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# Prophylactic *Sargassum duplicatum* inhibit joint damage in adjuvant arthritic rats exposed to cold stress through inhibition of NF-KB activation

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**Abstract:** Activation of NF-κB play important roles in inflammatory process of rheumatoid arthritis (RA). Cold stress cause increased body heat production by thermogenesis achieved by accelerating uncoupling oxidative phosphorylation. We aimed to investigate whether *Sargassum duplicatum* was able to reduce the extent of joint damage through inhibition of NF-κB activation in adjuvant arthritis an animal model of RA.

Male adjuvant arthritic rats (age=10-12 wk; n=10/group) were exposed to cold stress (5°C for 15 min/day for 7 days) with/without *Sargassum duplicatum* extract (400 mg/kg BW/day po) given from 7 days prior to cold stress until the end of the experiment. The control group did not receive either cold exposure or *Sargassum duplicatum*. At the end of the experiment plasma malondialdehyde (MDA) level as biomarker of oxidative stress was measured using spectrophotometry method, joint NF-κB levelwas measured by counting NF-κB positive cells in imunohistochemistry and the severity of joint damage was measured by the histopathological scoring system.

The results showed that cold stress significantly increased plasma MDA level (4.2666±0.1940 μM vs. 2.9297±0.1499 μM; p≤0.05; p≤0.005), increased joint NF-κB level (58.8±10.871 vs. 43.5±6.223; p≤0.001), and increased joint histopathological score (median: 4vs3; p≤0.001). *Sargassum duplicatum* extract administration in cold stress treated group significantly decreased plasma MDA level (2.2569±0.0792 μM vs. 4.2666±0.1940 μM; p≤0.001), joint NF-κB positive cells (24.3±4.832 vs. 58.8±10.871; p≤0.001) and joint histopathological score (median: 3vs4; p≤0.001).

Conclusion: Prophylactic *Sargassum duplicatum* extract significantly reduced joint damage through inhibition of NF-κB expression and reduction of oxidative stress in adjuvant arthritic rats exposed to cold stress.

**Key words:** Sargassum duplicatum, adjuvant arthritis, MDA, NF-κB, cold stress.

## Introduction

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology, leads to inflammation in the joints and subsequent destruction of cartilage and bone. Oxidative stress is elevated in RA patient implying that reactive oxygen species (ROS) are the possible mediator of tissue damage. In many joint diseases,

proinflamatory factors such as cytokines and prostaglandin, ROS and nitric oxide (NO) are released at sites of inflamation. In arthritic patient, increased ROS were found at the sites of inflamation[1].

Enviromental factor are known to affect rheumatoid arthritis. Low temperature is known to increase the level of joint pain[2,3]. Cold stress cause increase body heat production, increase redox imbalance and produce oxidative stress implying elevated ROS[4]. ROS are known to activate cellular redox sensitive transcription factor, including nuclear factor κB (NF-κB), that upregulate genes encoding pro-inflamatory cytokines and enzymes[6]. The main ROS produced by chondrocytes are nitric oxide (NO) and superoxyde anion ($O_2^-$). Oxygen dan nitrogen radicals damage cellular element in cartilage and components of the extracellular matrix either by activation latent metalloproteinases or by directly and/or indirectly reducing matrix synthesis, reducing the sulfation of newly synthesized glycosaminoglycans and inducing apoptosis[1].

Adjuvant arthritis, an animal model of RA is useful tool for investigating the underlying mechanism of the disease and for the screening of new therapeutic approaches. Nuclear Factor-kappaB (NF-κB) is a key transcription factor regulating proinflammatory cytokines involves in pathogenesis and progression of RA and ROS is likely triggers a cascade of events that activates NF-κB[5,7,8].

*Sargassum* species has been reported to produce metabolites of structural classes such as terpenoids, polysaccharides, polyphenols, sargaquinic acids, sargachromenol, plastoquinones and steroid wich possess some therapeutic activities. *Sargassum duplicatum* extract has anti inflammatory effect and antioxidant effect that may quench ROS[9]. We aimed to investigate whether cold stress in adjuvant arthritis increased oxidative stress as shown by elevation of plasma malondialdehyde (MDA) and activation of NF-κB resulted in accelerated joint damage. Furthermore we aimed to determine whether treatment with *Sargassum duplicatum* extract reduces joint damage associated with reduction of plasma MDA level and NF-κB expression.

## Materials and methods

### Animal study

Male Wistar rats (130-170 g, age 10-12 wk) were procured and housed in animal research centre of the Laboratory of Cellular and Molecular Biology, Faculty Sciences, Brawijaya University, Malang, Indonesia. The rats were fed standard chow diet and water ad libitum. The study was conducted in accordance to Institution guidelines for animal research and Ethical Clearence Committe of Brawijaya University.

Adjuvant arthritis *Rattus norvegicus* was induced by 0.1 ml Complete Freund Adjuvant (CFA) injection at the base tail of the rats intradermally. After 14 days, the rats were given a second injection at the right and left paw. The experiments were started 7 days after the second injection where distinct symptoms of adjuvant arthritis can be seen including swolen and redness of the paw, ankle and limitation of movement.

For this study we used 3 groups (n=10/group) of adjuvant arthriti*s*. The first group (control group) did not received either cold stress nor *Sargassum duplicatum* The second group were exposed to cold stress (5$^o$ C for 15 min for 7 days). The third group were exposed to cold stress and given *Sargassum duplicatum* starting from 7 days prior the exposure to cold stress until the end of the experiment. At the end of experiment all rats were sacrificed and blood and hind joints were removed immediately for further investigation.

### Preparation of Sargassum extract

*Sargassum duplicatum* was obtained from Madura island Indonesia, washed in fresh water to remove salt, sand and epiphyte and dried using fresh dryer and milled in our laboratory. Dried *Sargassum duplicatum* (128 g) was macerated with 2L of 85% ethanol for 2 days. The extract was filtrated and collected in a bottle and evaporated using rotary vaccum evaporator in an oven at 30$^o$ C until concentrated and dried to the final weight of 1.2205 gram.

### Measuring MDA level

The level of oxidative stress was calculated by measurement of plasma MDA level using spectrophotometer, (Northwest NWK-MDA01 kit, Lot number MDA-2154) by measuring thiobarbituric acid reactive substances produced during lipid peroxidation [8]. Ten µL BHT reagent was added to microcentrifuge vial together with either 250 µL calibrator or sample followed by 250 µL acid reagent. Finally 250 µL TBA

reagent was added, vortex vigorously (5-count) and incubated for 60 min at 60°C. At the end of the incubation period they were centrifuged at 10,000 g for 2-3 min. Spectrophotometer reading was done at absorbance of 532 nm.

**Histological slide preparation**

Rat joints were fixed in 8% paraformaldehyde (pH 7.2) for 12 h, then incubated in 20% EDTA (pH 7.2) for 3 days at room temperature to decalcify the bone. They were then processed and embedded in paraffin. Histological sections were cut at 4 μm thick using microtome onto poly-L-lysine coated slides.

**Measurement of joint NF-κB cellular expression by immunohistochemistry**

For immunohistochemistry experiment the slides were dewaxed, brought to water. The antigen was unmasked by immersion in 95°C citrate buffer (pH 6) for 5 min twice and left at room temperature for 20 min. They were then washed 3 times in destilled water (5 min each) and incubated in 0.1% trypsin 0.1% $CaCl_2$ for 20 min, then in 0.05% saponin for 40 min. To block endogenous peroxidase the slides were then immersed in 3% $H_2O_2$ and incubated for 10 min then washed 3 times in PBS (pH 7.4) for 5 minutes. Non-specific protein binding was blocked with 1% Normal Goat Serum (NGS; Novus Biologicals, Littleton, Colorado, USA) for 1 h. After blotted the serum dry using tissue, the slides were incubated in rat primary antibody anti-NF-κB (Novus Biologicals, USA) at 4°C overnight. The following day the slides were washed 3 times with PBS (pH 7.4) for 5 min and incubated in the biotin labelled secondary antibody (Novus Biologicals, Littleton, Colorado, USA) at room temperature for 60 min. They were then washed 3 times in PBS (pH 7.4) for 5 min and incubated in SA-HRP (Novus Biologicals, Littleton, Colorado, USA) for 40 min. They were washed again 3 times in PBS (pH 7.4) for 5 min. Positive reaction was identified by incubation in Diaminobenzidin (DAB; Novus Biologicals, Littleton, Colorado, USA) for 3 min [9]. They were then washed 3 times in destilled water and counterstained with Mayer's hematoxylin (Sigma-Aldrich, St Louis, Missouri, USA). After washing with tap water they were dehydrated in ethanol and xylene and permanently mounted in DPX (Sigma-Aldrich, St Louis, Missouri, USA). NF-κB expression level were measured by counting the number of NF-κB positive cells indicated as brown in colour.

**Joint Histopathological Score**

The hematoxylin and eosin staining[10] was used for evaluation of arthritis severity. The histopatological scoring system was used as follows: 1=normal synovium with occasional mononuclear cells; 2=the presence of two or more synovial lining cells and perivascular infltration of leucocytes; 3=hyperplasia of synovium and dense infiltration of leucocytes; 4=synovitis, pannus formation and cartilage/subcondral bone erosions[11].

**Statistical Analysis**

For comparison between groups, ANOVA test was applied for plasma MDA level and joint NF-κB positive cells, whereas Mann Whitney test was used for joint histopathological score.

## Results

Plasma MDA level was inceased in arthritic rats exposed to cold stress compared to no exposure to cold (4.2666±0.1940 μM vs. 2.9297±0.1499 μM; $p \leq 0.05$). Prophylactic *Sargassum duplicatum* treatment in the group exposed to cold stress decreased plasma MDA level compared to the non-treated group (2.2569 ±0.0792 μM vs. 4.2666±0.1940 μM; $p \leq 0.05$). The level of plasma MDA in prophylactic *Sargassum duplicatum* treatment group was similar to no exposure to cold group (2.2569±0.0792 μM vs. 2.9297±0.1499 μM).

The number of joint NF-κB positive cells were incease in arthritic rats exposed to cold stress compared to no exposure to cold (58.8±10.871 μ vs. 43.5±6.223; $p \leq 0.05$). Prophylactic *Sargassum duplicatum* significantly decreased joint NF-κB positive cells in cold stressor group compared to non-treated group (24.3±4.832 vs. 58.8±10.871; $p \leq 0.05$). The number of NF-κB positive cells in prophylactic *Sargassum duplicatum* treatment group was lower compared to no exposure to cold group (24.3±4.832 vs. 43.5±6.223 ;$p \leq 0.05$).

Joint histopathological score inceased in arthritic rats exposed to cold stress compared to no exposure to cold (median: 4 vs. 3). Prophylactic *Sargassum duplicatum* significantly decreased joint histopathological score (median: 3 vs. 4) similar to the no cold exposure group.

Means and standard deviation of plasma MDA levels and joint NF-κB positive cells were shown in Table 1. Medians, minimal and maximal values of joint histopahological score were shown in Table 2.

**Table 1.Mean and Standard Deviation of Plasma MDA level (µM) and joint NF-κB positive cells**

| Group | MDA plasma level (µM) | joint NF-κB positive cells |
|---|---|---|
| I | 2.9297 ±0.1499 | 43.5±6.223 |
| II | 4.2666 ±0.1940 | 58.8±10.871 |
| III | 2.2569 ±0.0792 | 24.3±4.832 |

Note : SD = standard deviation

Group I : Rats with adjuvant arthritis
Group II : Rats with adjuvant arthritis exposed to cold stress
Group III : Rats with adjuvant arthritis exposed to cold stress and prophylactic treatment with *Sargassum duplicatum*

**Table 2. Medians, minimal and maximal values of joint histopahological score**

| Group | joint histopahological score | | |
|---|---|---|---|
| | median | minimal | maximal |
| I | 3 | 3 | 4 |
| II | 4 | 4 | 5 |
| III | 3 | 2 | 3 |

Note :

Group I : Rats with adjuvant arthritis
Group II : Rats with adjuvant arthritis exposed to cold stress
Group III : Rats with adjuvant arthritis exposed to cold stress and prophylactic treatment with *Sargassum duplicatum*

Histopathology of the joints of normal rats, adjuvant arthritic rats, adjuvant arthritic rats exposed to cold stress, adjuvant arthritic rats exposed to cold stress treated with *Sargassum duplicatum* were shown in Figure 1.

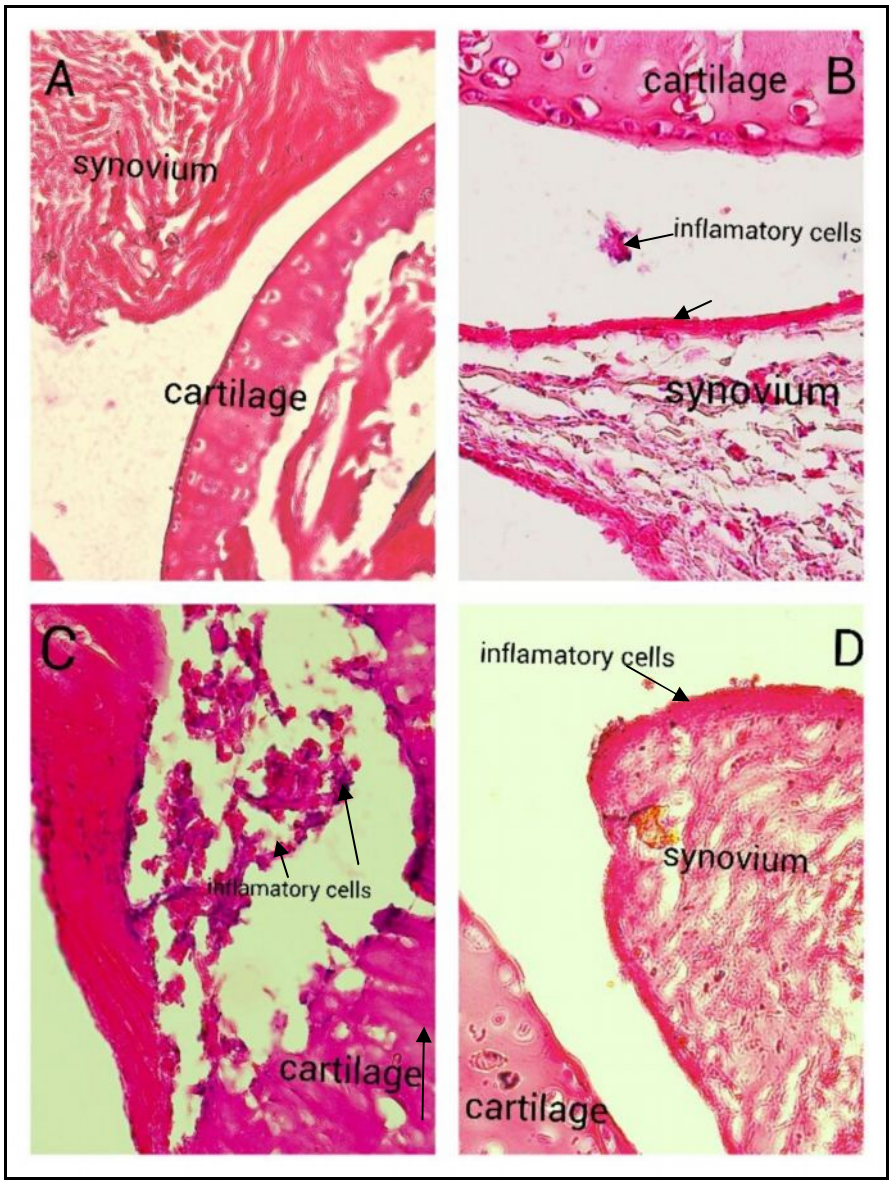


**Fig. 1:Joint tissue histopathology of normal rat (A), adjuvant arthritic rat (B) and adjuvant arthritic rat exposed to cold stress (C). and adjuvant arthritic rat exposed to cold stress treated with *Sargassum duplicatum* (D). Hematoxylin eosin staining. Magnification 40x.**

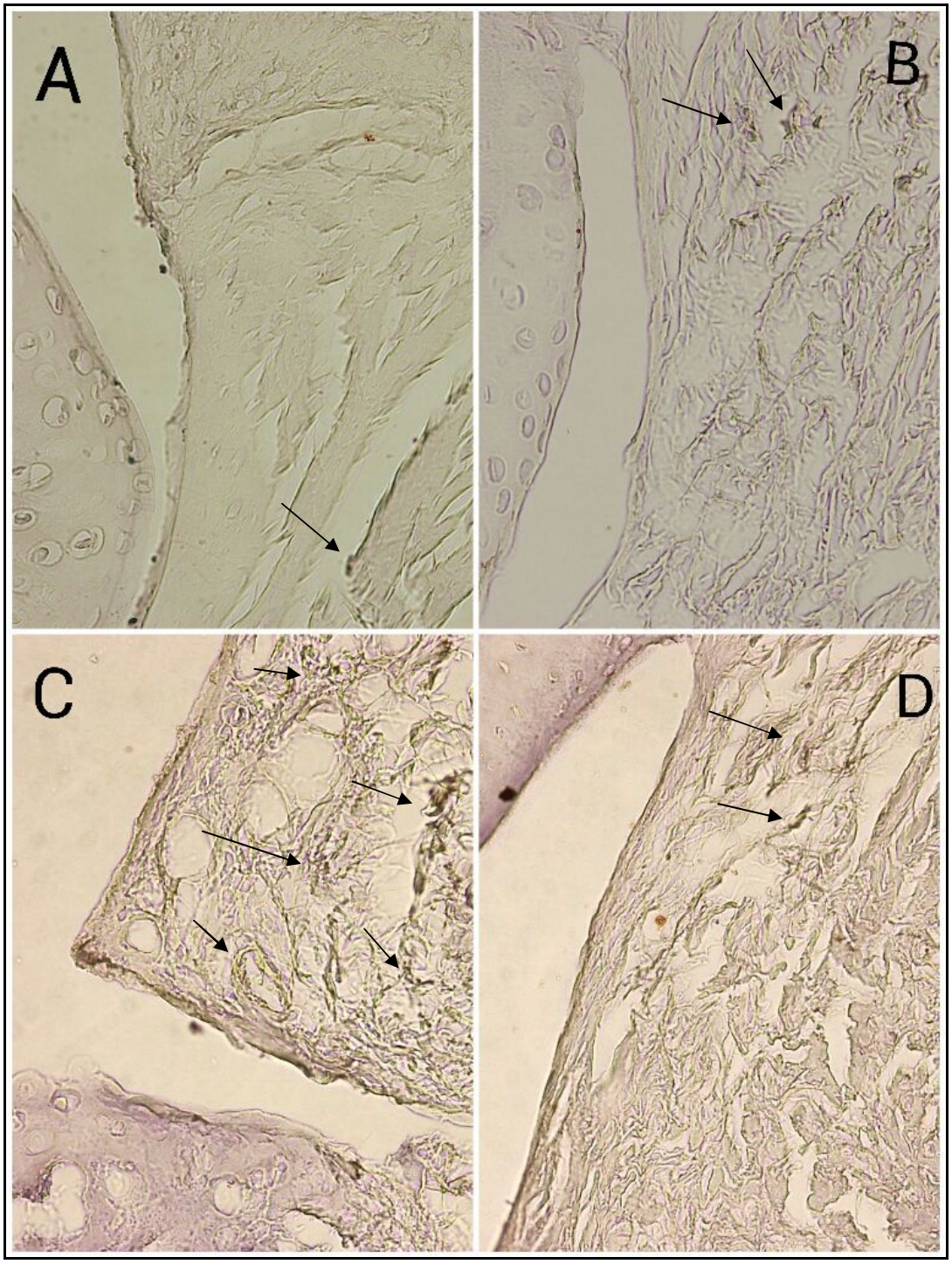


**Fig. 2: Immmunohistochemistry of joint tissue stained for NF-kB in normal rat (A) adjuvant arthritic rats (B), adjuvant arthritic rats exposed to cold stress (C) andadjuvant arthritic rats exposed to cold stress treated with *Sargassum duplicatum* (D). Positive cells are shown in black colour (arrows). Magnification 40x.**

*Statistical Analysis* using ANOVA test showed cold stress significantly inceased plasma MDA level ($p = 0.005$) and *Sargassum duplicatum* significantly decreased upregulating plasma MDA level of arthritic rats ($p = 0.001$). Cold stress significantly inceased joint NF-κB positive cells ($p = 0.001$), and *Sargassum duplicatum* significantly decreased upregulating joint NF-κB positive cells of arthritic rats ($p = 0.001$).

Mann Whitney test showed that cold stress significantly inceased plasma joint histopathological score ($p = 0.001$) and *Sargassum duplicatum* significantly decreased histopathological score ($p = 0.001$) of arthritic rats.

## Discussion

ROS are produced as an inevitable by product of electron transfer in oxydative phosphorylation during aerobic metabolism. In RA, oxidative stress has been described as an important mechanism that underlies destructive proliferative synovitis[15-16]. During inflamatory stages of RA, infiltration of immune activated cells in the synovium actively generated ROS via NADPH (nicotinamide adenine dinucleotide phosphate) oxidase system (Nox). Cold stress cause increased body heat production by thermogenesis achieved by accelerating uncoupling oxidative phosphorylation in inner membrane of mitochondria. The energy generated is shunted from ATP synthesis to heat production. Increasing oxidative phosphorylation increases ROS production[17]. Membrane lipid peroxidation is induced by free radicals or ROS and can be measured by plasma MDA assay. The result of this study showed that the plasma concentration of MDA was significantly increased in rats with adjuvant arthritis exposed to cold stress compared to rats with adjuvant arthritis without cold stress exposure. This indicated that cold stressor increased membrane lipid peroxidation, it can be measured by increased MDA.

*Sargassum* species are tropical and sub tropical brown seaweed. These are nutritious and rich of bioactive compound such as vitamins, minerals, carotenoid, dietary fiber and protein. Sulfated polysaccharide from algae possess important pharmacological activities such as antioxidant, anticoagulant, antiproliferative, antitumoral, antiinflamatory and antiviral. *Sargassum duplicatum* compound which acted as free radical scavenger might have the potential ability to prevent increase oxidative stress and combat inflammatory diseases[13,15,18,T9,20,21].

We found in this study that plasma concentration of MDA was significantly increased in rats with adjuvant arthritis exposed to cold stress and prophylactic treatment with *Sargassum duplicatum* significantly decreased the MDA level. The finding suggests the possibility that free radical scavenger from Sargassum was able to protect rats from lipid peroxidation and reduced oxidative stress and suggests the potential benefit of treatment with *Sargassum* in RA[4,13,14,15,].

NF-κB is a key transcription factor for regulating almost of all the pro-inflamatory factors during the progression of RA patogenesis, and a potential target for anti-arthritis therapy[22]. ROS are known to activate cellular redox sensitive transcription factor. The main ROS produced by chondrocytes are NO and superoxyde anion ($O_2^-$). High level of NO can cause damage to tissue as well as acute and chronic inflamation due to the stimulation of protein and enzymes responsible for an inflamatory response[18]. ROS has been reported to activate NF-κB. The activation of NF-κB up regulates genes encoding proinflamatory cytokines and enzyme[22,23]. Upregulation of NF-κB also has inhibitory effect on cartilage generation and interfere with the differentiation of mesenchymal stem cell into chondrocytes. In animal model of rheumatoid arthritis, NF-κB activation may contribute hyperplasia by increasing inflammation and inhibiting apoptosis[4]. This study showed further that cold stress significantly increase joint cellular NF-κBexpression possibly through increasing ROS production because of uncoupling oxidative phosphorylation in increasing heat production. ROS activates NF-κB which was required for induction of inflamatory cytokines in primary synovial fibroblast.

We showed that *Sargassum duplicatum* extract inhibit arthritic cellular joint expression of NF-κB indicating the presence of reduction or normalisation of NF-κB associated with reduction of arthritic severity shown in this study. Further investigation is needed to confirm whether the presence of increase of ROS production with cold stressor and whether increase ROS production is associated with increase NF-κB activation.

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Using histological scoring system we showed that cold stressor increase the severity of arthritis. Normal synovium is a delicate tissue that lines the joint capsule and the synovium integrity is important for joint function. Cold stress increase leucocyte infiltration and transforms the synovium into an agressive, tumor like structure called pannus which invades and erodes the joint. NF-κB activation may also facilitate synovial hyperplasia by promoting proliferation and inhibiting apoptosis of RA fibroblast-like synovial cells[24].

The biochemical extract from seaweed are such as fucoxhantin, astaxantin, alkaloids, sulfated polysaccharides (esspecially fucoidans) and polyunsaturated fatty acid are associated with the immune-

regulation of NO production. *Sargassum duplicatum* extract can inhibit the secretion of NO[18]. In this study we showed that *Sargassum duplicatum* extract inhibit the severity joint damage of arthritis in our adjuvant arthritis rat model exposed with cold stressor associated with reduced oxidative stress that shown by reduced plasma MDA and cellular joint NF-κB. It is likely that *Sargassum duplicatum* extract which functioned as an antioxidant queched ROS produced in oxydative phosphorilation during cold stressor expossure which subsequently decreased the secretion of NO. The presence of lower availability of NO secretion then limits the secretion of matrix metalloproteinases enzyme. Treatment with *Sargassum duplicatum* extract in adjuvant RA is associated with reduced NF-κB joint cellular expression. Reduction of cellular expression of NF-κB in this study might be due to the presence of reduced leucocyte infiltration which also indicating the presence of reduced matrix metalloproteinases secretion and thus potentially play important role in reduction of joint erosion and ultimately reduced joint damage[18,19,20,21].

## Conclusion

In this study we found that cold stress increase severity in arthritis adjuvant associated with oxidative stress lipid peroxidation, inflamation and NF-κB expression. Prophylactic *Sargassum duplicatum* reduced the severity of arthritis adjuvant associated with caused by cold stress through inhibition of lipid peroxidation, reduced inflammation and NF-κB expression and protect joint damage

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