Effects of Skeletonema Costatum's Powder on the Knee Joint's Diameter and the Degree of Pain of Male Rat Sparague Dawley Type Induced by Complete Freund's Adjuvant

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Abstract: Osteoarthritis is a degenerative disease caused by an abnormal inflammatory cytokine response. Complete Freund's Adjuvant (CFA) is a chemical substance that induces osteoarthritis. Bioactive compounds in Skeletonema costatum act as antioxidants, which could lessen inflammation. This study aims to determine how Skeletonema costatum affects the knee joint's diameter size and the degree of pain induced by CFA in rats. The study used thirty adult male rats (Sparague dawley), 2-3 months old, weighing 200-300 grams, divided into five groups: negative control (K1), positive control/piroxicam (K2), and intervention groups (K1, K2, and K3). All groups were induced with CFA, and the intervention began six weeks after induction. The negative and positive groups received normal saline and piroxicam (10 mg/kg BW/day) for 21 days, respectively. Skeletonema costatum was given to the intervention groups at doses of 60 mg/kg BW/day (K3), 90 mg/kg BW/day (K4), and 120 mg/kg BW/day (K5) for 21 days. The knee joint diameters and the degree of pain were assessed using a micrometer screw and a hot water tail-flick assay on days 0, 7, 14, and 21. The results were examined using the paired-t test, Kruskall-Wallis, and post hoc Mann-Whitney. Skeletonema costatum lowered the diameters and pain levels of rats' knee joints before and after the trial (paired t-test, $p<0.05$). All dosage groups demonstrated a beneficial effect (Kruskal Wallis, $p<0.05$); the higher the dose of Skeletonema costatum, the greater the effect on reducing the knee joint's diameter and pain. A multivariate analysis showed the reduction of knee joint diameter and pain after Skeletonema costatum's intervention was statistically greater compared to the control/placebo group. However, this effect was lower than in the piroxicam group ($p< 0.05$). Skeletonema costatum may have an anti-inflammatory effect in rats by lowering the size of the knee joints and the degree of pain induced by CFA. Given the potential of Indonesian marine products and the trend toward marine drug treatments, further studies are required to investigate the inflammatory effects of Skeletonema costatum.

1 INTRODUCTION

Osteoarthritis (OA) is a chronic degenerative disease that commonly affects older people and someone who uses their joint too much, which causing joint inflammation. Osteoarthritis usually affects the interphalangeal, hip, knee, spinal, and temporomandibular joints (Kasper, 2015; Gs M, 2014; Poulet, 2017; Gupta, 2017). Common symptoms of OA include pain and limitation of daily activities due to cartilage damage.

The incidence of OA under the age of 40 is minimal and is commonly due to trauma. The prevalence of OA increases between the age of 40-60 years. Globally, it is estimated that 9.6% of men and 18% of women over the age of 60 have symptomatic osteoarthritis. According to the *World Health Organization* (WHO) in 2010, OA affects more women than men in all age groups (2.95 women per 1000 population and 1.71 men per 1000 population). In women, the age group of 65-74 years has the highest incidence, about 13.5 per 1000 population per year. In men, the incidence in people over 75 years of age is about 9 per 1000 population per year. In Indonesia, the prevalence of OA based on radiological findings is 15.5% in males aged 40-60 years and approximately 12.7% in females. According to WHO, the elderly population in Indonesia is expected to increase by 414% compared

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to 1990 by 2025 due to the increase in life expectancy in Indonesia.

The development of OA is influenced by many risk factors such as: continuous mechanical stress occurs in multiple parts of joints, inducing the release of pro-inflammatory mediators and degradation enzymes such as IL-1, IL-6, IL- 1 β, NO, and TNF-α, resulting in joint inflammation (McCance, 2014).

Inflammation results from an imbalance between pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory mediators induce the formation of *reactive oxidative stress* (ROS), leading to exacerbated inflammation. Another consequence of the release of pro-inflammatory mediators is that the chondrocyte components in the turnover process become imbalance and the joint undergoes excessive destructive processes leading to chondrocyte apoptosis (McCance, 2014). Increased chondrocyte apoptosis reduces the number of proteoglycans in cartilage. Bonds between collagens weaken due to decreased synthesis of type II collagen and increased collagen degradation.

Molecules resulting from the breakdown of collagen and proteoglycans are destroyed by synovial macrophages causing an increase in the number of pro-inflammatory cytokines. These cytokines bind to chondrocyte receptors and trigger the release of *metalloproteinase* (MMP), inhibiting type II collagen production and continuing cartilage degradation. In response to mechanical and biochemical stimuli, chondrocytes overproduce its MMP, collagenase, *stromelysin,* and gelatinase, causing greater joint damage. *Metalloproteinase* also trigger the release of ROS, namely hydrogen peroxide, *hypochlorite ion*, *hydroxyl radical,* or *superoxide anion*[5]. Chondrocytes normally produce small amounts of *nitric oxide* (NO) and *superoxide anion*. These two ions will form *peroxynitrite* and *hydrogen peroxide*. *Hydrogen peroxide* can convert its form to *hydroxyl radical*, forming *lipid peroxide* in chondrocytes and causing further degradation (Gupta, 2017).

Our cells can naturally reduce excess ROS by using antioxidants. Antioxidants are divided into two groups: enzymatic and non-enzymatic. Antioxidants use *superoxide dismutase* (SOD), *catalase* (CAT), *glutathione peroxidase* (GPX) and some fat- and water soluble small molecules to prevent the formation of ROS. It acts as a ROS scavenger by repairing damage that has occurred (Gupta, 2017; Sun AR, 2017). However, when the antioxidant capacity is reduced, ROS damage the extracellular matrix, nucleus, and cell membrane, leading to cell death, causing dead cells to release oxidative molecules, triggering the release of synovial macrophages, pro-inflammatory cytokines, ROS and MMP, which lead to chronic inflammation (Sun AR, 2017).

Initial treatment of OA uses an oral *non-steroidal anti-inflammatory drug* (NSAID) such as piroxicam. It aims to reduce the inflammatory response that occurs in the joints. Additionally, corticosteroid injections, oral administration of glucosamine and chondroitin sulfate can reduce joint inflammation. It acts as an anti-inflammatory agent, causes a scavenging effect, and plays a role in lipid peroxidase mechanism by exploiting the mechanism of reducing ROS production (Gunawan SG, 2016). Because NSAID therapy adversely affects the gastrointestinal system when taken long term (Gunawan SG, 2016). Another alternative from a marine alga, *Skeletonema costatum,* may help reduce the inflammatory effects of OA.

Antioxidants are protective agents which inhibit disease progression and the formation of ROS from chondrocytes, thus reducing the progression of OA. *Skeletonema costatum* is a microalga belonging to the class *Coscinodiscophyceae* and the *Skeletonemacea* family (Miyashita K, 2009). This microalgae contains antioxidants such as carotenes and unsaturated fatty acids. The types of carotene they contain include *fucoxanthin* and astaxanthin (Miyashita K, 2009; Foo SC, 2017). The total *fucoxanthin* contained in this microalgae is 0.36 ± 0.00 with a total carotene of 0.97 ± 0.24 (Foo SC, 2017). While levels of omega 3: 0.911 – 3.738%; omega 6: 15.591 – 38.002% and omega 9: 0.292 – 15.112% (Erlina A, 2004). The antioxidants in *Skeletonema costatum* act as anti-inflammatory agents by reducing intracellular ROS, SOD, and NO levels, increasing type II collagen synthesis and enabling joint repair.

In the present study, CFA induction can induce inflammation due to OA after 6 weeks after intra-articular injection. No research explains the anti-inflammatory activity by *Skeletonema costatum,* based on data sources, so it is necessary to research to examine the effect of *Skeletonema costatum* on the diameter of the knee joint and the value of pain in rats induced by *Complete Freund's Adjuvant* (CFA).

2 METHOD

This type of research was conducted using a laboratory experimental method with a pre-post-study design and a post-test-only control group design. The research was conducted in January-February 2020 at the Nutrition Laboratory of the Center for Food and Nutrition Studies, Gadjah Mada University, and obtained research ethics permit from

the Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Gadjah Mada University with protocol number KE/0851/07/2019.

The inclusion criteria used in this study were adult male rats of the *Sprague Dawley strain*, aged 3-4 months and weighing 200-300 grams, the rats were obtained from the same breeding grounds with the same food and drink, the rats were healthy and had no marked defects with the animal trying to move actively, the fur looks clean and doesn't fall out. Meanwhile, the exclusion criteria were sick and dead rats during the study.

The grouping of the sample was carried out using the simple random sampling method. The size of the research sample is determined by the *Federer* formula.

Thirty adult male rats (*Sparague dawley*), 2-3 months old, weighing 200-300 grams, were utilized in the study and were divided into five groups: negative control (K1), positive control (K2), and intervention groups (K1, K2, and K3). CFA was used to induce all the groups, and the intervention started six weeks after induction. Normal saline and piroxicam 10 mg/kg BW/day for 21 days were administered to the negative and positive groups. *Skeletonema costatum* was administered to the intervention groups for 21 days at various dosages of 60 mg/kg BW/day (K3), 90 mg/kg BW/day (K4), and 120 mg/kg BW/day (K5). On days 0, 7, 14, and 21, the groups joint diameter and pain levels were measured using a micrometer screw and a hot water tail-flick assay. A paired-t test, Kruskall-Wallis, and post hoc Mann-Whitney were used to examine the results. On the 21st day, the groups were terminated.

SPSS software version 20.0 for Windows (SPSS Inc. Chicago, USA). Paired-t test (data are normally distributed) and Wilcoxon test (data are not normally distributed) were performed to confirm the difference in mean size of right knee joint diameters in rats before and after treatment (Sastroasmoro, 2011)

To confirm the decrease in joint diameter and comparison of pain response between groups, the non-parametric Kruskal-Wallis tes was performed because the requirements of the Anova test were not met. To identify significant differences in each group, we performed Mann-Whitney as a follow-up test to Kruskall Wallis.

3 RESULTS

3.1 Measurement of Rat Right Knee Joint Diameter

Right knee joint diameters in rats were measured using a screw micrometer calibrated based on the *Clinical Assessment of Experimentally Induced Osteoarthritis Rat Model In Relation To Time* to see OA before and after treatment. Table 1 observed diameter of the right knee joints of rats in the five groups.

Based on the test results, the right knee joint diameter of rat in the negative control group (K1) tended to increase. On the other hand, the positive control group (K2) (piroxicam 10 mg/kg BW) and all dose treatment groups (K3, K4, K5) decreased the diameter of the right knee joint of rats. A decrease in the diameter of the rat's right knee joint began to be seen on day 7.

Table 1: Rat's right knee joint diameter on days 0 to 21

Treatment Group	Mean diameter of right knee joint ± SD (mm)			
	Day-0	Day-7	Day-14	Day-21
K1	9.07±0.45	9.13±0.35	9.39±0.26	9.49±0.23
K2	8.62±0.33	6.48±0.35	3.97±0.44	2.50±0.23
K3	8.64±0.28	8.23±0.07	7.37±0.29	5.40±0.20
K4	8.77±0.50	8.00±0.07	6.51±0.29	4.58±0.21
K5	8.70±0.25	7.53±0.41	5.11±0.20	3.11±0.22

The paired-t test in table 2 was performed to confirm size group mean difference in the diameter of the right knee joint (data for all groups were normally distributed). A Kruskall-Walis test was then performed to confirm significant comparisons between groups (data not normally distributed until day 7). Then proceed to the Mann-Whitney test to check for significant differences between groups (table 4).

Table 2: Test results *paired -t-test* on groups rat's right knee joint diameter.

Group Pair	P Value, Confidence Interval Value (CI) 95%		
	Days 0 and 7	Days 0 and 14	Days 0 and 21
K1 before – K1 after	0.527 _	0 .029 *	0 .029 *
K2 before – K2 after	0,000 *	0.000 *	
K3 before – K3 after	0 .021 *		
K4 before – K4 after	0.013 *		
K5 before – K5 after	0.004 *		

Table 2 shows that joint diameter remained at day 7 and increased significantly at day 14 and 21 in group K1 ($p<0.05$). For K2 and all test groups (K3, K4, and K5) the mean joint diameter was significantly decreased on day 7, 14, and 21 ($p<0.05$). From the data in tables 1 and 2, we concluded, *Skeletonema costatum* (60 mg / kgBB; 90 mg / kgBB; 120 mg / kgBB) significantly reduced the size of diameter of right knee joint as seen in the piroxicam-treated group.

Table 3: *Mann -Whitney post hoc test* in groups of rat's right knee joint diameter.

Intergroup Relations		P value		
		Decline (0-7 days)	Decline (0-14 days)	Decline (0-21 days)
K1	K2	0.004*	0.004*	0.004*
	K3	0.004*	0.004*	0.004*
	K4	0.004*	0.004*	0.004*
	K5	0.004*	0.004*	0.004*
K2	K3	0.004*	0.004*	0.004*
	K4	0.004*	0.004*	0.004*
	K5	0.005*	0.004*	0.008*
K3	K4	0.004*	0.004*	0.004*
	K5	0.004*	0.004*	0.004*
K4	K5	0.004*	0.004*	0.004*

The results of the Kruskall-Walis test revealed that there was a significant difference ($p<0.05$) in the mean reduction in diameter of the right knee joints of rats between groups in all groups. Table 3 shows a significant ($p<0.05$) mean reduction in diameter of the right knee joint of rats in each group. The negative control (K1) is significantly different from the positive control (K2) or test group (K3, K4, K5). Although the right knee joint diameters of rats are decreased, Table 3 shows that the mean decrease in diameter in the various test groups is significant in the positive control group (K2). In the study groups, significant differences in joint diameter reduction were observed between the 60 mg/kg BW (K3), 90 mg/kg BW (K4), and 120 mg/kg BW (K5).

3.2 Measuring Pain Response of Rats

Assessment of the degree of pain in rats using the hot water tail-flick assay. The longer the rat lifts its tail from the water bath, the better the pain response. Below is a table of the observed pain scores of the rats in the five groups.

Table 4: Pain response rats on days 0 to 21.

Treatment Group	Rat pain score ± SD (seconds)			
	Day-0	Day-7	Day-14	Day-21
K1	0.42±0.07	0.32±0.04	0.25±0.40	0.23±0.03
K2	0.45±0.92	0.92±0.06	1.08±0.06	1.98±0.05
K3	0.45±0.05	0.51±0.02	0.66±0.03	0.78±0.05
K4	0.48±0.07	0.62±0.03	0.86±0.04	0.96±0.02
K5	0.45±0.03	0.80±0.04	0.95±0.04	1.47±0.22

Based on table 4, the pain scores of rats in the negative control group (K1) tended to decrease or showed no improvement in pain response with tail raising. In the positive control group (piroxicam 10 mg/kg BW) and all dose treatment groups (60 mg/kg BW; 90 mg/kg BW and 120 mg/kg BW), there was an increase in the tail-lifthing time which began to appear on the day 7.

Paired-t test (normally distributed data on days 7 and 21) and Wilcoxon (non-normally distributed data on day 14) were used to count difference in mean pain response before and after treatmnet. Tables 5 and 6 show the averages of various test results.

Table 5: Test results paired -t-test on groups pain response rat for days 0 and 7 and days 0 and 21

Group Pair	P value	
	Days 0 and 7	Days 0 and 21
K1 before – K1 after	0,000 *	0.003 * _
K2 before – K2 after		0.000 *
K3 before – K3 after		
K4 before – K4 after		
K5 before – K5 after		

Table 6: Wilcoxon test results on groups pain response rat for days 0 and 14.

Group Pair	P value
	Days 0 and 14
K1 before – K1 after	0.027 * _
K2 before – K2 after	0 .024 *
K3 before – K3 after	0.027 * _
K4 before – K4 after	0.027 *
K5 before – K5 after	0.028 *

Tables 5 and 6 for the negative control group (K1) show a reduction in time pain response on days 7, 14, or 21. This is linear with the data of measurement of the right knee joint diameter of rats (table 1) which illustrates enhanced inflammation in the K1 group.

Otherwise, the means pain scores before and after treatment, the positive control group (K2) or all test groups (K3, K4, and K5) were significantly different ($p<0.05$) in improving tail lift time in rats that indicates the pain response is improved.

The other test is to compare the average of decreased score pain on days 7, 14, and 21 using the Kruskall-Wallis test. The data for day 14 are not normally distributed and therefore did not meet the requirements for the Anova test. According to the results of the Kruskall-Walis test, there was a significant difference between groups in all post treatment observation periods ($p<0.05$). To see which groups differed, Mann-Whitney performed a post hoc test.

Table 7: Further test results post hoc Mann-Whitney on the pain response group.

Intergroup Relations		P value		
		Day-7	Day-14	Day-21
K1	K2	0.004*	0.004*	0.004*
	K3	0.003*	0.004*	0.004*
	K4	0.004*	0.004*	0.004*
	K5	0.004*	0.004*	0.004*
K2	K3	0.003*	0.004*	0.004*
	K4	0.004*	0.004*	0.004*
	K5	0.012*	0.005*	0.004*
K3	K4	0.003*	0.004*	0.003*
	K5	0.003*	0.004*	0.004*
K4	K5	0.004*	0.004*	0.004*

Table 7 shows this significant difference in pain score in all groups. Regarding the effective reduction of right knee joint diameter in rats, this study shows that the groups (K3, K4, and K5) are significantly different from the negative control group (K1) or the positive control group (K2). There are also significant differences between the *Skeletonema costatum* groups (60 mg/kg BW (K3); 90 mg/kg BW (K4), and 120 mg/kg BW (K5)).

4 DISCUSSION

This study showed that the negative control group has a decrease in pain response and an increase in the diameter of right knee joint rat, that is, there was inflammation caused by OA by *Complete Freund's Adjuvants* (CFA). While in the positive control group (piroxicam 10 mg/ kg BW) and in all treatment groups (*Skeletonema costatum* 60 mg/ kg BW; 90 mg/kg BW; 120 mg/ kg BW) there was an increase in pain response along with a decrease in the size of the right knee joint diameter of the rats($p<0.05$).

This study, also demonstrated that *Skeletonema costatum* has the effect of reducting joint diameter and improving pain response before and after treatment (*paired t-test*, $p<0.05$). Although efficacy was seen in all groups of *Skeletonema costatum*, it has been reported that the higher the *Skeletonema costatum* dose, the greater the reduction in joint diameter and the greater the improvement in pain response. Reduction in right knee joint diameter of rats and improvement of pain response after administration of *Skeletonema costatum* was better than negative control, but anot as good as using piroxicam compared with positive control.

It reduces inflammatory markers since *Skeletonema costatum* acts as an anti-inflammatory agent. This is achived by inhibiting the release of cytokine by macrophages and neutrophils because *Skeletonema costatum* contains fatty acids, trace elements, and antioxidants. Antioxidants act as inhibitors of inflammation by reducing the formation of pro-inflammatory cytokine (Health L, 2008). The inhibition of inflammation was supported by researchers who staid that the methanol extract of *Skeletonema costatum* produced the highest phenolic content compared with the hexane extract which was 0.644 mg / *gallic acid equivalent* (GAE)/g, where the phenolic content of the hexane extract wass 0.392 mg GAE/g (Health L, 2008). In addition, it said that the free radical scavenging activity of the *Skeletonema costatum* methanol extract is 59% at a concentration of 3.2 mg/ml (Lenin T, 2015).

The anti-inflammatory and antioxidant effects may be related to vitamins contained in *Skeletonema costatum*. Vitamins are trace minerals that play a role in metabolism, cell repair, immunity, and more. Vitamin content of *Skeletonema costatum*, among other things; vitamin A 141 µg, vitamin D 11 µg, vitamin E 108 µg, vitamin K 5.5 µg, vitamin B1 710 µg, B2 37 µg, B6 134 µg, B12 117 µg, vitamin C 59 µg and vitamin PP 511 µg (Roeck-holtzhauer, 1991). Vitamins A, C, and E have antioxidant roles in reducing *reactive oxidative stress* (ROS). According to the 2012 Framingham Cohort Study, vitamin E can reduce the progression of OA in men, but has no significant effect on the incidence of OA (Roeck-holtzhauer, 1991). At the same time, high doses of vitamin C can slow the progression of OA induced by surgery in *guinea pigs* because vitamin C has a protective effect on cartilage by stimulating collagen and proteoglycans (Roeck-holtzhauer, 1991).

In this study, the best anti-inflammatory effect of *Skeletonema costatum* was achieved after day 21 and using the highest dose of 120 mg/kg BW. Consistent with study Andari (2016) it was also found that *lemuru* fish oil which is rich in omega-3 and omega-6 had the best time to reduce *TNF-α* in joint cartilage

induced by CFA on day 21. In agreement with the study of Bahtiar, it was shown that the longer the administration of *Skeletonema costatum*, the higher the antioxidant level in the tissue and the better the ability of *Skeletonema costatum* to inhibit the inflammatory process (Bahtiar A, 2011).

To the researchers' knowledge, there are currently no publications on the benefits of *Skeletonema costatum* as an anti-inflammatory agent in OA. The results of this study could lead to a pilot study on the efficacy of *Skeletonema costatum* as an anti-inflammatory agent. In this study, the anti-inflammatory effects of *Skeletonema costatum* were not yet comparable to the administration of piroxicam as the standard, but using a higher dose of *Skeletonema costatum,* the effect was improved with a decrease in the diameter of the right knee joint diameter in rats. It can be observed in pain response. This could inform the next study to determine the dose of *Skeletonema costatum* to be tested.

5 CONCLUSIONS

Based on the study and results, it can be concluded that the administration of *Skeletonema costatum* reduced the diameter of the right knee joint in rats and improved the pain response before and after treatment (*paired t-test*, $p<0.05$). Although effects were observed in all groups of *Skeletonema costatum* groups, higher dose of *Skeletonema costatum* reported improved reduction in joint diameter and improvement in pain response (closer to the positive control). The mean reduction in right knee joint diameter and improvement in pain response in rats following administration of *Skeletonema costatum* is superior to the negative control (no treatment), but not as good as piroxicam (10 mg/kg BW) as a positive control ($p<0.05$).

Further investigation into the inflammatory effects of *Skeletonema costatum* needs to be carried out especially to determine the therapeutic dose of *Skeletonema costatum,* the time needed to reach maximum therapeutic levels, and the toxicity level of *Skeletonema costatum.* Apart from that, given the potential of Indonesia, which is rich in marine products, and the trend toward marine drug treatments, further studies are essential to study the effect of *Skeletonema costatum* on other types of diseases and other organs, for example, metabolic syndrome and cancer.

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