer. The TFC was expressed as mg QE/mg extract.

Antioxidant assay

To ethyl acetate extract solution of *S. polycystum* (2 mL) with a concentration range of 800 - 2400 µg/mL in methanol, 2 mL of DPPH solution was added, vortexed, and incubated for 1 hour. The absorbance was measured with a UV-Vis spectrophotometer at 516 nm; ascorbic acid was used as a positive control at concentrations of 4-16 µg/mL in methanol. Furthermore, the IC_{50} values of the *S. polycystum* extract and ascorbic acid were determined using a linear regression equation.

Inflammatory inhibition activity

Male mice Deutschland, Denken, and Yoken (DDY), 6-8 weeks old and weighing 20-35 grams, were acquired from the Faculty of Veterinary Medicine, Bogor Agricultural Institute (IPB), acclimatized for one week before testing, and divided into five treatment groups (n = 6). The negative control group received CMC Na (0.1% w/v). The positive control group received mefenamic acid (30 mg/kg BW). The treatment groups received *S. polycystum* at 200, 400, and 800 mg/kg BW doses. One hour after oral administration of the test material, carrageenan solution (1% w/v) was injected subcutaneously into the left hind paw of the mice. Observation and measurement of paw edema volume were carried out every 1 hour using a plethysmometer for 8 hours. The percentage change in the volume of paw edema was calculated using the following formula (12):

$$Edema\ percentage\ (\%) = (V_t - V_o)/V_o \times 100$$

Vo is the paw volume of pre-carrageenan injection (mL) and Vt is the paw volume of post-carrageenan injection at *t* time (mL).

The percentage change in edema volume was used to calculate the Area under the Curve (AUC) as follows (16):

$$AUC_n = ([\%E_n + \%E_{n-1}] \times [t_n - t_{n-1}]) / 2$$

AUC_n is the AUC at *n* hours, $\%E_n$ is the percentage of edema at time *n*, $\%E_{n-1}$ is the percentage of edema at time n-1, t_n is the time at n, and t_{n-1} is the time at n-1. The AUC value from 0 to 8 hours was used to calculate the percentage of inflammatory inhibition using the following equation (16):

$$Inflammatory\ inhibition\ (\%) = ([AUC_c - AUC_t]/AUC_c) \times 100$$

AUC_c is the average AUC of mice treated with the test substance, and AUC_t is the average AUC of the negative control mice.

Statistical analysis

Inflammatory inhibition effect test data was carried out using one-way analysis of variance (ANOVA) and continued with Tukey's post hoc test based on cut-off values significance at $P < 0.05$.

Results

The maceration of 1.450 g of *S. polycystum* powder with ethyl acetate (1:4 w/v) yielded 4.52 g of the extract with a brownish-green color (0.31% yield). The antioxidant testing of *S. polycystum* extract resulted in an IC_{50} value of 605.241 ± 26.533 µg/mL, which was more significant than that of the positive control (5.017 ± 0.407 µg/mL). The results of its TPC and TFC were 23.509 ± 0.109 mg GAE/g extract and 15.186 ± 0.046 mg QE/g extract, respectively. Subcutaneous injection of carrageenan could cause significant edema in the paw of mice. The percentage volume results of mice paw edema at mefenamic acid and *S. polycystum* (200, 400, and 800 mg/kg BW) groups were considered significantly distinct from the negative control ($P < 0.05$) during the 1st to 8th hour after carrageenan induction (Table 1, Figure 1a).

The percentage (%) of edema volume was used to determine the AUC value. A decrease in the AUC value indicated the presence of edema inhibition activity with the following calculation results: negative control (455.57 ± 27.58) > *S. polycystum* 200 mg/kg BW (214.44 ± 19.16) > positive control (178.61 ± 15.51) > *S. polycystum* 800 mg/kg BW (178.38 ± 8.00) > *S. polycystum* 400 mg/kg BW (174.82 ± 17.62).

The inflammatory effect of the positive control (mefenamic acid) was not significantly different compared to *S. polycystum* 200 mg/kg BW ($P = 0.144$), 400 mg/kg BW ($P = 0.520$), and 800 mg/kg BW ($P = 0.866$). Meanwhile, the inflammatory inhibition of *S. polycystum* 200 mg/kg BW was significantly different from 400 mg/kg BW ($P = 0.010$), and 800 mg/kg BW ($P = 0.034$). There was no statistically significant difference between *S. polycystum* 400 mg/kg BW and *S. polycystum* 800 mg/kg BW ($P = 0.923$).

Discussion

Based on the IC_{50} value (605.241 ± 26.533 g/mL), the ethyl acetate extract of *S. polycystum* can be assumed as a weak antioxidant, although it still requires more accurate testing. These results differed from the results of Arsianti et al. (298.32 g/mL), Hidayati et al. (102.4 ± 0.056 g/mL), and Gazali et al. (68.89 ± 5.36 g/mL) (17-19). This difference might be influenced by variations of algae growth conditions (location, light intensity, water salinity, water quality, etc) and differences in the extraction method, such as the ratio of solvent, maceration temperature, agitation or sonication, and duration of maceration.

Pramesti et al mentioned that the total phenolic contents of the ethyl acetate extracts of *S. duplicatum*, *S. crassifolium*, and *S. polycystum* had the highest antioxidant activities compared to hexane and methanol extracts (20). The total phenolic content value of the ethyl acetate extract of *Sargassum polycystum* was 23.509 ± 0.109 mg GAE/g, which was lower than the ones of Arsianti et al (548.61 mg GAE/g extract), Hidayati et al (120.29 ± 0.404 mg GAE/g extract), and Gazali et al (1.348.18 ± 2.57 mg GAE/g

Table 1. Effects of various doses of *Sargassum polycystum* at different times on the volume percentage (%) of post-carrageenan-induced paw edema

Time	Negative control	Positive control	*Sargassum polycystum* 200 mg/kg BW	*Sargassum polycystum* 400 mg/kg BW	*Sargassum polycystum* 800 mg/kg BW
0 h	54.50 ± 1.44[b]	52.18 ± 6.66	53.49 ± 5.76	52.85 ± 15.29	52.33 ± 14.14
1 h	55.41 ± 0.85[b]	53.93 ± 2.00	42.30 ± 3.02[ab]	38.19 ± 1.70[ab]	38.70 ± 1.13[ab]
2 h	57.14 ± 0.98[b]	28.32 ± 2.11[a]	37.38 ± 1.49[ab]	29.38 ± 2.93[a]	34.01 ± 1.70[ab]
3 h	58.31 ± 0.98[b]	23.85 ± 2.98[a]	32.43 ± 2.69[ab]	23.28 ± 2.54[a]	23.79 ± 1.94[a]
4 h	59.77 ± 1.06[b]	21.30 ± 2.57[a]	24.60 ± 3.27[a]	18.78 ± 2.93[a]	18.25 ± 2.60[a]
5 h	62.88 ± 2.65[b]	17.15 ± 2.95[a]	18.17 ± 2.64[a]	16.23 ± 2.72[a]	15.73 ± 2.08[a]
6 h	60.27 ± 5.15[b]	11.01 ± 1.82[a]	15.70 ± 2.70[a]	12.68 ± 2.12[a]	11.50 ± 1.53[a]
7 h	57.57 ± 4.21[b]	6.58 ± 2.32[a]	13.23 ± 2.78[ab]	7.65 ± 3.25[a]	10.18 ± 1.42[a]
8 h	52.28 ± 3.01[b]	3.26 ± 2.28[a]	7.75 ± 2.94[a]	4.38 ± 2.27[a]	6.33 ± 1.88[a]

Mice paw edema volume expressed as mean ± SD (n = 6).
a = significantly different from the negative control ($P < 0.05$ with Tukey post hoc test).
b = significantly different from the positive control ($P < 0.05$ with the Tukey post hoc test).

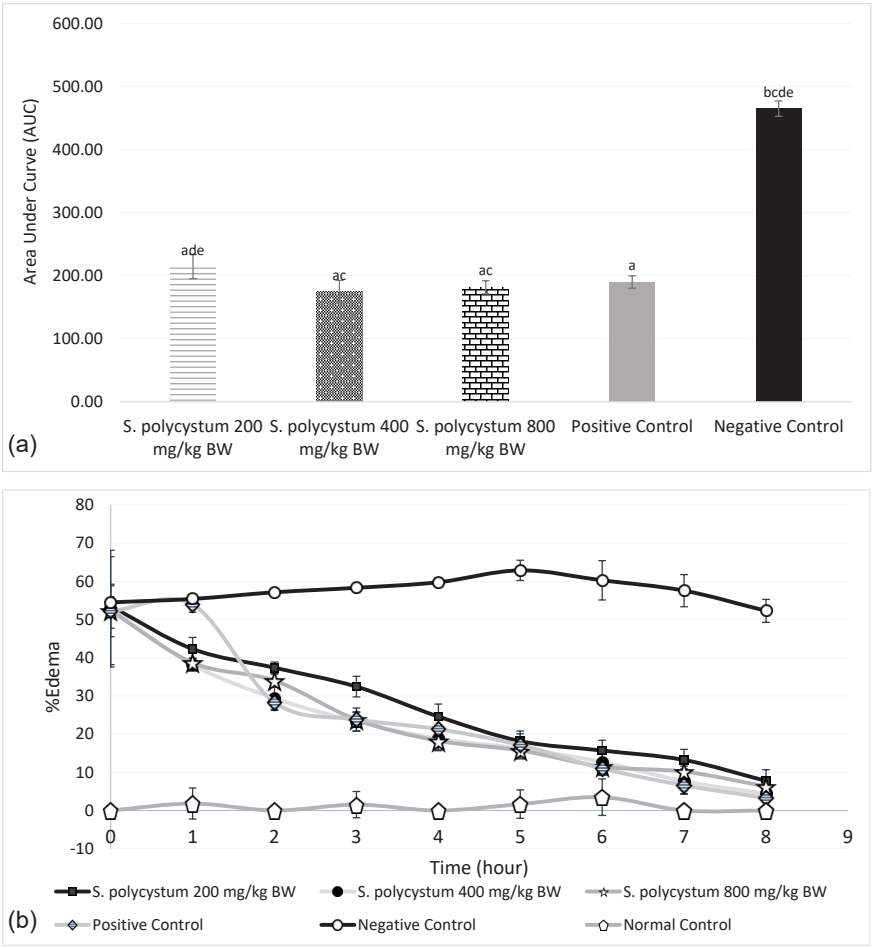

Figure 1. *Sargassum polycystum* treatment of mice paw edema for 8 hours following carrageenan injection. **(**a) Percentage of paw edema volume. (b) Area Under Curve (AUC) diagram of changes in paw volume of treated mice. All doses of *S. polycystum* provided edema inhibition activity, which was significantly different from the negative control group. Data were presented in mean ± SD (n = 6). a = significantly different ($P < 0.05$) from the negative control. b = significantly different ($P < 0.05$) from the positive control. c= significantly different ($P < 0.05$) from *S. polycystum* 200 mg/kg BW. d = significantly different ($P < 0.05$) from *S. polycystum* 400 mg/kg BW. e = significantly different ($P < 0.05$) from *S. polycystum* 800 mg/kg BW.

extract) (17-19). Tryptophan, thiols, redox-active metal ions, and nucleotide bases are all interfering substances that decrease Folin reagents and inflate the final readings. The limitations of this Folin reagent may be overcome by the application of analytical techniques that are more focused on phenolic chemicals (21). Yield differences can be influenced by the environmental conditions in which it grows, species differences, reproductive phase, harvest time, and extraction methods (17-19,22,23).

Pramesti et al previously discovered a link between total phenolic content and antioxidant activity, where higher total phenolic content resulted in a more excellent antioxidant activity (20). Furthermore, Maulina et al also reported that temperature and drying methods resulted in different values of total phenolic content (24). The differences in maceration methods can also affect the value of total phenolic content (25).

In this study, total flavonoid content (15.186 ± 0.046 mg QE/g extract) was lower than Arsianti et al (40.06 mg QE/g extract) and Pratiwi et al (107.66 mg QE/g extract), while it was higher than Soleimani et al (3.79 ± 0.01 mg QE/g extract) (17,26,27). This difference in results might be due to the geographical location of the algae and differences in maceration methods. Furthermore, Obluchinskaya et al reported that the brown algae *Fucus vesiculosus* had a higher total flavonoid content and antioxidant activity at certain growth phases (28).

The mechanism of carrageenan in inducing inflammation locally in mice paws can be represented by a biphasic curve. Bradykinin, histamine, vasoactive amine compounds, and serotonin are released during the first phase of inflammation. In contrast, the second phase involves the release of other inflammatory mediators, such as leukotrienes, prostaglandins, and nitric oxide (NO) free radicals. Furthermore, NO can penetrate smooth muscle blood vessels and cause an increase in vascular permeability leading to plasma fluid and protein exudate and edema formation.

The potential of mefenamic acid as an anti-inflammatory agent is shown in Figure 1a, where mefenamic acid

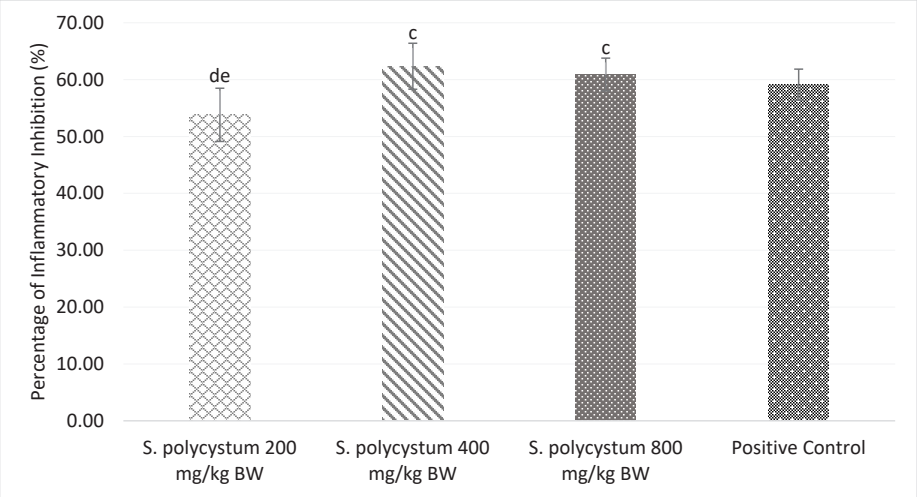

Figure 2. Diagram of the percentage of inflammatory inhibition in mice paw for 8 hours. The percentage of inflammatory inhibition of *S. polycystum* (200, 400, 800 mg/kg BW) and mefenamic acid (30 mg/kg BW) showed no significant differences. Data were presented as mean ± SD (n = 5). a = significantly different ($P < 0.05$) from the negative control. b = significantly different ($P < 0.05$) from the positive control. c = significantly different ($P < 0.05$) from *S. polycystum* 200 mg/kg BW. d = significantly different ($P < 0.05$) from *S. polycystum* 400 mg/kg BW. e = significantly different ($P < 0.05$) from *S. polycystum* 800 mg/kg BW.

significantly reduced the percentage of edema from the 2nd to 8th hour ($P < 0.05$) and in Figure 1b, where mefenamic acid produced a lower AUC than the negative control ($P < 0.05$). Positive control (mefenamic acid) is a NSAID that suppresses the inflammatory response by inhibiting the cyclooxygenase (COX) enzyme. Subsequently, COX is the enzyme that catalyzes the reaction of arachidonic acid to prostaglandins by stimulating proinflammatory cytokines, such as chemokines, IL-6, IL-1β, and TNF-α.

Sargassum polycystum (200, 400, and 800 mg/kg BW) also inhibited the inflammation of mice paw edema volume compared to the negative control group ($P < 0.05$). The percentages of inflammatory inhibition of *S. polycystum* 200, 400, and 800 mg/kg BW, as well as mefenamic acid 30 mg/kg BW were 53.80 ± 4.68%, 62.35 ± 4.05%, 60.90 ± 2.88%, and 59, 11 ± 2.74%, respectively. Based on the Tukey's post hoc analysis (Figure 2), the inflammatory inhibition of *S. polycystum* 200 mg/kg BW ($P = 0.144$), 400 mg/kg BW ($P = 0.520$), and 800 mg/kg BW ($P = 0.866$) were not significantly different from mefenamic acid.

Conclusion

Sargassum polycystum from Ria Beach, West Nusa Tenggara, Indonesia, has anti-inflammatory activity with the highest inhibition percentage of 62.35 ± 4.05% at a dose of 400 mg/kg BW. Due to the moderate levels of total phenolics and flavonoids in the ethyl acetate extract of *S. polycystum,* other bioactive substances besides phenolic and flavonoid compounds are believed to have a significant role in providing anti-inflammatory benefits. The safety and identification testing, isolation of causative compounds, and precise mechanism of action require further study.

Authors' contribution

EAS designed the project and the main conceptual ideas and wrote the original manuscript, while SIG performed all of the experiments, analyzed the data, and wrote and edited the manuscript.

Conflict of interests

The authors have stated that there is no conflict of interest.

Ethical considerations

This experimental animal study was approved by the Ethics Committee of the School of Medicine and Health Science of Atma Jaya Catholic University with the number: 13/04/KEP-FKIKUAJ/2022.

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