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Research Article

Halimeda opuntia Green Seaweed Possesses Anti-Inflammatory and Analgesic Activities

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Abstract

In the present study, the anti-inflammatory and analgesic activities of the green seaweed *Halimeda opuntia* were performed. Here, we report on the anti-inflammatory and analgesic activities of the 50% ethanol extract of this seaweed. Using a Swiss Albino mouse model, carrageenan-induced paw edema was used to evaluate the anti-inflammatory activity, while hot plate, formalin-, and acetic acid-induced writhing were used to test the analgesic efficacy. In an earlier test for acute toxicity, the extract was given to mice at a concentration of 500 mg/kg body weight (BW), and the animals showed no detrimental effects. The extract revealed an anti-inflammatory effect by reducing paw edema as compared to the control and diclofenac groups in the carrageenan-induced paw edema method. The highest inhibition of 17.19% was recorded at hour four. In the analgesic study, the extract lengthened the response latency of the mice on the hot plate, and a time-dependent analgesic effect was observed. The extract showed promising results in both the early and late phases of pain removal in the formalin-induced method. The inhibition rate exceeded 80%. In the acetic acid-induced method, the *H. opuntia* extract reduced the number of writhings induced by intraperitoneal injections of acetic acid and about 14.7% inhibition was observed. These findings unequivocally demonstrate that the *H. opuntia* extract has anti-inflammatory and analgesic properties, which will pave the way to using this species as a therapeutic drug target.

Keywords: *Halimeda opuntia*; Analgesic; Anti-Inflammatory; Marine Algae; Green Seaweed

Introduction

It is explicitly stated that marine organisms are unexplored sources of incredibly diverse natural compounds [1, 2]. In the last century, a great deal of use has been made of the molecules that are produced by terrestrial life. The discovery of substances and enzymes that can heal diseases, expedite commercial manufacturing and processes, facilitate lab testing and diagnostics, and mark the start of a new age are highly sought after. Marine organisms thrive in completely different conditions where increased salinity, both high and low temperature, high pressure, and complex symbiotic and predator-prey relationships are present. As a result, to adapt, they create a wide variety of fascinating biomolecules with special qualities and purposes, such as metabolites, proteins, enzymes, and polymers. Because of this, researchers are working to create novel medications using marine resources to treat a variety of illnesses [3-7].

Seldom are the about 193 seaweeds of the Bay of Bengal, which are located inside Bangladesh's coastal borders, examined, and researched for their uses in the industrial and pharmaceutical industries [8]. Halimeda is a genus that belongs to the phylum Chlorophyta and includes warm temperate to tropical calcium carbonate depositing macroalgae [9]. An extensive range of bioactivities [10], including antibacterial [11-13], antifungal [11-14], antioxidant [15-18], cytotoxic activity [11,18,19], anti-plasmid activity [11], antityrosinase activity [17], wound healing activity [19], interferon ß production promoting activity [20], antiviral activity against acyclovir-resistant HSV-1 and -2 [21], antileishmanial activity [22], etc., have been reported previously on *Halimeda opuntia*.

In light of these bioactivities, we have chosen to evaluate the analgesic and anti-inflammatory properties of the green seaweed *H. opuntia*, which was obtained from the Bay of Bengal. The most prevalent conditions for which non-steroidal anti-inflammatory medicines (NSAIDs) are administered include several chronic inflammatory illnesses and pain management; however, NSAIDs can have several negative effects, including gastrointestinal ulcers, renal failure, and cardiovascular events [23]. Therefore, it is necessary to look for alternative medications in nature to improve health issues.

In this study, we took a part to unveil the pharmacological properties of green seaweed *H. opuntia* as a source of possible marine-derived pharmacological origin. We used Swiss-albino mice as a model organism to explore our goal, and by applying some well-known analgesic and anti-inflammatory test methods (Hot-plate test, formalin-induced, and acetic acid-indued test), we confirmed that *H. opuntia* is a potent source of an agent with anti-inflammatory and analgesic activity, which would have a potential pharmacological use as a therapeutic drug.

Materials and Methodology

Sample Collection

Seaweed sample was collected in November 2019 from the shallow water of the eastern side of Chera Island (CheraDwip), an extension of St. Martin's Island, Bangladesh, by maintaining the local guidelines of seaweed collection. As previously described [14], it was cleaned with pure seawater and submerged in 50% ethanol for preservation. Based on its morphology [24] and the AlgaeBase database (https://www.algaebase.org), the collected seaweed was later identified as *H. opuntia* (Linnaeus) J.V. Lamouroux. The seaweed was removed from the ethanol after roughly a month, cleaned with filtered water, segmented, and dried by air and oven at 37ºC before being pulverized with a mortar and pestle to a powder. The powder was kept cold at -20ºC, until that had to be used. It should be noted that the Bangladesh Navy, an authorized body, granted us permission to collect seaweed samples and utilize them for scientific research.

Extract Preparation

50 mL of 50% ethanol was combined with 5 g of the powdered material. For 5-7 days, the mixture was shaken in a tabletop shaking incubator at 150 rpm and 25ºC. Next, Double Rings of 11.0 cm filter paper were used to filter the entire mixture (Qualitative, 102). After that, the solvent eventually evaporated resulting in an extract of 50% ethanol.

Phytochemical Screening

The 50% ethanol extract of *H. opuntia* was subjected to qualitative phytochemical screening. The presence of tannins, steroids, flavonoids, saponins, and phenolic compounds was tested according to the methods described by Alam et al. 2011 [25]. The necessary reagents for the screening were: ferric chloride solution for tannins, chloroform, and sulfuric acid for steroids (Salkowski's test), 2 N sodium hydroxide for flavonoids, ability to form stable foam for saponins, Lead Acetate test for phenolic compounds.

Experimental Animals

The experiments used male Swiss Albino mice, aged 6-7 weeks, weighing 25–35 grams. At four weeks old, they were purchased from the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR'B). The mice were kept in the typical laboratory conditions, which included a 12-hour light-dark cycle, a temperature of 240C, and a relative humidity of 55–65% [25]. After purchase, they were kept in this condition for ten days so that they became accustomed to the lab atmosphere. They have unfettered

access to pure water and specifically made rodent food. Every three days, the wood flakes bed was replaced, and hygiene was checked. They were not given meals during the experimentation days as long as the experiment was not completed. Since the drugs were injected into the sub-plantar region of the mice, which only causes extremely little discomfort, no moderate anesthetic was given to any of the groups of animals because the experiment required continuing movement surveillance. The International Centre for Diarrheal Disease Research, Bangladesh's (ICDDR,B) relevant ethical committee gave its approval for the treatment of the animals, which was done in compliance with ARRIVE criteria.

Acute Toxicity Test

To evaluate the acute toxic effects and safety of the algal extracts, the acute toxicity test was carried out with modifications from Alam et al. 2011 [25]. For the investigation, Swiss Albino mice weighing between 25-35 g were employed. The mice were given 500 mg/kg (0.5 mg/10 g) of the ethanol extract powder (dissolved in pure water at a concentration of 50 mg/mL) one night before their body weight measurement. Every two days, the animals were given the extract, and in the interim, they were closely monitored for any signs of toxicity, including breathing difficulties, altered behavior, reactions of the sensory nerve system, gastrointestinal problems, etc. For around ten days, the observation was carried out every day. The weight of the mice was measured on days 01, 04, 06, 09, and 10 as an indication of their health status.

Test for Anti-inflammation

Paw Edema Approach Induced by Carrageenan

Using a mouse model, the anti-inflammatory test was carried out in vivo [25]. Twelve Swiss Albino mice altogether were split up into three groups, each with four mice. The mice were 5-6 weeks old. Injection of 0.1 mL of 1.0% carrageenan in normal saline (0.9% w/v NaCl) was performed into the right hind paw's sub-plantar area. The mice were given the ethanol extract 30 minutes before to the carrageenan injection. Different groups were treated as follows:

Group I (Control): Carrageenan and saline water (10 mL/kg BW)

Group II (Positive control): Carrageenan and Diclofenac (10 mg/kg BW)

Group III (Extract): Carrageenan and ethanol extract (500 mg/kg BW)

Using a Plethysmometer, the paw value was recorded at 0.5 (0th H), 1 (1st H), 2 (2^{nd} H), 3 (3^{rd} H), 4 (4^{th} H), 5 (5^{th} H) and 6 (6^{th} H) hours following the injection of saline, diclofenac, or extract. The left hind paw served as a reference for the non-inflamed paw for comparison. The average percentage increase in paw volume over time was calculated in relation to the control group. Percent inhibition was calculated using the formula below:

% Inhibition of paw edema = [(Vc – Vt) / Vc] □ 100

Where V_c and V_t represent the average paw volume of the control and treated animal respectively [25].

Analgesic Test

Hot plate approach

In light of the hot plate's high temperature (50±0.50C), mice exhibited a central nociceptive response [26]. Using a mouse model, the hot plate method (Lanhers et al., 1992, [27], modified by Mahomed and Ojewole, 2004 [28]) was used to evaluate the extract's analgesic effect. There were 3 groups of mice designated as group-I (n = 4), group-II (n = 4), and group-III (n =4) for control, positive control, and test sample group respectively. Each group received a particular treatment i.e. control (distilled water, 0.1 ml/10g), positive control (Diclofenac sodium 10mg/kg), and the test sample (50% ethanol extract, 500 mg/kg). To prevent paw damage, the animals were placed on Eddy's hot plate and held at a temperature of 50±0.50C for a maximum of 20 seconds. Jumping, licking, biting, and paw vibration were all considered legitimate forms of response. Twenty-second total response times were recorded before the sample was administered orally at 0 hr, and then at 0.5, 1, 2, 3, and 4 hr afterwards. The experimental scheme was mentioned in Fig. 3A.

The percent analgesic score (PAS) was calculated as:

$PAS = [(T_b - T_a) / T_b] \times 100$

Here, Tb = All responding moments (within 20 seconds) before drug administration, Ta = All responding moments (within 20 seconds) after drug administration (value of T_b and $T_a < 20$).

Formalin-Induced Approach

To be used as an edematogenic agent in the formalin-induced approach, a 0.5% (v/v) formalin solution was produced [25]. Three sets of experimental mice were created. Each group received different treatment regimens as follows:

Group I (Control, n = 4): Saline water (10 mL/kg BW), orally.

Group II (Positive control, n = 4): Diclofenac at 1 mg/mL concentration (10 mg/kg BW), orally.

Group III (Experimental, n =4): Ethanol extract dissolved in milli-Q water at 50 mg/mL concentration (500 mg/kg BW), orally.

Following a half-hour period of oral medication administration, each mouse underwent a subcutaneous injection of 20 µL of freshly prepared 0.5% (v/v) formalin solution into its right hind paw. In the injected paw, biting and licking were interpreted as

pain reflexes. After receiving a formalin injection, responses were noted between 0-5 minutes (early phase) and between 15-30 minutes (late phase). The experimental scheme was shown in Fig. 4A. Percent inhibition was calculated as:

% inhibition = $[(N_b – N_a) \div N_b] \times 100$

Here, Nb is the number of responses before the drug administration and Na is the number of responses after the drug administration.

The percent (%) inhibition was calculated based on the values of the control group.

Acetic Acid-Induced Writhing Approach

Acetic acid-induced writhing in mice is another way to evaluate the analgesic effects of the extract, a method described by Ahmed et al., 2004 [29]. The mice were divided into 3 different groups to administer different treatments as follows:

Group I (Control, n = 4): Saline solution (10 ml/kg), orally.

Group II (Positive control, n = 4): Diclofenac at 1 mg/mL concentration (10 mg/kg BW), orally.

Group III (Experimental, n =4): Ethanol extract dissolved in milli-Q water at 50 mg/mL concentration (500 mg/kg BW), orally.

Following a half-hour group-specific treatment, each mouse in the various groups received an intraperitoneal injection of 0.6% acetic acid solution (10 µL/gm BW). After that, each mouse was put under observation in a glass beaker. The number of abdominal constrictions, or writhes, that happened five to fifteen minutes after the acetic acid injection was tallied. An anti-nociceptive response was characterized as a marked decrease in writhing in the tested animals when compared to the control group. The experimental procedure was mentioned in Fig. 5A.

Percent inhibition was calculated as:

% inhibition = $[(Nb – Na) \div Nb] \times 100$

Here, N_b is the number of writhing before drug administration and Na is the number of writhing after drug administration.

Statistical Analysis

Statistical analyses of the results obtained from the experiments were performed in Microsoft Excel 2013 (Windows 10 Pro). Data obtained from the acute toxicity test, anti-inflammatory test, and analgesic tests were expressed as Mean ± SD (n = 4, and/or 5). Unpaired T-tests were performed for the study and the significance level was set as $p < 0.05$.

Results

Phytochemical Screening

A 50% ethanol extract of the *H. opuntia* sample was used to assess the presence of several phytochemicals. In this investigation, the only substances discovered to be significantly present were steroids and phenolic compounds (Fig. 1A).

Acute Toxicity Test

The mice's body weights (Fig. 1B) indicate that there was no discernible harmful effect of the ethanol extract throughout the experimentation days, nor were the mice's weights significantly altered. Abnormal behavior and zero fatalities were noted. The weight of the mice in the extract group grew daily in a linear fashion in comparison to the mice in the control group and a significant increase in body weight was observed on the day 09 ($p < 0.01$) and day 10 ($p < 0.001$) when compared among the tested group on day 01 (Fig. 1C). Every mouse has access to clean water and identical rodent food. Thus, to proceed with the study, the dose of 500 mg extract/kg BW of the mice was deemed safe for the analgesic and anti-inflammatory tests.

Figure 1.

A

Tested for	Tannins	Steroids	Flavonoids	Saponins	Phenolic compounds
50% EtOH extract of *H. opuntia*	-	+++	-	-	++

(-) not detectable; (+) low quantities; (++) moderate quantities; (+++) high quantities.

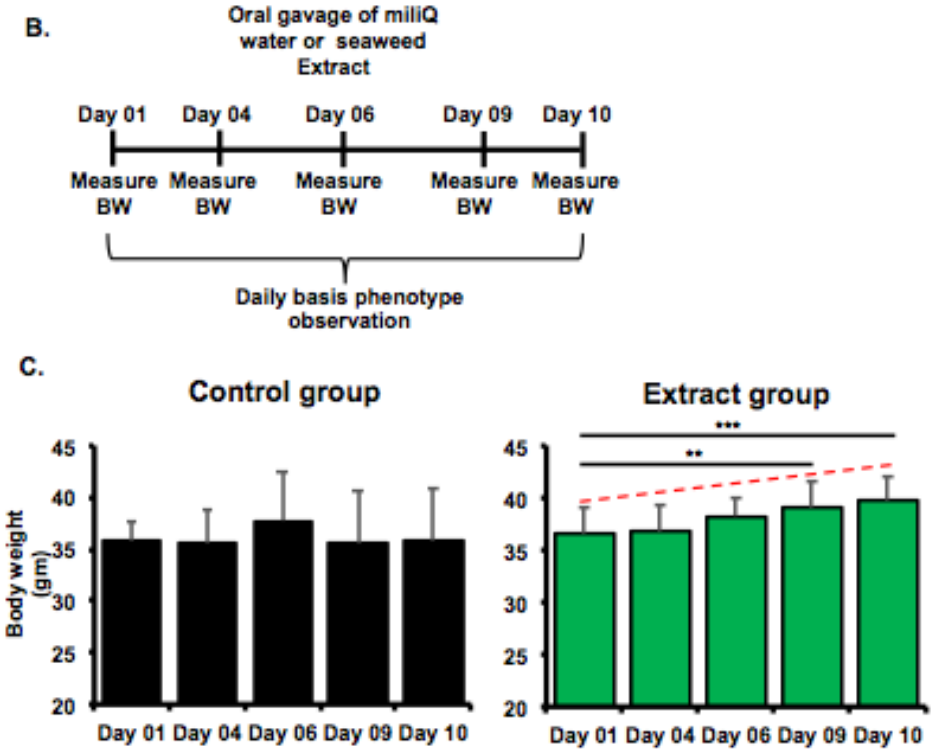

Figure 1: Phytochemicals screening and acute toxicity study. **A.** Qualitative analysis of the phytochemicals in 50% ethanol extract of *H. opuntia*. **B.** Illustration of acute toxicity study. **C.** Acute body weight gain of the subjected Swiss-Albino mice. Phenotype of the mice was observed for ten consecutive days and body weight (gm) were measured and showed as mean ± standard deviation (n = 4). Unpair T-test was performed, and significance levels were set as *** $p < 0.001$; ** $p < 0.01$.

Test for Anti-inflammation

Paw Edema Approach Induced by Carrageenan

Carrageenan-induced paw edema method was performed according to the scheme (Fig. 2A). The values calculated for each group were presented in Fig. 2B. In the control group, the paw volume was increased gradually and reached the maximum value at the 4th hour. Diclofenac treatment showed the best value at the 4th hour. But in the mice of the experimental extract group, paw volume was not increased sharply, which indicated the anti-inflammatory activity of the 50% ethanol extract of *H. opuntia*. The percent (%) inhibition value was calculated based on the value of the control group. The percent inhibition (% inhibition) for both the diclofenac and the extract group was highest at the 4th hour (21.87% and 17.19% respectively) (Fig. 2C). At this point, the result comparison between the control and the extract group was found to be significant ($p < 0.01$). The comparison between the control and the diclofenac group also showed significant decrease ($p < 0.01$) in paw volume as expected. Thus, our study suggests that *H. opuntia* possesses potential anti-inflammatory activity.

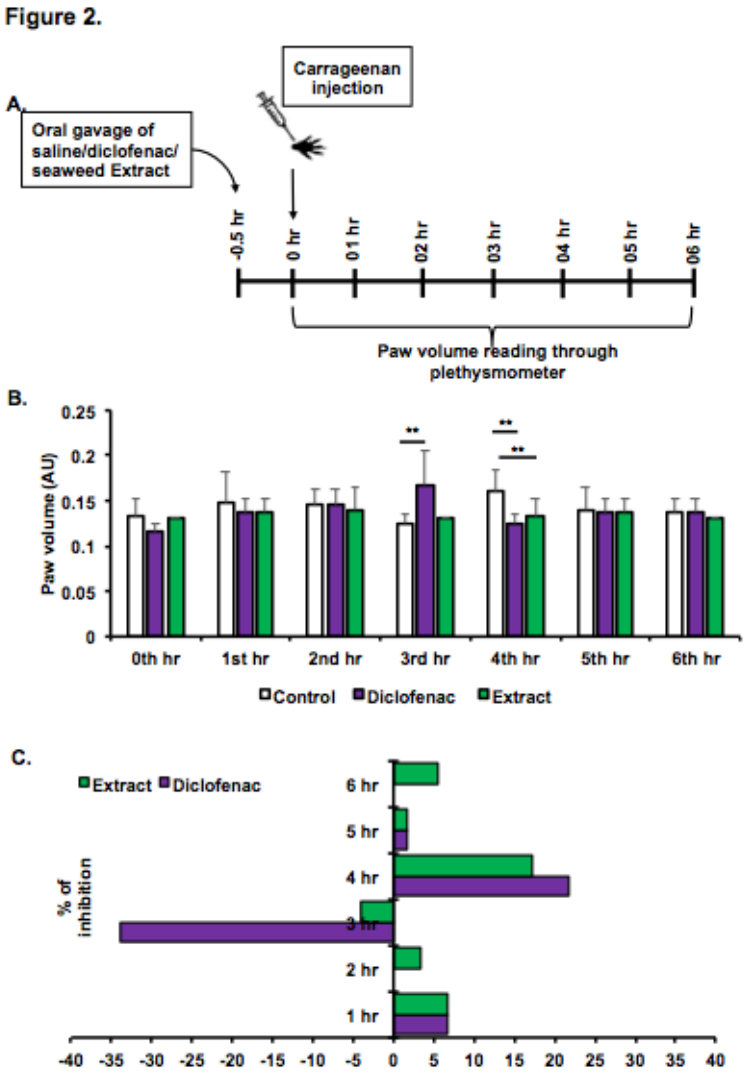

Figure 2: Carrageenan-induced paw edema and anti-inflammation activity test using Swiss-albino mice. **A**. Experimental scheme of carrageenan-induced paw edema study for anti-inflammation activity test. **B**. Anti-inflammatory effect of the *H. opuntia* extract over time. Paw volume measurements over time using plethysmometer was shown as mean ± SD (n=4). Unpair T-test was performed, and significance levels were set as ** $p < 0.01$, * $p < 0.05$. AU = arbitrary unit. **C**. Percent (%) of inhibition of paw edema between the positive control (Diclofenac) (n=4) and the tested sample (Extract) group (n=4).

Analgesic Test

Hot Plate Approach

Increased temperature stimulates a central nociceptive response in biological system [26]. To verify the central nociceptive response of the tested sample, we conducted hot plate test study in vivo. The response of mice to certain temperature and time of the hot plate test (mean ± SD; n = 4/5) were presented in Fig. 3B for the ethanol extract of *H. opuntia* and the other groups. The algal extract exhibited a profound analgesic effect as there was significant decrease in response in the mice of the extract group when compared with the control ($p < 0.01$) and the diclofenac group ($p < 0.001$) after 3rd hr of algal extract administration. Statistically significant decreased response was also observed after the 2nd hr, and 4th hr of administration when compared with control group and the diclofenac group (Fig. 3B).

The *H. opuntia* extract consistently performed better than the other two groups in increasing the response latency till the last hour except for 3rd hour. More precise analgesic effect was observed when the percent analgesic score was calculated based on the value of 0 hour for each group. The *H. opuntia* extract treated group showed a promising pain-relieving tendency at a percentage of 36.12% and 62.70% after 2 hr and 4 hr of oral administration of the extract, respectively. On the other hand, the positive diclofenac treated group showed 24.66% and 42.46% of pain relieve on the same time point as compared to algal extract treated group. Data of the control group was omitted due to negative value. The *H. opuntia* extract treated group showed around 13% and 21% more efficacy to relieve pain after 2 hr and 4 hr of oral administration of the extract, respectively, when compared with diclofenac positive control group (Fig. 3C). These data indicate *H. opuntia* as a potent source of analgesic or anti-central nociceptive agent.

Figure 3.

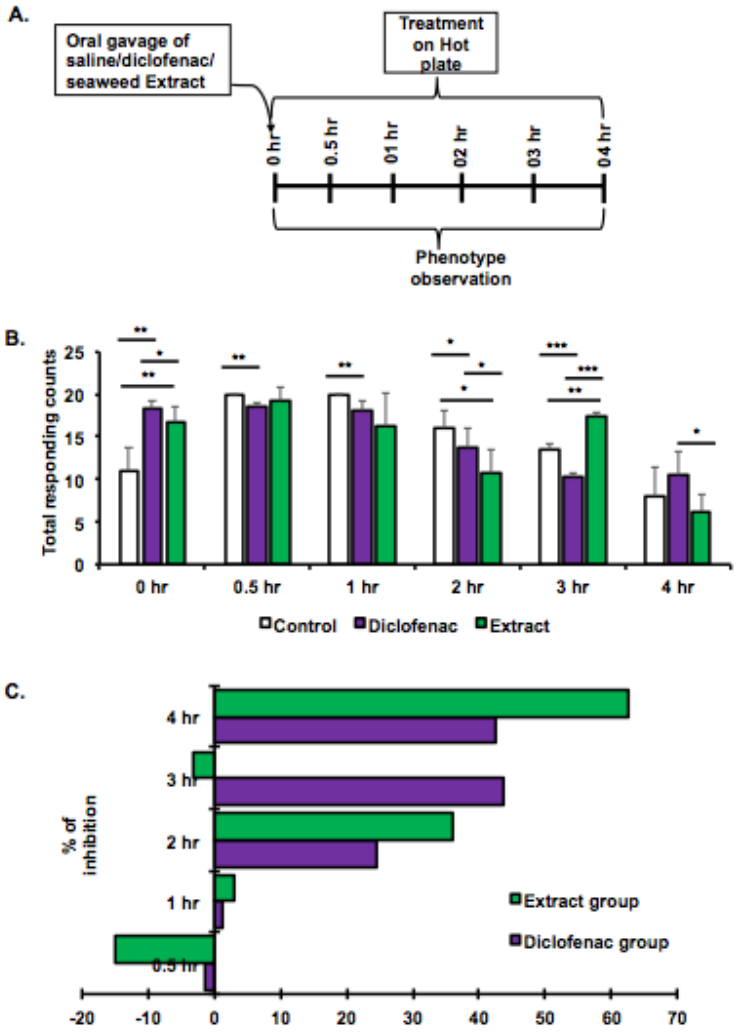

Figure 3: Determination of analgesic effect of *H. opuntia* in Swiss-albino mice through Hot-plate method. **A**. Experimental scheme of Hot-plate method. **B**. Total responding moments upon hot plate exposure to each group of mice in a time dependent manner. Data are expressed as mean ± SD (n = 4); SD – standard deviation; Unpair T-test was performed, and significance levels were set as *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. **C**. Percent (%) of inhibition of pain between the positive control (Diclofenac) (n=4), and the tested sample (Extract) group (n=4).

Formalin-Induced Approach

Formalin induces pain by affecting nociceptors in the early phase whereas the late phase is associated with inflammatory reactions [30-31]. To further explore the role of the *H. opuntia* extract during early phase of nociceptors and also in the late phase related to inflammatory reactions, we performed formalin-induced paw edema test. The algal extract gave a contrasting finding in both phases as compared to control group and diclofenac treated positive control group. More strikingly, the experimental group significantly reduced the number of formalin-induced nociceptive responses (licking and biting in the injected paw) when compared with the control (early phase, $p < 0.01$; late phase $p < 0.001$), and the diclofenac group (late phase $p < 0.05$) (Fig. 4B). The percent (%) inhibition of pain analysis showed 80.18% (early phase) and 92.21% (late phase) of pain relieve in the *H. opuntia* extract administered group. On the other hand, the diclofenac treated group showed 51.35% (early phase) and 61.70% (late phase) of pain relieve tendency that were around 29% (early phase) and 31% (late phase) less than the *H. opuntia* extract administered group (Fig. 4C). These results suggests that *H. opuntia* possesses anti-nociceptive role both at the analgesic stage, and inflammatory reactions stage and thus exerting strong reservoir of analgesic agents.

Figure 4.

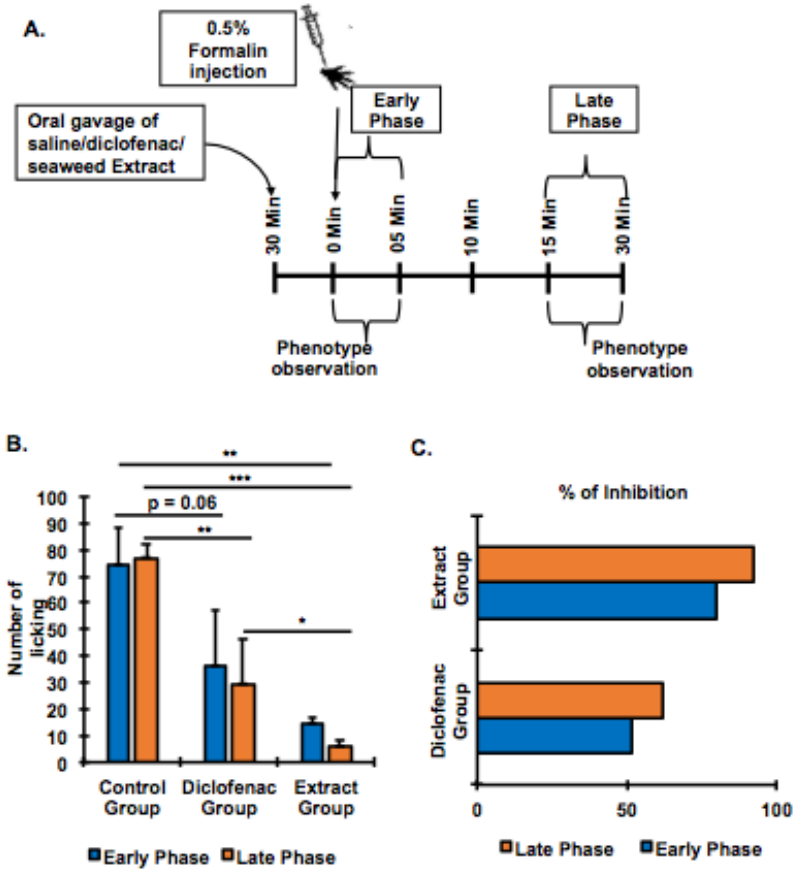

Figure 4: Determination of phase specific (early or late phase) analgesic effect of *H. opuntia* on Swiss-albino mice through 0.5% formalin-induced method. **A**. Experimental scheme of formalin-induced method. **B**. Number of licking and biting response in the paw of mice induced by formalin. Values are expressed as mean ± SD (n = 4); SD – standard deviation; Unpair T-test was performed, and significance levels were set as *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. C. Percent (%) of inhibition of pain between the positive control (Diclofenac) (n=4) and the tested sample (Extract) group (n=4).

Acetic acid-Induced Approach

To further extend our understanding in the analgesic effect of the algal extract, we performed acetic acid-induced test since acetic acid elicits pain sensation by activating localized inflammatory reactions [32]. Oral administration of the 50% ethanol extract of *H. opuntia* reduced the number of writhing (abdominal constriction) in the mice of the extract group (Fig. 5B & C) when compared with the control (1.20-fold) and the diclofenac (1.23-fold) group which ultimately demonstrated the peripheral analgesic effect of the extract.

Discussion

The adverse effects of synthetic drugs have led to a growing interest in exploring new natural sources of medicinal pharmaceuticals. Marine algae are rich sources of numerous medicinal pharmaceuticals. Our research led us to the discovery of *H. opuntia* seaweed as a possible analgesic and anti-inflammatory agent, which may result in the development of medicinal drugs.

In this study, phytochemical screening revealed the presence of phenolic compounds and steroids in the 50% ethanol extract of *H. opuntia*. High amounts of both were found (Fig. 1A). The extract was found to be safe at 500 mg/kg body weight in the acute toxicity test on mice. Next, in comparison to the control and diclofenac groups, the extract significantly reduced paw edema in the carrageenan-induced paw edema approach (Fig. 2B & C). Three techniques were used to assess the extract's analgesic properties: the hot plate method, the formalin-induced method, and the acetic acid-induced method. The administration of the extract lengthened the mice's response latency to a heat challenge in their paws when using the hot plate method (Fig. 3B & C). In the formalin-induced method, the extract's analgesic efficacy was shown in both the early and late phases of the pain mechanism (Fig. 4B & C). The extract's mediocre peripheral antinociceptive efficacy was evidenced by the slight reduction in writhing in the acetic acid-induced method (Fig. 5B & C).

Figure 5.

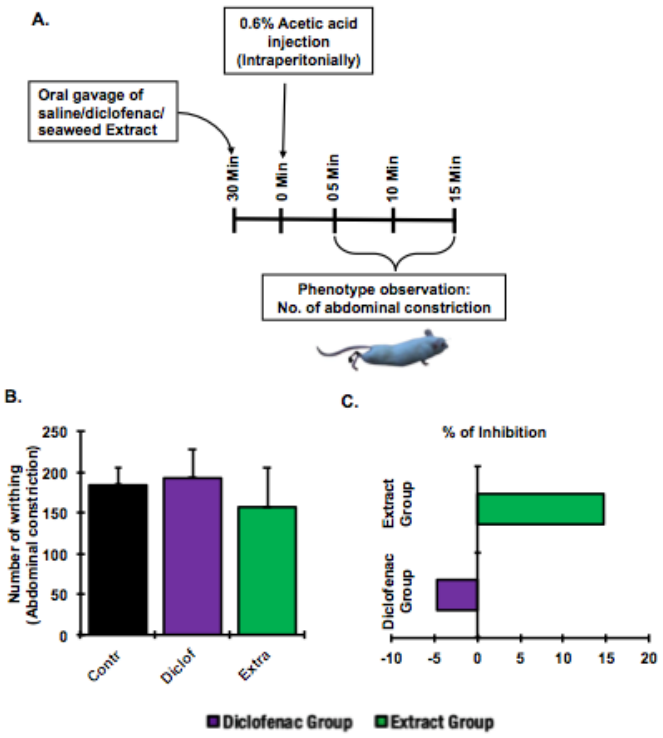

Figure 5: Determination of abdominal analgesic effect of *H. opuntia* on Swiss-albino mice through acetic acid-induced writhing method. **A**. Experimental scheme of 0.6% acetic acid-induced writhing method. **B**. Fold difference of the number of writhing (abdominal constriction) response in the abdomen by mice induced by acetic acid. Values are expressed as mean ± SD (n = 4); SD – standard deviation; **C.** Percent (%) of inhibition of pain between the positive control (Diclofenac) and the tested sample (Extract) group (n=4).

In this investigation, phytochemical screening was done primarily to decipher the extract's anti-inflammatory properties. Steroids and phenolic compounds were found in large amounts during phytochemical screening (Fig. 1A), which is also consistent with previous findings [18,33]. The outcome aligned with other studies performed previously on the genus Halimeda [16,18,34,35]. Additionally, carotenoids and chlorophyll a and b are present in *H. opuntia* extract [36-37]. Pigments and phenolic compounds may aid in the DPPH radical scavenging process [38-40]. Effective DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activities of the 50% ethanol extract of *H. opuntia* employed in this work for the analgesic and anti-inflammatory tests suggest the extract contains strong antioxidant molecules [18]. In addition, antioxidants have anti-inflammatory properties [41-43]. The antioxidant properties of seaweed compounds can greatly reduce tissue deterioration and speed up the healing of wounds [44]. Other Halimeda species were also shown to exhibit antioxidant-mediated anti-inflammatory responses; for instance, halitunal from Halimeda tuna has a potent antiviral action against the mouse corona virus A59 in a manner akin to this [1]. In addition to our research, some previous studies also suggested substantial presence of various bioactive compounds in *H. opuntia* such as hexadecenoic acid, heptadecane, 1-eicosanol, 1-dodecanol, 3,7,11-trimethyl, phytosterol, alkaloids, halimedatetrasetat, halimedatrial, udoteal, rhipocephalin, rhipocephenal, etc. [10]. Thus, it implies that *H. opuntia* is a potentially rich source of bioactive chemicals.

To assess the extract's hazardous effect when taken orally, the acute toxicity test must be carried out before the anti-inflammatory and analgesic assays. After around two weeks of monitoring, the 50% ethanol extract of *H. opuntia* used in this study, was determined to be non-toxic because no anomalous behavior or death was noted. There were no alterations in behavior, respiration, agility, gastrointestinal effects, or responses of the sensory nerve system to an oral dose of 500 mg extract/kg BW. Hence, 500 mg/Kg BW was a safe dose for the mice in this study's subsequent experiments. This discovery will aid in formulating the extract's composition in the future in-vivo study.

An approved approach for assessing a chemical compound's or extract's anti-inflammatory effectiveness is the carrageenan-induced paw edema method [45]. Carrageenan injection provokes the formation of edema at the site of injury by fostering the release of inflammatory mediators such as prostaglandins, histamine, bradykinins, leukotrienes, and serotonin [46]. At the site of action, these mediators also result in pain and other inflammatory signs. Compounds that inhibit these mediators or the enzymes that manufacture them must block their biological pathways that cause inflammation to have an anti-inflammatory impact. Many inflammatory mediators can be induced by carrageenan; histamine and serotonin are released during the early phase (within an hour), and prostaglandins, cyclooxygenase products, and kinins are released during the late phase (after an hour) [47].

The anti-inflammatory study's outcome for *H. opuntia*'s 50% ethanol extract was remarkable. In the experimental mice, the paw volume was shown to vary within a small range (about 0.13). Paw volume values, however, were significantly larger in the diclofenac and control groups of mice (Fig. 2B). which unequivocally demonstrated the extract's anti-inflammatory properties. Following the application of the extract, the percent inhibition peaked at hour four. Prostaglandins mediate the ultimate stage of the inflammation caused by carrageenan. Therefore, the extract might contain substances that affect prostaglandins. There are a lot of steroids in the extract used in this study. Steroids have been proven to have anti-edema activity in several investigations [48-52]. Furthermore, *H. opuntia* possesses a highly diverse fatty acid profile including polyunsaturated fatty acids [53]. These compounds can lower the production of proinflammatory prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) by acting as competitive inhibitors of lipoxygenase and cyclooxygenase [54]. Seaweeds' active ingredients can induce a transcriptional modulation of NFkB-p65 and IL-1ß in the rat brain region [56], as well as mediate the down-regulation of pro-inflammatory cytokines TNF-a and IL-1ß in paw tissue [55]. Robust anti-inflammatory and antinociceptive effects during inflammatory pain have been attributed to the overexpression of Heme-oxygenase-1 (Nrf2-mediated) in some investigations [57-59]. Induction of HO-1 considerably reduces inflammation. The anti-inflammatory properties of *H. opuntia* may be mediated by a combination of these factors. It's also impossible to rule out the possibility of new anti-inflammatory agents from this species. To identify those molecules, more investigation is needed.

Analgesic activity refers to the pain-relieving property. All body tissues, with the exception of the brain, are innervated by nociceptors, which are sensory nerve endings that are activated by intense mechanical, chemical, or thermal stimuli. Chemicals that excite nociceptors, such as kinins, prostaglandins (PGs), and potassium ions, can be released from tissue damage [60]. Analgesic medicines are pain-relieving medications. It is reasonable to anticipate a relationship between antinociceptive and anti-inflammatory responses. The anti-inflammatory and antinociceptive properties of lectins extracted from the seaweed Pterocladiella capillaceae and Caulerpa cupressoides have been linked in past studies [61-62]. In this work, the hot plate method, formalin-induced method, and acetic acid-induced writhing method were used to investigate the analgesic effect of the extract.

One of the paradigms commonly used to evaluate central nociceptive activity is the hot plate test, which measures the complex response to an acute, non-inflammatory nociceptive input

[63]. Compared to diclofenac, the extract in this investigation showed a superior ability to increase response latency. The extract's analgesic efficacy varied with time. The percentage analgesic score was 46% in the second hour and 62% in the fourth. Any agent that causes a prolongation of the hot plate latency must be acting centrally [64]. Spinal and supra-spinal receptors mediate the analgesic effect of heat stimulation [65]. According to a study, the sulfated polysaccharides isolated from seaweed had antinociceptive activity when heated to a high temperature [66]. This effect may be attributed to an increase in endogenous opioids or the activation of the opioid receptor. The green seaweed *H. opuntia* is a rich source of sulfated polysaccharides that are soluble in water. It also includes bioactive chemicals called ulvan and fucoidan, which have anti-nociceptive properties [67]. Thus, more research on *H. opuntia*'s sulfated polysaccharides may be very worthwhile [68].

Formalin's direct influence on nociceptors is reflected in the early phase of formalin-induced pain, while prostaglandin synthesis appears to be a prerequisite for the late phase of inflammatory pain [30-31]. In the formalin-induced pain method, the seaweed extract significantly reduced (early phase $p < 0.01$ and late phase $p < 0.001$) licking activity in both phases when compared to control (Fig. 4B & C). This suggests that the algal extract contains bioactive compounds that function centrally in the early stages and on inflammatory mediators in the later stages. In the late stage of the formalin test, histamine, serotonin, prostaglandins, nitric oxide, and bradykinin are secreted [31]. However, the μ/d/?-opioid receptor, the nitric oxide/cyclic GMP/protein kinase G/ATP-sensitive potassium channel (NO/cGMP/PKG/ K+ATP) pathway, and the active heme oxygenase-1 (HO-1) pathway are all required for the polysulfated polysaccharide, an additional distinctive active component of *H. opuntia*, to mediate the relief of pain in the temporomandibular joint induced by formalin [69]. Furthermore, in the rat trigeminal ganglion and subnucleus caudalis, polysulfated polysaccharide-mediated enhanced release of IL-10, an anti-inflammatory cytokine, likewise controls the inflammatory process in the temporomandibular hypernociception [69]. As the overexpression of Heme-oxygenase-1 has been linked to strong anti-inflammatory and antinociceptive effects in formalin-induced inflammatory pain [57], it is possible that the active ingredients in *H. opuntia* function in this route, which is consistent with some other studies using seaweed [59,69]. In addition, the active ingredients in *H. opuntia* may regulate the TRPA1 (Transient receptor potential cation channel), which gets activated during formalin-induced pain [70]. Therefore, the existence of active chemicals that may alter the activities of various ion channels, enzymes, mediators, and cells may be associated with the promising effect of 50% ethanol extract of *H. opuntia* in the anti-inflammatory and antinociceptive activities in the present investigation.

The writhing approach generated by acetic acid is a useful tool for assessing an analgesic drug's peripheral action. By inducing a localized inflammatory response, this approach causes pain perception. It does this by releasing arachidonic acid from tissue phospholipid and using cyclooxygenase to convert it to prostaglandins [71]. In the end, the circumstance results in elevated levels of endogenous mediators like IL-8, bradykinin, substance P, PGI2, IL-1b, and TNF-a in peritoneal fluids, as well as lipoxygenase products, PGE2 (Prostaglandins E2), and PGF2a (Prostaglandins F2a) [72]. The function of mast cells in this context is critical [73-75]. By enhancing capillary permeability, an elevated prostaglandin level in the peritoneal cavity exacerbates pain and inflammation [76]. The seaweed extract used in this study for an acetic acid-induced writhing test in mice showed a marginal reduction in writhing number compared to the control and the diclofenac group (Fig. 5B & C). A few extract ingredients might have ancillary effects that lessen the writhing reaction. They preferably block mast cells or the prostaglandin pathway [71,74]. In the acetic acid-induced writhing method, extracts and active components (lectin and sulfated polysaccharides) from green, brown, and red seaweeds revealed intriguing results; our seaweed may follow suit [59,61,62,66,77-83].

Conclusion

As a result of its strong central antinociceptive activity and passable peripheral antinociceptive activity, our study's findings suggest that *Halimeda opuntia* may have anti-inflammatory and analgesic properties (Fig. 6). Our study eventually concluded that because of its wide geographic distribution and the resulting variety of environmental constraints, the seaweed *H. opuntia* can produce potent metabolites and active compounds with notable pharmacological action. More investigation will uncover the presence of stronger bioactive compounds in this intriguing seaweed, which will aid in the understanding of the intricate processes behind its analgesic and anti-inflammatory benefits in connection to a range of ailments associated with aging.

Figure 6.

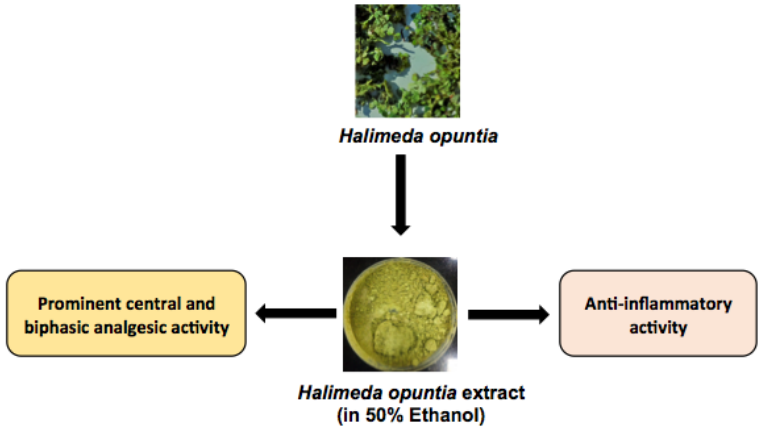

Figure 6: Summary of the in vivo findings of *H. opuntia*. *In vivo* study of *H. opuntia* showed prominent anti-inflammatory and analgesic activities in murine model.

Authors' Contributions: MAA- performed all experiments, collected and analyzed the data, wrote the initial draft of manuscript; **FR**- supported the experiment; **MNH-** helped to collect the seaweed sample; **MAH**- reviewed and edited the manuscript; **MMA**- conceived the idea, designed the total research project and research grant, supervised the work, performed the experiments, collected the seaweed sample, analyzed the data, wrote, and edited the final manuscript.

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Ethics Approval and Consent to Participate: All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) and have therefore been performed in accordance with the ethical standards. In addition, all experimental methods are reported in accordance with ARRIVE guidelines.

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