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Research Article

Mechanism of the Bioactive *Sargassum cristaefolium* in Inhibiting Inflammatory Mediators in a Nitroglycerin-Induced Migraine Model in Rats

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ABSTRACT

Background: Migraine headaches are a form of sterile neurogenic inflammation. The sterile inflammatory process of the trigeminal nerve releases the vasoactive neuropeptide CGRP which stimulates the release of inflammatory mediators. In the incidence of migraine there is an increase in TNF-α and IL-10. *Sargassum cristaefolium* ethanol extract contains flavonoids, alkaloids, triterpenoids, steroids, and tannins, which has analgesic and anti-inflammatory function. **Method:** *Sargassum cristaefolium* was extracted using maceration method with 70% ethanol as solvent. Animal models were divided into 5 groups and given NTG induction 5 times with 1 day intervals, treated for 3 weeks. All data were analyzed using IBM SPSS version 26.0. **Results:** *Sargassum cristaefolium* ethanol extract - CGRP levels β: -0.26, p: 0.17; *Sargassum cristaefolium* ethanol extract - CGRP expression β: -0.04, p: 0.85; *Sargassum cristaefolium* ethanol extract - TNF-α levels β: -0.63, p: 0.01; *Sargassum cristaefolium* ethanol extract - TNF-α expression β: -0.40, p: 0.04; *Sargassum cristaefolium* ethanol extract - IL-10 levels β: 0.77, p: 0.00; *Sargassum cristaefolium* ethanol extract - IL-10 expression β: 0.45, p: 0.01. **Conclusions:** A significant path between the administration of *Sargassum cristaefolium* ethanol extract and a decrease in TNF-α and an increase in IL-10. But the effect of giving *Sargassum cristaefolium* ethanol extract on CGRP levels did not have a significant relationship.
Key words: Inflammatory mediator, Migraine, Nitroglycerin, *Sargassum cristaefolium*.

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INTRODUCTION

Migraine is a headache with pain attacks lasting 4-72 hours, unilateral, throbbing, with moderate to severe pain intensity, aggravated by activity, and may be accompanied by nausea and vomiting, and/or photophobia phonophobia.[1] Genetically susceptible and influenced by population area, climate change, socioeconomic status and lifestyle.

Many studies state that migraine headaches are a sterile form of neurogenic inflammation of the trigeminal nerve that releases vasoactive neuropeptides such as Calcitonin Gene Related Peptide (CGRP) which causes vasodilation of blood vessels and activates endothelial cells, mast cells and platelets to release vasoactive substances. CGRP will bind to its receptors on meningeal mast cells and stimulate the release of inflammatory mediators, causing sterile inflammation of neurons.

The pathophysiology of migraine is multifactorial, with most studies showing increased peripheral circulating concentrations of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IL-8 and the anti-inflammatory cytokine IL-10. During the ictal period IL-1β, IL-6, IL-8 and TNF-α were increased, while the anti-inflammatory cytokines IL-4 and IL-5 decreased and IL-10 increased compared to during the interictal period.[2]

As many as 80% of patients use analgesics to treat headaches. NSAIDs are drugs that are widely used in migraine sufferers because they can provide anti-inflammatory, analgesic, and antipyretic effects. Analgesic therapy is the most widely prescribed drug in the world, although these drugs often cause serious side effects. Therefore, it is necessary to look for new alternative drugs derived from natural ingredients that are relatively easy to obtain and come from plants that have analgesic and anti-inflammatory functions.

Indonesia is a country that has a very wide sea area, about two-thirds of the country's territory is ocean. Seaweed or better known as seaweed or algae is one of the most abundant biological resources in Indonesian waters, which is about 8.6% of the total marine biota. The bioactive substances contained in the *Sargassum cristaefolium* algae include flavonoids, alkaloids, tannins, terpenoids and steroids. Flavonoids act as anti-inflammatory by inhibiting the secretion of pro-inflammatory mediators. Alkaloids have a function as analgesics, while tannins, terpenoids and steroids function as anti-inflammatory.

MATERIALS AND METHODS

Ethics, consent and permissions

Ethics and experimental procedures were approved by the Faculty of Veterinary Medicine, Universitas Airlangga Surabaya Indonesia and the testing service unit of the Faculty of Pharmacy, Universitas Widya Mandala Surabaya Indonesia.

Characteristics of research subjects

Method Cohort using male Rattus norvegicus wistar strain, animals were randomized to treatment conditions. The treatment group involved the induction of NTG 10 mg/kg. It was administered intraperitoneally 5 times.

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Extracts obtained from the leaves and stems of brown algae originating from the waters of Ra`as Island, Sumenep Regency, were extracted using the maceration method with 70% ethanol solvent and thickened with a rotary evaporator at 40°C and 1% Na CMC solvent was given to rats orally using probe. The bioactive components contained in brown seaweed *Sargassum cristaefolium* use a test method based on which includes:[3]

Flavonoid test

2 mL of *Sargassum cristaefolium* extract was added with 1 mL of 2N NaOH. The presence of flavonoid content was indicated by the formation of a yellow color in the extract.

Alkaloid test

2 mL of *Sargassum cristaefolium* extract was added with 2N HCl, and a few drops of Dragendorff's test were added to the Meyer and Wagner tubes. The presence of alkaloids was indicated by the formation of a yellowish white precipitate in the Meyer tube, a brown precipitate in the Wagner tube and an orange-red precipitate with Dragendorph's reagent.

Triterpenoids/Steroid test

Dissolve 4 g of *Sargassum cristaefolium* extract in 25 mL of ethanol and then filtered. Then 0.5 mL of Sargassum cristaefolium extract added 2 ml of chloroform and aquades (1:1). The layer formed was dried on a hot plate and 1 drop of Lieberman-Burchard reagent was added. The presence of triterpenoids is indicated by the formation of a red color and a blue-green color for steroid content.

Tannin test

1 mL of *Sargassum cristaefolium* extract was added with 1 mL of 5% FeCl3. The presence of tannin content is indicated by the formation of a dark blue or blackish green color.

Saponin test

2 mL of *Sargassum cristaefolium* extract added 2 ml of distilled water and shaken in a test tube for 15 minutes. The presence of saponins was indicated by the formation of a 1 cm foam layer.

Statistical analysis

All data were analyzed using IBM SPSS version 26.0. The significance level for statistical analyzes $p < 0.05$ was considered statistically significant.

Procedure

The experiment was started after adaptation for 7 days, given NTG induction 5 times, 1 day apart, treatment for 3 weeks. Experimental animals were divided into 5 groups, namely:

K- = Negative control group + placebo

K+ = Positive control group + NTG + placebo

KP1 = Treatment group (1) + NTG + *Sargassum cristaefolium* ethanol extract dose 250 mg/kg body weight

KP2 = Treatment group (2) + NTG + *Sargassum cristaefolium* ethanol extract dose 500 mg/kg body weight

KP3 = Treatment group (3) + NTG + *Sargassum cristaefolium* ethanol extract dose 750 mg/kg body weight

RESULTS

The test results for the content of Flavonoids, Alkaloids, Terpenoids, Steroids, Tannins and Saponins in the ethanolic extract of *Sargassum cristaefolium* were carried out using the spectrophotometric method. Phytochemical test results on brown algae *Sargassum cristaefolium* showed positive results for the presence of flavonoid compounds, steroids/triterpenoids, alkaloids, and tannins (Table 1).

The results of the description test for levels and expressions of CGRP, TNF-α and IL-10 in the form of average and standard deviation can be seen in Table 2.

NTG induction increased the mean plasma CGRP levels by comparing the K- and K+ groups. The administration of *Sargassum cristaefolium* ethanol extract reduced CGRP levels by comparing the K+ group to the P1, P2 and P3 groups. The average comparison between groups of CGRP levels can be seen in Figure 1 and Figure 2.

NTG induction increased the mean plasma TNF-α levels by comparing the K- and K+ groups. The administration of *Sargassum cristaefolium* ethanol extract reduced TNF-α levels by comparing the K+ group to the P1, P2 and P3 groups. The average comparison between groups of TNF-α levels can be seen in Figure 3 and Figure 4.

NTG induction increased the mean plasma IL-10 levels by comparing the K- and K+ groups. The administration of *Sargassum cristaefolium* ethanol extract reduced IL-10 levels by comparing the K+ group to the P1 group. The administration of *Sargassum cristaefolium* ethanol extract increased IL-10 levels by comparing the K+ group to the P2 and P3 groups. The average comparison between groups of IL-10 levels can be seen in Figure 5 and Figure 6.

The results of the regression and significance values between *Sargassum cristaefolium* ethanol extract on levels and expression of CGRP, TNF- α and IL-10 can be seen in Table 3.

DISCUSSION

The ethanolic extract of *sargassum* contains bioactive flavonoids, alkaloids, triterpenoids, steroids, tannins and saponins. Flavonoid compounds are thought to have antioxidant, antitumor, antiviral, anti-inflammatory and antibiotic activities.[4] Flavonoids have strong antioxidant abilities because their chemical structure contains more than one phenol group and has a conjugated double bond. The ability to bind and prevent the formation of free radicals indicates that flavonoids apart from being analgesics, antioxidants also have anti-inflammatory functions.[5] Alkaloids function as antibiotics, analgesics, anti-inflammatory and blood circulation.[6] Tannins are active secondary metabolites which are known to have several properties, namely as astringent, antidiarrheal, antibacterial, anti-inflammatory and antioxidant. Terpenoids and steroids function as anti-inflammatory.[5,7]

A study showed that the extraction method and the type of solvent did not affect the flavonoid content. This is presumably because in its structure, flavonoids have polar and nonpolar parts with almost the same parts.[8] In this study, 70% ethanol was used because the selection of ethanol as a solvent has the ability to find compounds in a wide polar range, from polar to non-polar compounds, is non-toxic and effective in finding active ingredients.

The dose of *Sargassum cristaefolium* commonly used by humans is 18 grams/50kg BW, with an average human weight of 50 kg. The conversion factor from 70 kg adult human to 200 gram rat is 0.08.[9] Therefore, in this study, the optimal dose of *Sargassum cristaefolium* ethanol extract for rats was 500 mg/kg BW, minimum dose 250 mg/kg BW, maximum dose 750 mg /kg body weight and given by oral procedure.

In this study, the results of the phytochemical test of *Sargassum cristaefolium* were positive for flavonoids, alkaloids, triterpenoids, steroids, and tannins but did not show the presence of saponins. The highest levels are found in terpenoids and steroids that function as

Table 1: Phytochemical test results of *Sargassum cristaefolium*.

Phytochemical test results			
Parameters	**Fresh**	**Dry**	**Powder**
Alkaloid	++	+	+
Flavonoid	++	+	+
Triterpenoid/ Steroid	++	++	++
Tanin	+	+	+
Saponin	_	_	_

Table 2: Descriptive test results mean and standard deviation of CGRP, TNF-α and IL-10 levels in experimental animal plasma.

	Mean ± SD					
	CGRP		TNF-α		IL-10	
Group	level	expression	level	expression	level	expression
K-	80.30 ± 7.47	6.28 ± 1.93	228.79 ± 104.51	5.25 ± 1.99	55.41 ± 3.30	2.35 ± 1.10
K+	115.38 ± 8.36	7.98 ± 2.52	395.68 ± 156.99	6.48 ± 2.05	57.41 ± 4.08	3.97 ± 1.69
P1	102.57 ± 8.92	5.73 ± 3.11	186.88 ± 94.92	4.25 ± 0.91	56.74 ± 3.65	3.27 ± 1.03
P2	70.26 ± 9.35	4.87 ± 3.45	130.20 ± 79.12	4.08 ± 1.75	65.52 ± 5.32	3.47 ± 1.48
P3	84.92 ± 20.78	7.48 ± 3.09	138.70 ± 22.80	4.07 ± 1.10	69.90 ± 3.70	5.98 ± 3.41

Description:

K- = Negative control group + placebo

K+ = Positive control group + NTG + placebo

KP1 = Treatment group (1) + NTG + *Sargassum cristaefolium* ethanol extract dose 250 mg/kg body weight

KP2 = Treatment group (2) + NTG + *Sargassum cristaefolium* ethanol extract dose 500 mg/kg body weight

KP3 = Treatment group (3) + NTG + *Sargassum cristaefolium* ethanol extract dose 750 mg/kg body weight

Table 3: Regression and significance values between Sargassum cristaefolium ethanol extract on levels and expression of CGRP, TNF-α and IL-10.

No.	Variable	Beta (β)	R^2	Nilai P
1.	*Sargassum cristaefolium* ethanol extract -> CGRP levels	-0.26**	0.07	0.17
2.	*Sargassum cristaefolium* ethanol extract -> CGRP expression	-0.04**	0.00	0.85
3.	*Sargassum cristaefolium* ethanol extract -> TNF- levels	-0.63**	0.55	0.01*
4.	*Sargassum cristaefolium* ethanol extract -> TNF-α expression	-0.40**	0.37	0.04*
5.	*Sargassum cristaefolium* ethanol extract -> IL-10 levels	0.77	0.60	0.00*
6.	*Sargassum cristaefolium* ethanol extract -> IL-10 expression	0.45	0.20	0.01*

Description:

Beta (β) : Regression coefficient, shows the relationship of the independent variable to the variable dependent (increase or decrease)

R2 : The coefficient of determination, the contribution of the independent variable to the variation (up and decrease) dependent variable

P value : The value of the probability of significance, declared significant/significant if the p value <0.05

Sign* : Significant or meaningful

Sign** : Negative relationship

anti-inflammatory. This is in line with research conducted by Riwanti which stated that *Sargassum polycystum* obtained from Madura was positive for alkaloids, steroids/triterpenoids, flavonoids, polyphenols, and tannins.[10] Lailiyah's research showed that the phytochemical test results of *Sargassum cristaefolium* methanol extract obtained from Madura allegedly contained steroids that had antioxidant and anti-inflammatory activities.[3] Another study by Gazali found that the ethanol extract of *Sargassum sp* obtained in the Aceh area contained higher levels of terpenoids and alkaloids.[6]

Flavonoids are efficacious as analgesics whose mechanism of action is to inhibit the work of the cyclooxygenase enzyme which reduces the production of prostaglandins, thereby reducing pain.[11] CGRP is widely distributed in the body, central and peripheral nervous system, as a very potent and long-lasting vasodilator at all levels of the vascular system and has a major role in sensory nerve transmission.[12,13] Cerebral vascular dysfunction caused by abnormal levels of neuropeptides such as CGRP, plays an important role in migraine. After the vascular active substances are not balanced, there is vasomotor dysfunction, vasodilation and pain in migraine.[14]

In this study, *Sargassum cristaefolium* has not been able to reduce plasma CGRP levels and CGRP expression in the brain tissue of experimental animals. The above findings indicate that the analgesic

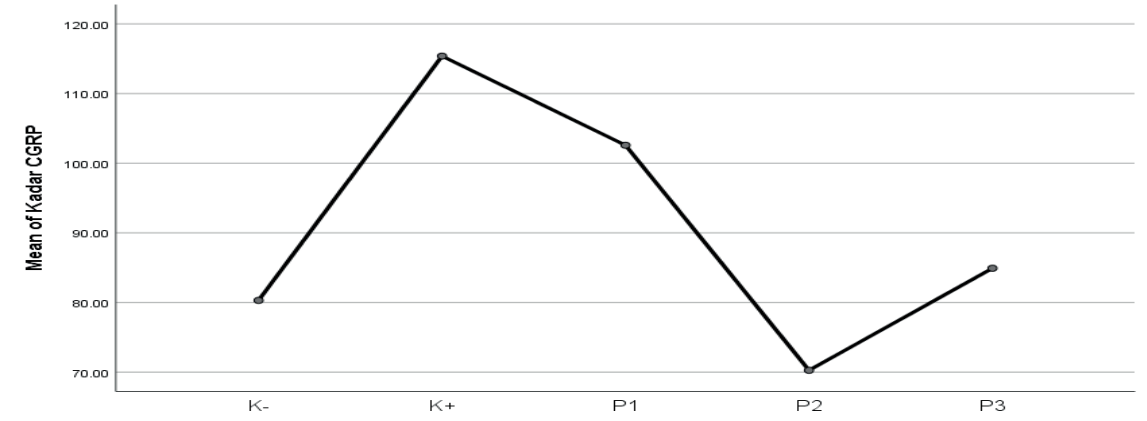

Figure 1: The average comparison between groups of CGRP levels.

Description:

K- = Negative control group + placebo

K+ = Positive control group + NTG + placebo

KP1 = Treatment group (1) + NTG + *Sargassum cristaefolium* ethanol extract dose 250 mg/kg body weight

KP2 = Treatment group (2) + NTG + *Sargassum cristaefolium* ethanol extract dose 500 mg/kg body weight

KP3 = Treatment group (3) + NTG + *Sargassum cristaefolium* ethanol extract dose 750 mg/kg body weight

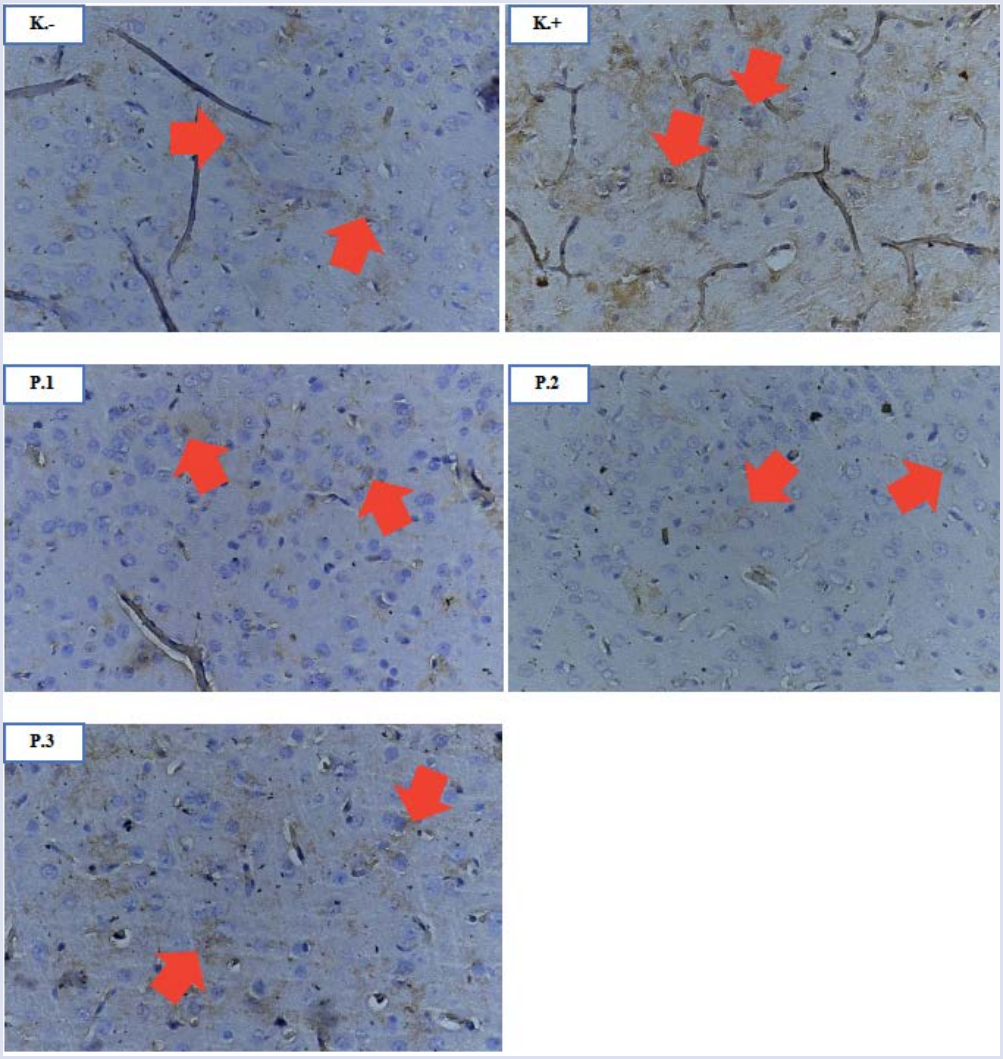

Figure 2: Comparison of CGRP expression in brain tissue groups (K-, K+, P.1, P.2, P.3). Red arrows indicate the presence of serotonin expression in the cerebrum which is indicated by the presence of a brown chromogen color. IHC. 400x.

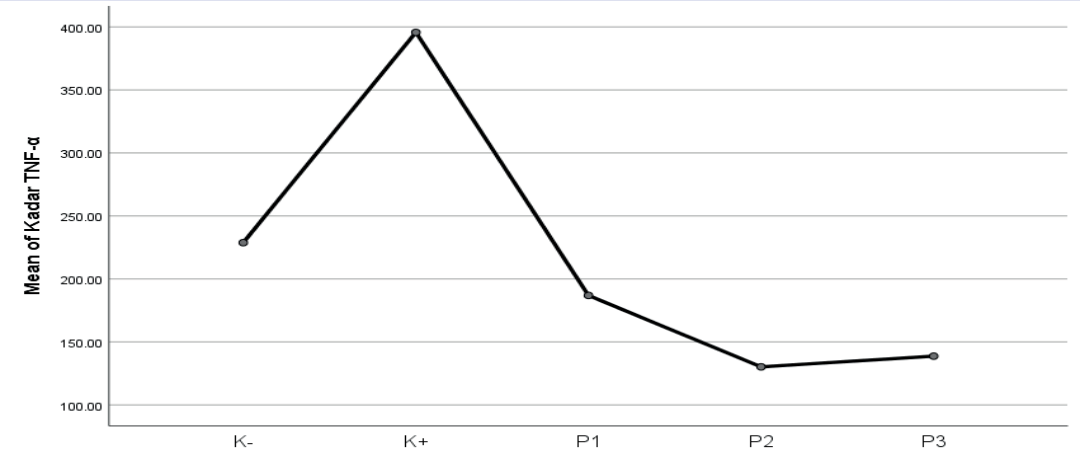

Figure 3: The average comparison between groups of TNF-α levels.

Description:
K- = Negative control group + placebo
K+ = Positive control group + NTG + placebo
KP1 = Treatment group (1) + NTG + *Sargassum cristeafolium* ethanol extract dose 250 mg/kg body weight
KP2 = Treatment group (2) + NTG + *Sargassum cristaefolium* ethanol extract dose 500 mg/kg body weight
KP3 = Treatment group (3) + NTG + *Sargassum cristaefolium* ethanol extract dose 750 mg/kg body weight

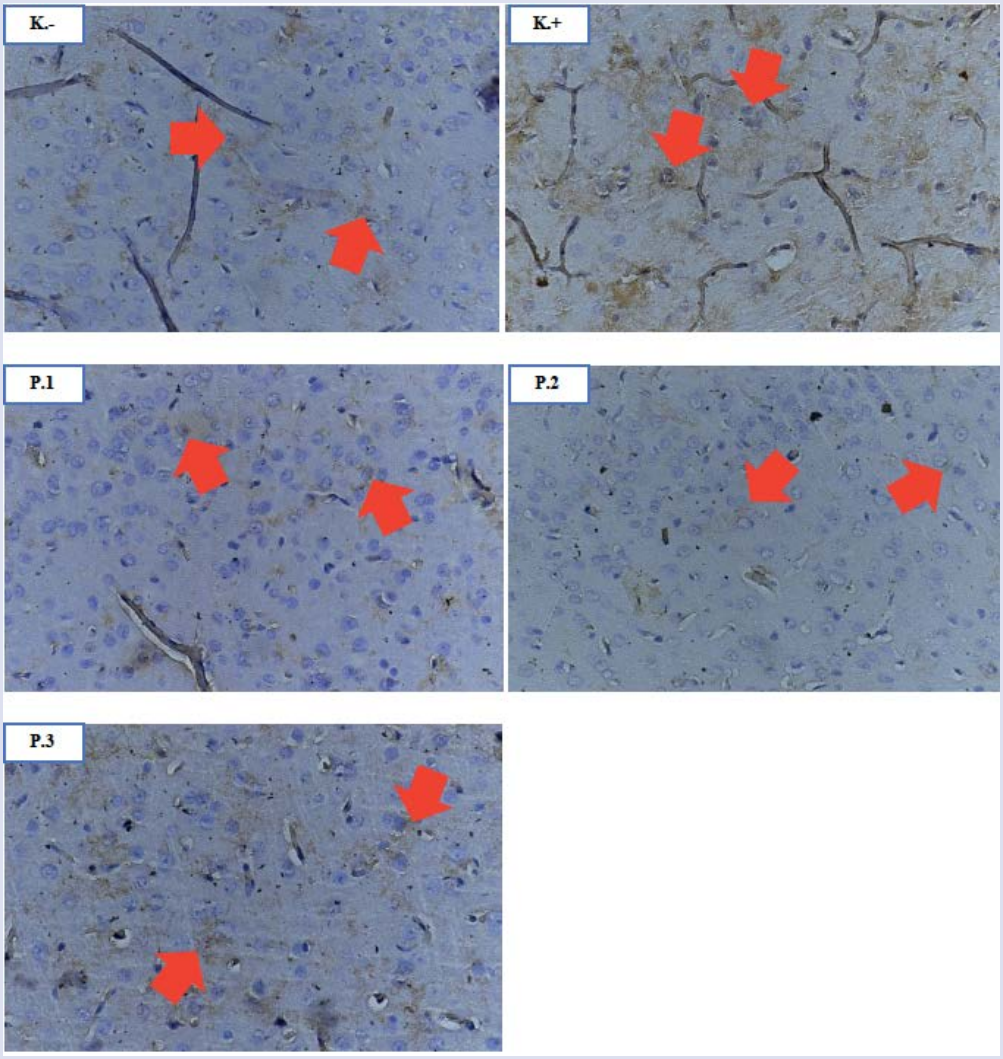

Figure 4: Comparison of TNF-□ expression in brain tissue groups (K-, K+, P.1, P.2, P.3). Red arrows indicate the presence of serotonin expression in the cerebrum which is indicated by the presence of a brown chromogen color. IHC. 400X.

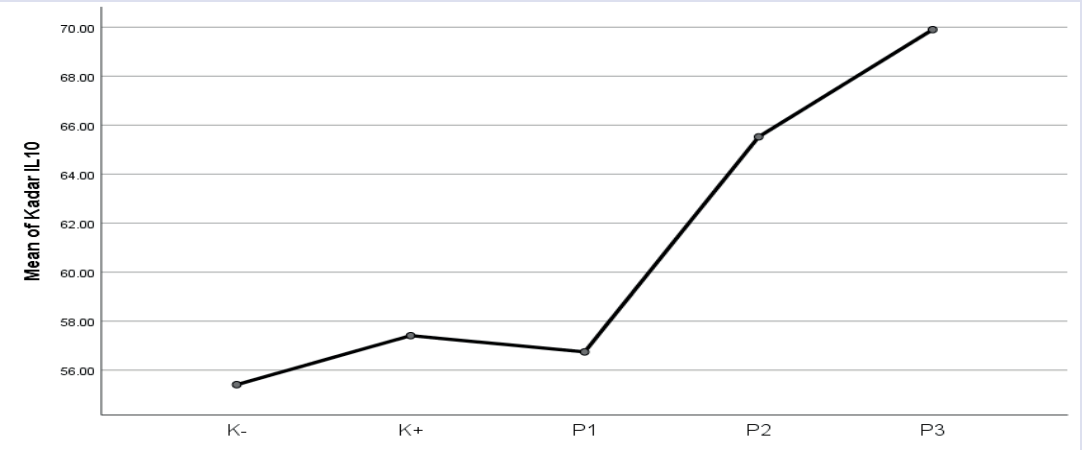

Figure 5: The average comparison between groups of IL-10 levels.
Description:
K- = Negative control group + placebo
K+ = Positive control group + NTG + placebo
KP1 = Treatment group (1) + NTG + *Sargassum cristaefolium* ethanol extract dose 250 mg/kg body weight
KP2 = Treatment group (2) + NTG + *Sargassum cristaefolium* ethanol extract dose 500 mg/kg body weight
KP3 = Treatment group (3) + NTG + *Sargassum cristaefolium* ethanol extract dose 750 mg/kg body weight

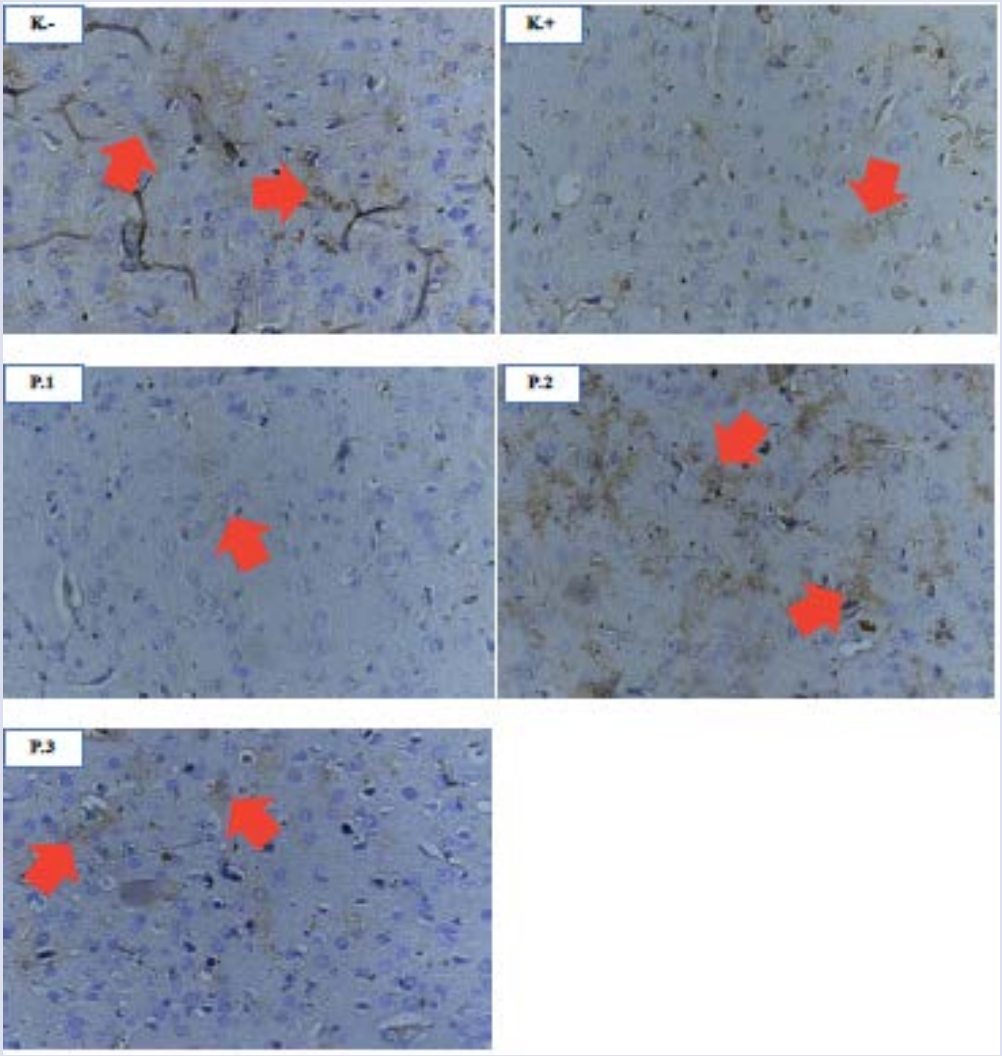

Figure 6: Comparison of IL-10 expression in brain tissue groups (K-, K+, P.1, P.2, P.3). Red arrows indicate the presence of serotonin expression in the cerebrum which is indicated by the presence of a brown chromogen color. IHC. 400x.

effect of flavonoids on *Sargassum cristaefolium* is lacking, possibly related to the adjustment of vasoactive substances, neurotransmitters and doses of *Sargassum cristaefolium* extract, although there is a decrease in CGRP levels in migraine experimental animals after administration of *Sargassum cristaefolium* extract.

Study by Abbey showed that the secretion of CGRP by trigeminal nerve stimulation can be inhibited directly by the flavonoid-containing methanol extract of *Theobroma cocoa* bean. In the TMJ inflammation model, CGRP release from trigeminal neurons can be inhibited by flavonoids. It was stated that the flavonoids in *Theobroma cacao* only suppressed the release of stimulated CGRP but did not inhibit basal CGRP secretion.[15]

IL-10 downregulates proinflammatory cytokines such as TNF-α, IL-6 and IL-1. One study suggested the anti-inflammatory activity of flavonoids through their potential downregulation of transcriptional downregulation of cyclooxygenase-2, prostaglandin E2, and TNF-α.[16] Another study showed the lowest TNF-α expression level in wound skin given *Sargassum illicifolium* extract containing alkaloid compounds compared to the control group during the healing process through RT-PCR analysis.[17]

Another study showed that the ethanolic extract of *Sargassum cristaefolium* could inhibit the proinflammatory cytokines TNF-α and IL-6 in skin lesions of rats treated with UVR by reducing epidermal thickness. Anti-inflammatory cytokine IL-10 levels were higher in mice treated with UVR with *Sargassum cristaefolium* ethanol extract due to its faster regenerative healing properties.[18]

IL-10 is produced by monocytes and astrocytes in brain tissue and is found in plasma in migraine sufferers, IL-10 functions as a macrophage inhibitor, reduces antigen presentation, and inhibits the production of IFN-γ, IL-6 and TNF-α. IL-10 plays a role in neutralizing macrophage pathology in migraine by inhibiting the secretion of IFN-γ and TNF-α.[2] In the serum of migraine sufferers, there was an increase in the level of TNF-α and an increase in the level of IL-10. Elevated levels of IL-10 during migraine attacks in response balance the increased plasma proinflammatory cytokine levels during attacks.[18] However, excessive increases in IL-10 can suppress the immune response and worsen disease outcome. The IL-10/TNF-α ratio in the range of 1.3-1.9 has an anti-inflammatory effect.[19]

CONCLUSION

The results showed that the ethanol extract of *Sargassum cristaefolium* could reduce levels and expression of CGRP compared to the positive control in the treatment group at a dose of 500 mg/kgBW. decreased levels and expression of TNF- compared to the positive control in the treatment group at a dose of 500 mg/kgBW. The levels and expression of IL-10 increased compared to the positive control in the treatment group at a dose of 750 mg/kgBW. The relationship that has a positive direction is the administration of *Sargassum cristaefolium* ethanol extract and IL-10 levels. While the relationship that has a negative direction is the administration of *Sargassum cristaefolium* ethanol extract and CGRP levels; administration of *Sargassum cristaefolium* ethanol extract and levels of TNF- α. From the path analysis carried out, it was found that there was a significant path between the administration of *Sargassum cristaefolium* ethanol extract and a decrease in TNF- α; administration of *Sargassum cristaefolium* ethanol extract with an increase in IL-10. The effect of giving *Sargassum cristaefolium* ethanol extract on CGRP levels did not have a significant relationship.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

ABBREVIATIONS

BW: body weight: FeCl: Ferric Chloride; HCl: Hydrogen Chloride; IFN: Interferon; IL: Interleukin; LPS: Lipopolysaccharide; ml : mililiter; NaOH: Natrium Hidroksida; NA: Neurokinin A; NO: Nitric Oxide; NOS: Nitrous Oxide Systems; NSAID: Non-steroidal anti-inflammatory drugs; NTG: Nitroglycerin; RT-PCR: Real time polymerase chain reaction; SD: Standart Deviation; SP: Substance P; SPSS: Statistical Package for the Social Sciences; TNF: Tumor Necrosis Factor; UVR: Ultraviolet radiation.

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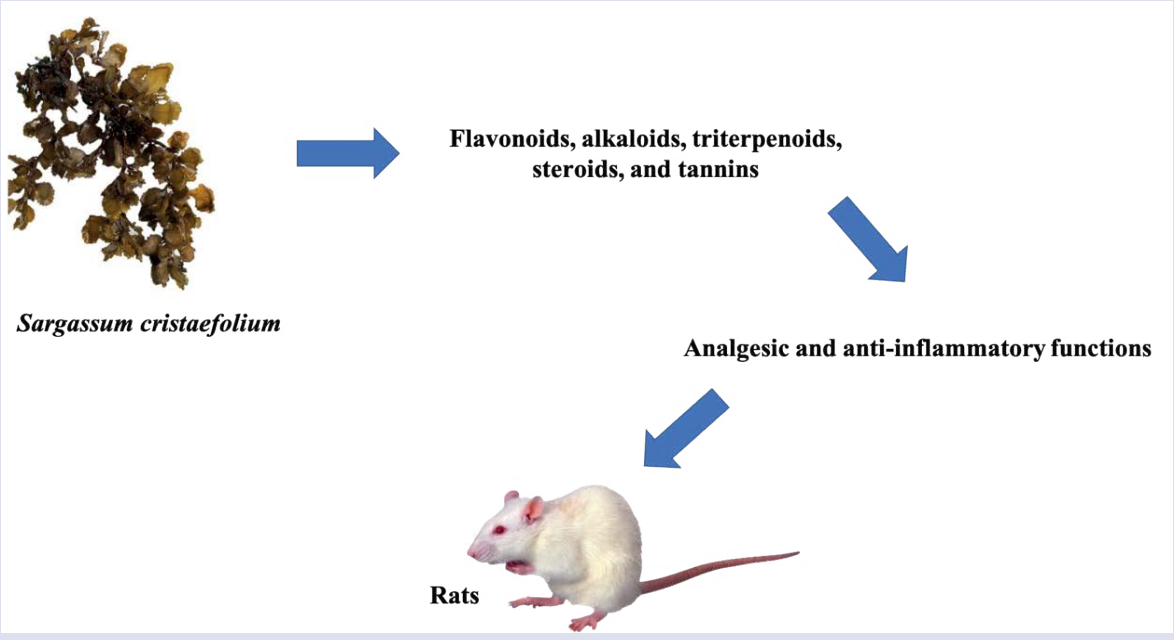
GRAPHICAL ABSTRACT



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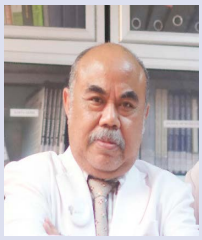

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