

IN-VIVO PHARMACOLOGICAL STUDIES OF *HYPNEA MUSCIFORMIS* FOUND IN THE COAST OF SAINT MARTIN ISLAND OF BANGLADESH

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ABSTRACT

Hypnea musciformis, a red seaweed prevalent in tropical and subtropical regions, has been linked to a multitude of therapeutic benefits. The aim of this study was to investigate the analgesic and anti-inflammatory properties of its extracts in 50% ethanol. Using the hot plate test, the acetic acid induced writhing test, and the formic acid induced test on Swiss albino mice, the analgesic potential of ethanol extracts was examined. Each test was administered at a dose of 500 mg/kg. Alternatively, an ethanol extract from carrageenan-induced paw edema was used in an anti-inflammatory experiment. Diclofenac is used as a reference standard in studies looking at the analgesic and anti-inflammatory effects of substances. In those animal models, our research demonstrates that *Hypnea musciformis* possesses potent anti-inflammatory and pain-relieving capabilities (percent inhibition found 28.22% on acetic acid method, 42.3% on Hot plate, and 48.7% on Formic acid).

KEYWORDS: St. Martin Island; *Hypnea musciformis*; Bioactive Compounds; Antinociceptive; Anti-inflammatory and Analgesic.

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Introduction

Pain and inflammation are nonspecific symptoms that can accompany many different diseases. Historically, non-steroidal anti-inflammatory drugs (NSAIDs) and opiates have been used to treat these symptoms, but they come with risks, including gastrointestinal (GI) distress, renal (kidney) damage, pulmonary (lung) depression, and even dependence (Damaj et al., 1999; Shojaii et al., 2015). Research into the possibility that medicinal natural substances could provide safer alternatives to synthetic anti-inflammatory and analgesic medications has recently increased.

The marine ecosystem, extending from the shoreline to the ocean floor, is home to a diverse variety of marine plant and animal life. Countless animals have made their home there. When compared to terrestrial ecosystems, marine habitats have the highest biodiversity (Beaumont et al., 2007; Palumbi et al., 2009). The coastal region of Bangladesh covers 19 districts and an area of 47,201 km² (32% of the country's total geographical area) (H. Ahmad, 2019). Seaweed, which can be found in abundance wherever there is salt water, is a good source of many nutrients. South of the Bay of Bengal, from the Sundarbans mangrove forest to St. Martin's Island, seaweed is abundant from October to April. There are 193 different types of seaweed that have been identified so far (Arulkumar et al 2020), of which 19 have any kind of

commercial potential. Among the many rapidly growing markets for seaweed are those for human use as food, medicine, hydrocolloid production, cosmetics, animal feed, fish feed, fertilizers, soil conditioners, etc. Marine resources are a great source of bioactive compounds. Red seaweeds have gained special interest as a good source of bioactive compounds. Red seaweeds are rich in phytochemicals and potent source of anti-tumor, anti-inflammatory, and analgesic substances (Araújo et al., 2017; Coura et al., 2015; De Sousa Oliveira Vanderlei et al., 2011; Pérez et al., 2016). Types of algae found off the coast of Bangladesh *Hypnea musciformis*, a type of carrageenophyte (a plant that produces the polysaccharide carrageenan), is widely employed in a variety of food, industrial, and medical applications due to its peculiar gelling characteristics (Mtolera & Buriyo, 2004; Rhein-Knudsen et al., 2015). A previous study reported that 500 µg/mL of *H. musciformis* powder was dissolved in a 5% ethanolic solution and, after extraction applied to black tiger shrimp, resulting in an increased shelf life (Arulkumar et al., 2020). The decreased levels of cholesterol and total lipids facilitate better outcome in cardiovascular patients (Bersot et al., 2003a). During pharmacological investigation it was found that blood lipids, cholesterol and triglycerides were shown to be decreased after the administration of a methanolic extract of *H. musciformis* in a mice model (Najam et al., 2010).

Several studies depict the therapeutic potentials of the red seaweed, *H. musciformis*. The antihypertensive effect of seaweeds was reported in the paper by (V. U. Ahmad et al., 1993). The effects of red seaweeds on neuropharmacological activities were investigated by different scientific methods. In case of Parkinsonism the increased level of dopamine could also be beneficial. In this study serotonin level was found to be decreased after the administration of *H. musciformis*. As a result it was thought that the regular use of seaweeds would relieve the symptoms of anxiety because the decreasing the concentration of serotonin which is beneficial in treating anxiety (Najam et al., 2010).

Unfortunately, *H. musciformis* has not been studied in sufficient detail to provide adequate nutritional data, and its exact dietary composition has not been documented in Bangladesh. In light of the foregoing, the present study assessed the analgesic and anti-inflammatory properties of *H. musciformis*. Due to these results, *H. musciformis* can be safely used to treat inflammatory disorders.

Materials and Methods

Sample Collection and Preparation

Hypnea musciformis, a type of red algae, was harvested in March 2021 from Saint Martin Island in the Bay of Bengal, where it had been growing in ideal conditions. *Hypnea musciformis* was gathered, cleaned with tap water, and then dried in the shade at room temperature rather than in direct sunshine, which could cause the active components to evaporate. Following drying, a 27g sample was ground into a powder using a mortar and pestle. This fine powder was kept in an airtight container at room temperature (25–27 °C). A shaking incubator (Model: JSSI-070C, JSR, Korea) was used to shock the crushed powder (20gm) in 200ml of 50% ethanol and keep it there for two hours at 25°C and 150 rpm. Following filtration, the solution was collected on sterile Whatman No. 1 filter paper (Qualitative, 102). Following the evaporation of the suspensions, 5 g of ethanol extract were produced (Basit et al., 2022; Karthikeyan et al., 2009).

In Vivo Assay

Experimental Animals

In the current investigation, male young Swiss-albino mice (n = 4 per group) between the ages of 4-5 weeks and weighing 20-25 grams were employed. The International Center for Diarrheal Disease Research, Bangladesh (ICDDR, B) supplied the animals used in this study from its animal Research Branch. Experiments involving animals were conducted in a pathogen-free environment at the Marine Biotechnology Laboratory of the Bangabandhu Sheikh Mujibur Rahman Maritime University in Bangladesh. All animals were kept in stainless steel cages with a controlled environment that included a constant temperature of (24.0 ± 0.5 °C), humidity of 55-65%, and a 12-hour light-dark cycle. When carrying out any of the investigations associated with the study of pain, strict adherence to the recommendations made by the International Association was ensured (Zimmermann, 1983).

Acute Toxicity Test

To determine the acute toxicity of a 500 mg/kg single dose of *Hypnea musciformis* ethanolic extract in mice, the

investigation was conducted orally. The study used 12 mice. Each animal was individually selected, and its weight was used to determine how much extract diluted in 50% ethanol would be given to it. After each mouse received an oral dose of the extract, they were monitored for harmful behavioral effects for three days. For the potential fatal long-term consequence, the animals were observed for a total of 9 days. The animals' body masses were recorded as part of the study.

Analgesic Study

To test the analgesic activity of the *Hypnea musciformis*, the following three experiments were performed sequentially.

Hot Plate Test

To determine whether or not the 50% ethanol extract of *Hypnea musciformis* had analgesic effects, a hot-plate test was conducted. Mice were screened by holding them in a glass beaker (600 ml) on top of a hot plate (thermostatically maintained at 50 ± 1 °C).

To prevent tissue damage in the paws, the maximum allowed hot-plate latency was reduced to 20s (Duan et al., 2006). Response latency was measured from the time an animal was placed on the hot plate until it acted in some way, such as licking its paws, holding its feet, shaking, or jumping off (in seconds). The longer it takes for a person to react, the more analgesic they are. After orally ingesting *Hypnea musciformis* extract, response latencies were measured immediately, then again 30, 60, 120, 180, and 240 minutes later (EDDY & LEIMBACH, 1953; Franzotti et al., 2000; Toma et al., 2003).

Acetic Acid Induced Method

According to the approach described by Januário et al., the analgesic efficacy of *Hypnea musciformis* extract was assessed (Januário et al., 2021). Three groups of mice, each with four mice, were selected at random. Mice in the test group were given extract orally at a dose of 500 mg/kg during the study. Diclofenac was used in the positive control group whereas saline water was used on the negative control group. Each mouse was injected intraperitoneally with a 0.7% (10 ml/kg) acetic acid solution 30 minutes after the extract was given to see if they would experience pain. Every mouse was observed for 30 minutes beginning 5 minutes after injection to record the number of times it contorted. The percentage of pain inhibition was calculated according to the following formula:

$$\% \text{ inhibition} = \left(1 - \frac{V_T}{V_C} \right) \times 100$$

Where V_T means number of writhing motions in drug-treated mice and V_C number of writhing motions in the control group of mice.

Formic Acid Induced Assay

After 30 minutes of treatment, mice in all three groups received an injection of 0.5% v/v formic acid into the sub plantar region of their right hind paw. Mice (n = 4) were given three different doses of an oral solution containing either 500 mg / kg of body weight of *Hypnea musciformis* extracted in 50% ethanol, 10 mg / kg of body weight of diclofenac, or normal saline (10 ml/kg). Following administration of formic acid, the animals were monitored in a 600 ml beaker for up to 30 minutes. Quantitative analysis of the nociceptive behavior elicited by formic acid was performed by timing the onset of

the behavioral response (lifting or licking) following formic acid injection during the neurogenic phase (between 0 and 5 minutes) and the inflammatory period (15 to 30 minutes).

Anti-inflammatory Activity

This study used a modified version of the carrageenan-induced mice hind paw edema test, the standard for assessing the anti-inflammatory properties of pharmaceuticals (Bersot et al., 2003b). There were a total of 12 mice used in this experiment: 4 in each of the three groups. A saline water solution (10 ml/kg of body weight) was administered to the control group (Groups I). Animals in Group II (the positive control) were given 10 mg/kg of Diclofenac, while those in Group III were given 500 mg/kg of an ethanol extract of the *Hypnea musciformis*. Sub plantar injection of carrageenan (1.0%) in normal saline (0.9% w/v NaCl) caused acute inflammation in the right hind paw of mice after 30 minutes. A plethysmometer was used to measure the volume of the paw at 0, 1/2, 1, 2, 3, 4, and 8 hours following the carrageenan injection. The proportion of inflammation suppressed was calculated using the average paw volume of control and treated mice (V_c and V_t) ($\%I = (V_c - V_t/V_c) 100$) And "I" refers to reducing inflammation.

Results

Effect of Ethanolic Extract on Acute Toxicity Studies

No mice were killed in an acute toxicity test using a 500 mg/kg oral limit dosage of *Hypnea musciformis* 50% ethanol extract. In both the short and long-term study periods, no fatal side effects were detected. Throughout the course of the 9-day research, no evidence of toxicity was seen in the animals. This suggests that the extract is likely safe at these levels, with an oral LD₅₀ that is higher than 500 mg/kg in mice.

Hot Plate Analgesic Test

The results of the hot-plate test in Group-I (the control), which received distilled water (10 ml/kg) orally; Group-II (the positive control, or standard), which received diclofenac sodium (10 mg/kg) orally; and Group-III (the test sample), which received algal extract (500 mg/kg BW) orally, are shown in Table 1. The *H. musciformis* group's results were compared to those of the control group.

According to the results from Table 1 at 30 and 240 minutes, the percent inhibition of doses of 500 mg/kg body weight were 15.66% & 42.30% for *H.musciformis*, respectively. The results were found to be statistically significant.

Table 1. Hot-Plat Test for *H.muciformis* ethanol extract. Values were expressed as mean \pm SD (n=4 animals per group). *P< 0.01, ***P< 0.001 vs. control. a= T-test between diclofenac and extract. b= T-test between control.

Group	0 min	30 min	60 min	120 min	180 min	240 min
Control	11 \pm 2.70	20.75 \pm 1.70	19.5 \pm 1.2 9	16 \pm 2.16	11.5 \pm 2.38	6.75 \pm 3.77
Diclofenac	18.25 \pm 0.95	18.5 \pm 0.57*a	18 \pm 1.15	13.75 \pm 2.2 1	10.25 \pm 0.5	10.5 \pm 2.8* a
Extract	17 \pm 1.41** b	17.5 \pm 0.57** b	18.5 \pm 1.9 1	14 \pm 0.81	13 \pm 1.25** b	7.5 \pm 1

% Inhibition						
	0 min	30 min	60 min	120 min	180 min	240 min
Diclofenac	3.93	10.84	5.26	14.06	10.86	19.23
Extract	10.52	15.66	2.63	12.5	6.52	42.30

Values were expressed as mean \pm SD (n=4 animals per group).

*P< 0.01, ***P< 0.001 vs. control.

a= T-test between diclofenac and extract. b= T-test between control.

Analgesic Activity Assessment by Acetic Acid-Induced Writhing Test

The effects of 50% ethanol extract of *H.musciformis* 0.7% acetic acid induced writhing in Swiss albino mice are summarized in Table 2. A dose-dependent and significant

reduction in the number of abdominal constrictions induced by intraperitoneal administration of 0.7% acetic acid was observed with oral administration of *H.musciformis*, at the doses of 500 mg/kg body weight showed significant result, when compared to the control.

Table 2. Effect of *H. musciformis* extract on acetic acid induced (0.7%) writhing in mice.

	Control	Diclofenac	<i>H. musciformis</i>
Mean Writhing	154.66±31.06*a	162.33±6.806	111±7.93***b
% Inhibition		4.95	28.22

Values were expressed as mean ± SD (n = 4 animals per group).

*P< 0.05, **P< 0.01, ***P< 0.001 vs. control.

a= T-test between diclofenac and extract. b= T-test between control.

Formic Acid Induced Method

In the case of formic acid-induced nociceptive behavior, the usual pain responses included an acute phase (lasting from 0 to 5 min, phase I), a relatively short quiescent interval, and a protracted tonic phase (15- 30 min, phase II). Phase I is mostly caused by direct chemical stimulation of peripheral nociceptors. Instead, phase II involves neuronal sensitization at the spinal cord because of inflammatory stimulation.

Phase I and II results are summarized in Table 3, which reveals that at a dose of 500 mg/kg, 50% ethanolic extract of *H. musciformis* significantly reduced neurogenic pain (inhibition of 48.21%) but had a lesser effect on inflammatory pain (inhibition of 68.28%) compared to the positive control (Diclofenac).

Table 3. Effect of *H.musciformis* extract on Formic Acid-induced pain method.

Phase	Group of mice with mean±SD		
	Control	Diclofenac	H. musci.
Early phase	60.5±14.52	48.33±29.92	31.33± 38.00*a
Late phase	77.75±4.57	12.66±2.51	24.66± 16.77***a
%Inhibition			
Early phase(0-5min)		20.11	48.21
Late phase (15-30min)		83.71	68.28

Values were expressed as mean ± SD (n=4 animals per group).

*P< 0.01, ***P< 0.001 vs. control.

a=T-test between control and extract.

Anti-inflammatory Activity

Mice given an intraperitoneal injection of carrageenan displayed a time-dependent increase in paw edema in a test designed to measure this. Among the control group, edema worsened the most by the third hour after carrageenan was

given. At 500mg/kg, *Hypnea musciformis* extract significantly decreased edema. At a dose of 500 mg/Kg body weight, the percentage of inhibition was reported to be 10.34% after 2 hours and 12.5% after 4 hours of investigation (Table-4).

Table 4. Effect of Carrageenan induced anti-inflammatory test for *H.musciformis* extract.

Mean \pm SD						
Group	0H	1H	2H	3h	4H	5H
Control	0.155 \pm	0.147 \pm	0.145 \pm	0.132 \pm	0.16 \pm	0.14 \pm
	0.033	0.035	0.017	0.020	0.024	0.024
Diclofenac	0.127 \pm	0.137 \pm	0.147 \pm	0.167 \pm	0.125 \pm	0.137 \pm
	0.023	0.01	0.017	0.037	0.010	0.015
Extract	0.15 \pm	0.132 \pm	0.130.016*a	0.122 \pm	0.14 \pm	0.137 \pm
	0.014	0.020		0.009*a	0.018	0.015
% inhibition						
Diclofenac	18.06	6.8	1.36	-26.51	21.87	7.14
Extract	3.22	10.2	10.34	7.58	12.5	7.14

Values were expressed as mean \pm SD (n=4 animals per group).

*P<0.05, **P<0.01, ***P<0.001.

a=T- test between diclofenac and extract.

Discussion

The root cause of symptoms brought on by illnesses that affect the body is inflammation. Vascular tissues have a complex biological reaction to harmful stimuli such as infections, damaged cells, physical and chemical harm, as well as immune reactions. In terms of humanity and health, understanding inflammation and related processes has been a huge puzzle. Increased cellular metabolism, the release of cellular-soluble inflammatory mediators, increased blood flow, vasodilation, extravasation of fluids and cellular influx, the formation of abnormal granulations, necrosis, and excessive tissue degeneration and exudation are the cardinal signs that indicate inflammation. All of them result in varied degrees of tissue damage and may even be fatal (Olela et al., 2020). Current treatment consists of steroidal and nonsteroidal anti-inflammatory medicines, which are associated with a number of adverse effects (Sun et al., 2018). *Hypnea musciformis*, it has been observed, contains a wealth of active pharmaceutical ingredients. Several types of bioactive activity, including analgesic and anti-inflammatory effects, were found in red algae. *H. musciformis*, a common species of seaweed, has been demonstrated in multiple studies on animal models to reduce blood lipids, including cholesterol and triglycerides. Decreased cholesterol and total lipids reduce the risk of numerous cardiovascular issues (Aziz et al., 2021; Balamurugan et al., 2017). Insightful new information was uncovered in the present investigation. The anti-inflammatory and analgesic properties of a 50% ethanol extract of *Hypnea musciformis* were studied and found to be quite potent.

Central analgesic action was evaluated using the hot-plate test and formic acid test, whereas peripheral analgesic activity was often measured using the acetic acid-induced writhing test (Azim et al., 2021; Sun et al., 2018).

Both central antinociception and supraspinal nociception were found to be active in the hot plate test. Since mouse paws are sensitive to heat even at temperatures that do not cause skin damage, the central antinociceptive mechanisms of the extract were detected by exposing the mice to a constantly heated plate and timing their reactions, which included jumping away from the heat, withdrawing their paws, and licking them. The time it took for these reactions to occur increased when centrally acting analgesics were administered (Yimer et al., 2020). Since this animal responds well to strong analgesics and causes minimal tissue damage when the mouse is removed from the hot plate after 20 seconds, it was chosen to test the extract's central analgesic potential. There is a time savings associated with using the model, and the results of the measurements are usually reliable.

The pain threshold was considerably (*P < 0.01, ***P < 0.001) raised by the extract test dose (400 mg/kg of body weight) beginning at 30 minutes into observation, as measured by an increase in reaction time compared to the positive control. At 30 minutes and 240 minutes of observation, the extract's analgesic effects were at their peak at 15.66% and 42.30 %, respectively, outperforming Diclofenac (10 mg/kg), which showed analgesic values of 3.93 % and 19.2 % at the same times. This extract's peak time for maximum impact was 240 minutes after dosing. This lag could be from the drug needing some time to enter the central compartment and distribute to the target site, or it could be the result of the drug's metabolism into active metabolites that possess analgesic efficacy with longer half-lives.

Chemically inducing visceral pain models like acetic acid-induced writhing has been linked to the production of cyclooxygenase, bradykinin, arachidonic acid, histamine, and substance P, which in turn excites pain nerve endings and

causes belly writhing (Lv et al., 2012). The number of writhes was much lower in the 50% ethanol extract of *H. musciformis* than in the positive control group (reference drug Diclofenac), as determined by statistical analysis ($P < 0.001$) (Table 2). One of these pain mediators may have been blocked by a 50% ethanolic extract of *H. musciformis*, leading to a decrease in writhing.

In mice, *H. musciformis* considerably mitigated the twitching brought on by acetic acid. The results suggested that, like with NSAIDs, the analgesic action might be partially mediated at the periphery of the body.

Formic acid produced paw pain is a well-established *in-vivo* model of pain that can be used to research analgesics. Different analgesics may have different effects in the early and late stages of the formic acid test, which is why it is important to conduct both phases. As a result, the test can be used to better understand how a potential analgesic would work to reduce pain. The anti-nociceptive effect of the *H. musciformis* extract may be related to its peripheral action, and it acts better than the reference medication (Diclofenac) receiving group, as shown by its inhibitory effect on the nociceptive response in the early and late phases of the formic acid test.

The induction of inflammation by carrageenan is a standard technique for identifying anti-inflammatory drugs with an oral bioavailability and a biphasic response. Histamine, serotonin, and kinins are released to mediate the first phase, and prostaglandins are released to mediate the second. When compared to diclofenac-treated control mice with carrageenan-induced paw edema, *H. musciformis* ethanol extract did not significantly reduce inflammation. Table 4 shows that an extract given at a constant dose of 500 mg/kg body weight showed the greatest inhibition after 1 and 2 hours, suggesting that its anti-inflammatory action may be attributable to blocking the release of histamine or kinins (Dray & Perkins, 1993).

Conclusion

The longevity of algae products in the pharmaceutical industry has been boosted by the curiosity of scientists. Finally, current research indicates that *H. musciformis* has potent analgesic and anti-inflammatory properties. Many endogenous inflammatory mediators, pain transmission, and mediators may be inhibited by the *H. musciformis* extract, according to these findings. Nonetheless, *H. musciformis* may be a source of novel bioactive chemicals, and the mechanism of each action should be investigated in order to unveil the pathway, as demonstrated by the substantial results. Using this scaffold, with some tweaks to improve the pharmacokinetic profile and to address difficulties linked to the adverse effects of conventional analgesic anti-inflammatory medications, this study would undoubtedly aid pharmaceutical researchers in conducting more organized and fertile drug discoveries.

Authors Contribution

MNH made the hypothesis, designed the experiments, supervised the work, analyzed the data, wrote and revised the final version of manuscript; MA conducted the experiments,

analyzed the data and wrote the initial draft of manuscript; SS did manuscript editing.

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