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Sargassum duplicatum Extract Reduced Artritis Severity Score and Periarticular Tissue Matrix Metalloproteinase-1 (Mmp-1) Expression in Ajuvan Artritis Exposed to Cold Stressor

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ABSTRACT

In rheumatoid arthritis, significant increase of matrix metalloproteinase-1 (MMP-1) expression results in destruction of cartilage's collagen, joint's bone and tendon. Cold stressor increase reactive oxygen species (ROS) production caused by increased oxidative phosphorylation and oxidant compounds can increase MMP-1 production. *Sargassum duplicatum* contains antioxidant compounds, including polyphenols (flavonoids and phlorotannin) and fucoxanthin.

The purpose of this study was to show the effect of *Sargassum duplicatum* extract (ESD) in reducing arthritis severity score and inhibiting periarticular tissue MMP-1 expression in adjuvant arthritic rat exposed to cold stressor.

In this study 30 male adjuvant arthritis (AA) rats divided into three groups: 1) without treatment; 2) exposed to cold stressor of 5**°** C for 15 minutes on day 8 - 14; 3) exposed to cold stressor and given oral ESD extract 400 mg / kg BW / day on day 1 - 21. At the end of the study, subjective arthritis severity score were assessed and periarticular tissue MMP-1 expression were analysed using immunohistochistry methods.

Mann Whitney statistical analysis showed exposure to cold stressor increase arthritis severity score significantly (p=0.002). Treatment with ESD reduce arthritis severity score significantly in AA rats exposed to cold stressors (p=0.001). Anova test results showed that MMP-1 expression were significantly increase in AA rats exposed to cold stressors compared to the group unexposed to cold stressors (p=0.001). ESD treatment significantly reduce the MMP-1 expression in AA rats exposed to cold stressors (p=0.001).

In conclusion this study showed that cold stressor increased arthritis severity score and MMP-1 expression in AA, while antioxidant content of ESD decreased arthritis severity score and MMP-1 expression.

Keywords: *Sargassum duplicatum*, arthritis severity score, MMP-1, cold stressor

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INTRODUCTION

Rheumatoid arthritis is a systemic inflammatory disease with heterogeneous symptoms in the form of synovial tissue inflammation and hyperplasia, production of autoantibodies (rheumatoid factors and Anti-Citrullinated Protein Antibodies (ACPA), cartilage and bone destruction and systemic abnormalities, including cardiovascular, pulmonary, psychological disorders and skeletal disorders.[1]

The prevalence of rheumatoid arthritis is 1% of the world's population and mainly affects women. Although the onset of rheumatoid arthritis usually occurs in the fourth and fifth decades of life, rheumatoid arthritis can occur at any age. The exact etiology and pathogenesis of this disease are still not known, but it is thought to be a combination of various factors, including genetic and environmental factors which result in inappropriate immunomodulation that causes inflammatory processes due to damage of synovial structures. Reactive oxygen species (ROS) have been reported to play an important role in the progression of the disease.[2]

Most of the joint damage of rheumatoid arthritis is believed to be caused by abnormal release of matrix metalloproteinases (MMPs) in the rheumatoid synovium stimulated by persistent inflammation. MMPs are controlled at various levels of transcription. They are synthesized as an inactive enzyme and activation occurs outside the cell. In vitro studies have shown a correlation between cartilage matrix degradation and increase in MMPs. mRNA and protein levels from various MMPs show increase in joint arthritis in both humans and animals.[3]

Cartilage consists of proteoglycans and type II collagen, tendons and bones mainly consist of collagen type I. In rheumatoid arthritis, inflammatory cytokines such as IL-1β and TNF-α stimulate the production of MMPs, enzymes that degrade all components of the extracellular matrix. MMP-1 (collagenase) has an important role in rheumatoid arthritis, and its levels are greatly increased in rheumatoid arthritis. The result is destruction of collagen from cartilage, bone and tendons from joints of patients with rheumatoid arthritis. MMP-1 is mainly produced by synovial cells lining of the joints.[4]

Research conducted by Macfarlane and friends states that there was a strong correlation between lack of sunlight, low temperatures and pain in patients with rheumatoid arthritis, but pain was not an inevitable result of weather conditions.[5] Other research conducted by Fernandes and colleagues states that a cold environment increased hyperlagesia in arthritis in experimental animals.[6]

Cold stressors increase the production of reactive oxygen species (ROS) caused by an increase in oxidative phosphorylation when increasing heat production to maintain body temperature. Increased ROS will activate Nuclear Factor-κB (NF-κB) thereby increasing the production of IL-1, TNF-α, and proinflammatory mediators[7,8], so it is thought to increase joint MMP-1 expression and the inflammatory process.

The antioxidant activity contained in brown seaweed (*Sargassum* sp) is a component of polyphenols (flavonoids and phlorotannin), fucoxanthin.[9,10] In this study *Sargassum duplicatum* was extracted with one-stage ethanol, because the research conducted by Putranti showed that this extract had the highest total phenol and flavonoid content.[10,11] The antioxidant content found in *Sargasum duplicatum* is

expected to reduce the ROS that is formed in adjuvant arthritis exposed to cold stressors.

This study aimed to show the role of *Sargasum duplicatum* extract in decreasing the arthritis severity score and periarticular tissue MMP-1 expression in adjuvant arthritic rats exposed to cold stressor.

METHOD

This research was an experimental laboratory study conducted in the Biochemistry Laboratory of Experimental Animal Unit, Faculty of Mathematics and Sciences, Brawijaya University, Malang, Indonesia. The study was conducted in accordance with the applicable Research Ethics at Brawijaya University, Malang, Indonesia.

This study used 30 *Rattus norvegicus* Wistar rats that were made into adjuvant arthritis by injecting 0.1 ml Complete Freund's Adjuvant (CFA) intradermally at the base of the rat's tail and given a booster 14 days later intradermally on the right and left foot. After 7 days adjuvant arthritis will develop in the form of swelling, redness, and pain in the feet joints.[12]

Rats were divided into 3 groups: group 1 was untreated adjuvant arthritic rats; group 2 were adjuvant arthritic rats exposed to cold stressor by being put into room 5^0C for 15 minutes every day for 7 consecutive days starting on day 8 to 14; the third group were adjuvant arthritic rats given *Sargassum duplicatum* extract at a dose of 400 mg / kgBW orally on day 1 to 21, and on day 8 to 14 cold stressor exposure at 5^0C for 15 minutes every day. At the end of the study (day 21) an assessment of subjective arthritic severity score and MMP-1 expression by immunohistochemistry was assessed in the rat ankle joint to all groups.

How to make *Sargassum duplicatum* extract

Sargassum duplicatum was cleaned, and then finely chopped and dried until the water content reaches 20-30%. *Sargassum duplicatum* was weighed as much as 116 grams and extracted by maceration with 1.5 L 85% ethanol for 2 days. The extract was then filtered to get the filtrate concentrated with a rotary vacuum evaporator at 40^0 C for about 2 hours. The concentrated extract then were washed with 100 ml of chloroform 3 times. The ethanol fraction was taken and dried with N_2 gas into an extract in the form of a paste weighing approximately 3% of fresh weight.[11]

Arthritis severity score examination

Arthritis severity score examination was a subjective assessment of the degree of inflammation in the feet of experimental animals which were classified based on the extent of edema and deformity that occurs in experimental animal feet. Assessment of subjective arthritis severity score was as followed.[13]

0 = No redness or swelling.
1 = Redness and mild swelling limited to the tarsal or ankle joint.
2 = Redness and mild swelling extending from the ankle to tarsal.
3 = Redness and swelling spread from the ankle to the metatarsal joint.
4 = Redness and severe swelling include the ankles and fingers or ankylosis of the limbs

Immunohistochemistry Examination of Rat Ankle Periarticular Tissue MMP-1

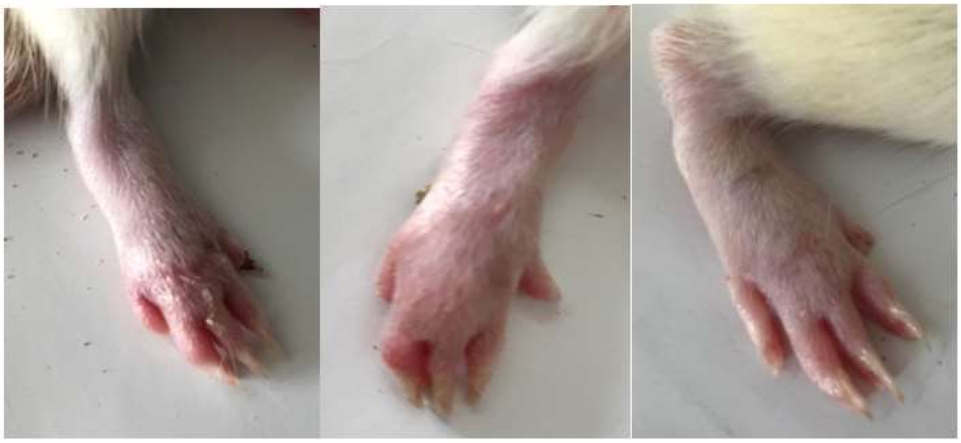
Histological preparations of de-neuralized rat ankle joints were put into a pH 6 buffer for 5 minutes at 95° C twice and allowed to stand at room temperature for 20 minutes, then washed 3 times with deionized water for 5 minutes followed by incubation in 0.1% trypsin, 0.1% CaCl2 for 20 minutes. The preparation was then washed with deionized water for 5 minutes 3 times, then incubated in 0.05% saponin for 40 minutes, 3% H_2O_2 was added and incubated for 10 minutes. The preparation then was washed with Phosphate Buffer Saline (PBS) pH 7.4 for 5 minutes 3 times, then blocked with non-specific proteins using 1% Normal Goat Serum for 1 hour, then blotted with tissue blocking serum, and then incubated with primary antibody anti rat MMP-1 at 4^0 C overnight.

The preparation was then washed with PBS pH 7.4 for 5 minutes 3 times then incubated in secondary antibodies labeled biotin for 60 minutes at room temperature, then washed with PBS pH 7.4 for 5 minutes 3 times and incubated with Streptavidin-Horseradish Peroxidase for 40 minutes. The preparation was washed with PBS pH 7.4 for 5 minutes 3 times and DAB (diaminobenzidin) was added for 3 minutes, then washed with deionized water for 5 minutes 3 times, counterstained with Mayer's hematoxyline for 10 minutes and washed with tap water. After that the preparation was mounted using a cover slip, and viewed using a light microscope at magnification 200 times and 400 times. The assessment was conducted by counting the number of positive cells per field of view at magnification 400 times. Observations were made in 4 field of view and then averaged.[14]

RESULTS

Picture of **Rat's** Feet

Rat's foot with adjuvant arthritis, rat's foot with adjuvant arthritis exposed to cold stressors and rat's foot with adjuvant arthritis exposed to cold stressor and received *Sargassum duplicatum* extract can be seen in Figure 1.



A B C

Figure 1: A. Rat's foot with adjuvant arthritis. B. Rat's foot with adjuvant arthritis exposed to cold stressor. C. Rat's foot with adjuvant arthritis exposed to cold stressor and received *Sargassum duplicatum* extract

The Results of Arthritis Severity Score Examination

The means of arthritis severity score in group of rats with adjuvant arthritis, group of rats with adjuvant arthritis exposed to cold stressors, and group of rats with adjuvant arthritis exposed to cold stressors and received *Sargassum duplicatum* extract can be seen in Table 1.

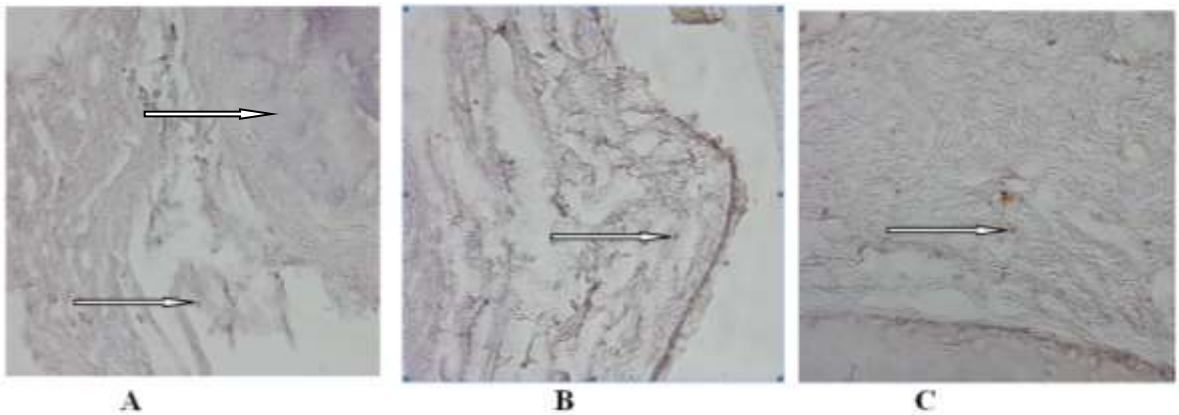
Table 1: Means of arthritis severity score in group of rats with adjuvant arthritis, group of rats with adjuvant arthritis exposed to cold stressor, and groups of rats with adjuvant arthritis exposed to cold stressor and received *Sargassum duplicatum extract*

GROUP	MEAN
I	3.5
II	4.6
III	2.6

Note:
Group I: Rats with adjuvant arthritis
Group II: Rats with adjuvant arthritis exposed to cold stressors
Group III: Rats with adjuvant arthritis exposed to cold stressors and received *Sargassum duplicatum* extracts

Mann Whitney test results showed a significant reduction (p=0.002) the mean of arthritis severity score in group of rats with adjuvant arthritis (mean=3.5) compared with group of rats with adjuvant arthritis exposed to cold stressors (mean=4.6). There was a significant reduction (p= 0.001) the the mean of arthritis severity score in group of rats with adjuvant arthritis exposed to cold stressors and received *Sargassum duplicatum* extract (mean=4.6) compared to group of rats with adjuvant arthritis exposed to cold stressors only (mean=2.6) .

The result of this study showed that cold stressor significantly increased the arthritis severity score in adjuvant arthritis, and *Sargassum duplicatum* extract significantly decreased the arthritis severity score.



A B C

Figure 2: A. MMP-1 immunohistochemistry of rat's ankle with adjuvant arthritis. B. MMP-1 immunohistochemistry of rat's ankle with adjuvant arthritis exposed to cold stressors. C. MMP-1 immunohistochemistry of rat's ankle with adjuvant arthritis exposed to cold stressors and received *Sargassum duplicatum extract*

Note: Cell that positively expressed MMP-1

Periarticular Tissue MMP-1 Expression Measurement

Images of rat ankle MMP-1 immunohistochemistry of rat with adjuvant arthritis, rat with adjuvant arthritis exposed to cold stressors, and rat with adjuvant arthritis exposed to cold stressors and received *Sargassum duplicatum* extract can be seen in Figure 2.

Mean and standard deviation of the sum of the MMP-1 periarticular tissue positive cells in the group of rats with adjuvant arthritis, groups of rats with adjuvant arthritis exposed to cold stressors and groups of rats with adjuvant arthritis exposed to cold stressor and received *Sargassum duplicatum* extract can be seen in Table 2.

Table 2: Mean and standard deviation the sum of MMP-1 periarticular tissue positive cells in group of rats with adjuvant arthritis, group of rats with adjuvant arthritis exposed to cold stressors and groups of rats with adjuvant arthritis exposed to cold stressors and received *Sargassum duplicatum extract*

GROUP	MEAN ± STANDARD DEVIATION (The Sum of Positive cells / Field of View)
I	37.4 ± 4.142
II	47.7 ± 8.820
III	28.9 ± 7.295

Note:

Group I: Rats with adjuvant arthritis

Group II: Rats with adjuvant arthritis exposed to cold stressors

Group III: Rats with adjuvant arthritis exposed to cold stressors and received *Sargassum duplicatum* extracts

The results of this study indicated that there was an increased in the mean of the sum of MMP-1 periarticular tissue positive cells in the group of rat with adjuvant arthritis exposed to cold stressors (mean ± SD = 47.7 ± 8.820) compared to group of rat with adjuvant arthritis that did not expose to cold stressors (mean ± SD = 37.4 ± 4.142). The administration of *Sargassum duplicatum* extract reduced the number of cells expressing MMP-1 in rat with adjuvant arthritis exposed to cold stressors (mean ± SD = 28.9 ± 7.295).

Anova test results showed there was significant increase (p=0.001) of the sum of periaricular tissue MMP-1 positive cells in the group of rats exposed to cold stressors compared to group of rats with adjuvant arthritis. There was a significant decrease (p=0.001) of the sum of periarticular tissue MMP-1 positive cells in group of rats with adjuvant arthritis exposed to cold stressor and received *Sargassum duplicatum* extract compared to group of rats with adjuvant arthritis exposed to cold stressor only.

So, cold stressor significantly increase the sum of periaricular tissue MMP-1 positive cells in adjuvant arthritis and *Sargassum duplicatum* extract significantly decreased periaricular tissue MMP-1 positive cells.

DISCUSSION

In this study, arthritis severity scores were significantly increased (p=0.002) in rat with adjuvant arthris exposed to cold stressors compared to rat with adjuvant arthritis that did not exposed to cold stressor. In the rats received cold stressor, the condition cause an increase in the formation of free radicals and oxidants in the body due to the body's mechanism in maintaining body temperature homeostasis by increasing heat production through the uncoupling of oxidative phosphorylation that occurs in the inner membrane of mitochondria. The energy produced occurs through the transfer from ATP production to heat production. The increase in oxidative phosphorylation that occurs gives the effect of an increase in the formation of free radicals and oxidants.[15]

Free radicals indirectly play a role in joint tissue damage, because free radicals also act as secondary messengers in the inflammatory response and cellular immune response in rheumatoid arthritis. T cell exposure to increased oxidative stress will make it refractory to many stimuli including growth and death and may cause a continuing abnormal immune response. Furthermore, free radicals can directly damage joint cartilage tissue, attack proteoglycan cartilage, and inhibit its synthesis. Oxidative damage from hyaluronic acid and products of lipoperoxidation, as well as oxidation of low-density lipoproteins and increased carbonyl derived from protein oxidation occured in patients with rheumatoid arthritis. Increased levels of 4-hydroxy-2-nonenal (4-HNE) have been found in serum or plasma and synovial fluid in patients with rheumatoid arthritis.[16] This situation triggers an increase in joint inflammation which has an impact on increasing arthritis severity score.

Sargassum duplicatum extract in this study was able to significantly reduce (0.001) the arthritis severity score in experimental animals with adjuvant arthritis exposed to cold stressor compared to those of adjuvant arthritis experimental animals who were exposed to cold stressors without administration of *Sargassum duplicatum* extract.

The ethanol extract content of *Sargassum duplicatum* consists of fucoxanthin, astaxanthin, carotenoids and polyphenols (eg phenolic acids, flavonoids, tannins). Flavonoid, one of the ingredients in ethanol extract of *Sargassum duplicatum* with antioxidant properties, that works by capturing free radicals so that oxidative stress due to free radicals will be reduced so that the process of joint inflammation is expected to be reduced too. Furthermore, besides functioning as an antioxidant, flavonoid also works as an anti-inflammation through inhibition of cyclooxygenase and lipoxygenase, by inhibiting arachidonic acid and secretion of lysosomal enzymes from the endothelial thereby inhibiting the proliferation and exudation of inflammatory cells. Inhibition of the release of arachidonic acid from inflammatory cells will cause the lack of arachidonic substrate available for the cyclooxygenase pathway and lipoxygenase pathway. Another mechanism of flavonoids as anti-inflammatory is by inhibiting the secretion of proinflammatory mediators[17,18] This condition will cause a decrease in the joint inflammatory process and furthermore will decrease arthritis severity score.

In this study *Sargassum duplicatum* was able to reduce the subjective evaluation arthritis severity score of adjuvant arthritis exposed to cold stressors through the mechanism as anti-oxidant and anti-inflammatory substance contained in it.

In this study there was a significant increase in periarticular tissue MMP-1 expression (p=0.001) in rats with adjuvant arthritis exposed to cold stressor compared to rats with adjuvant arthritis without treatment. This is likely due to an increase in ROS production due to increased oxidative phosphorylation as the body's defense mechanism to maintain optimal body temperature. This condition will cause oxidative stress.

In changing of cellular environments, the control of NF-kB pathway is important. The process of inflammation is the body's response to overcome various kinds of trauma and infections that attack the body. Proinflammatory response is controlled by NF-kB. In cannonical pathway, activation of NF-kB, signal received from tumour necrosis factor receptor (TNFR), interleukin-1 receptor (IL-1R) and also Toll Like receptor families. The inflammatory response mediated by active macrophages and other immune cells, causes an increase in transcription of TNF-α, IL-1 and other proinflammatory cytokines.[7] In a state of oxidative stress, NF-κB also stimulates the activity of proteolytic enzymes. In patients with rheumatoid arthritis, proteolytic enzymes play an important role in cartilage and bone destruction. Cytokines activate fibroblasts and macrophages in the synovial joints to produce cartilage-destroying enzymes, such as matrix metalloproteinases (MMPs), including MMP-1 and MMP-3 [7,19]

Matrix metalloproteinase-1 (MMP-1) is an extracellular proteolytic enzyme involved in the degradation of matrix and non matrix proteins. MMPs are produced by various connective tissue and proinflammatory cells including fibroblasts, cardiomyocytes, endothelial cells, macrophages, neutrophils, and lymphocytes. MMPs are secreted as zymogens, proteolytic solutions are needed for their activation. Activation of MMPs can be carried out by other extracellular proteolytic enzymes, increased levels of pro-oxidants and increased levels of reactive oxygen species [20,21]

MMP-1 and MMP-3 are the main MMPs produced by fibroblasts and macrophages in synovium with significantly higher levels in patients with rheumatoid arthritis compared with osteoarthritis sufferers. Not only do these MMPs damage collagen, proteoglycans and molecules, but they also activate other MMPs. The activity of MMP-1 and MMP-3 causes damage of the articular cartilage and subchondral bone, which causes joint deformity and severe pain in patients with rheumatoid arthritis.[22] Baseline values of MMP-1 serum MMP-3 levels are correlated to disease activity in early rheumatoid arthritis and predict functional conditions in untreated rheumatoid arthritis patients.[23] Thus, the increased periarticular tissue MMP-1 expression in adjuvant arthritis in this study is also one of the cause of increased arthritis severity score of rats with adjuvant arthritis exposed to cold stressor, because in these rats periarticular tissue MMP-1 expression is also increased.

In this study there was a significant decrease in periarticular tissue MMP-1 expression (p=0.001) in rats with adjuvant arthritis who were given cold stressors and *Sargassum duplicatum* extract compared to rats with adjuvant arthritis without treatment.

Sargassum duplicatum extract can prevent oxidative stress, because *Sargassum duplicatum* contains antioxidants. Seaweed is rich in herbal antioxidants. Brown seaweed (*Sargassum duplicatum*) contains antioxidant, such as polyphenols (flavonoids and phlorotannin). Polyphenols have hydroxyl groups in aromatic compounds that can give hydrogen atoms to oxidant compounds and have the ability as electron delocalization because they have conjugated double bonds, and also as stability of resonance structures. In addition, brown seaweed is also has flavonoids (polyphenols), terpenoids and alkaloids compounds. Furthermore, flavonoid compounds found in seaweed can reduce levels of malondialdehyde and increase the activity of superoxide dismutase that functions as antioxidant.[6,21,24] As a result, NF-κB activation can be inhibited and oxidative stress can be reduced, so the production of MMP-1 will also be reduced. It can be shown that there was significant decrease in the number of periarticular tissue MMP-1 positive cells in rats with adjuvant arthritis exposed to cold stressors and given *Sargassum duplicatum* extract compared to rats with adjuvant arthritis exposed to cold stressors only.

CONCLUSION

Cold stressor increased arthritis severity score and MMP-1 expression in periarticular tissue of rats with adjuvant arthritis. *Sargassum duplicatum* extract reduced arthritis severity score and periarticular tissue MMP-1 expression in rats with adjuvant arthritis exposed to cold stressors.

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