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Antinociceptive and Anti-inflammatory Effects of Methanolic Extract of *Laurencia caspica*

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Abstract

Introduction: Seaweeds are valuable resources for the discovery of efficient and safe drugs for pain treatment. In the present investigation, we evaluated the antinociceptive and anti-inflammatory properties of, methanolic extract of *Laurencia caspica*, a red algae, in mice models.

Methods: The analgesic effect of methanolic extract of *L. caspica* was assessed by hot-plate and acetic acid-induced writhing tests in male Swiss albino mice (weight = 20-25 g). The anti-inflammatory activity of methanolic extract of *L. caspica* was also evaluated by formalin-induced ear edema and xylene-induced paw edema tests.

Results: The total flavonoid content of the extract was estimated as 0.0537 mg quercetin/g extract. Both first and second phases of the nociception were significantly inhibited at a dose of 120 mg/kg of methanolic extract of *L. caspica*. The observed anti-inflammatory effect was dose-dependent. Acetic acid-induced writhing test and hot plate test showed that the extract significantly reduced pain in all evaluated doses (15, 30, 60, and 120 mg/kg). The antinociceptive activity of the methanolic extract was significantly reduced by naloxone (4 mg/kg). Moreover, the extract significantly reduced paw edema at the dose of 120 mg/kg in all the animals.

Conclusion: Methanolic extract of *L. caspica* exhibited central analgesic effect, as well as anti-inflammatory activity probably due to the presence of constituents like flavonoids and triterpenoids.

Keywords: Red algae, *Laurencia caspica*, Anti-inflammatory, Antinociceptive

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Introduction

The aquatic ecosystem is considered a rich source of natural products with extensive therapeutic applications.[1] Marine products, especially those derived from the secondary metabolism of marine organisms, are accepted as a potential source of pharmaceuticals for the management of various diseases.[2] Seaweeds consist of three phyla including green, red, and brown algae, and three families including *Chlorophyceae*, *Rhodophyceae*, and *Phaeophycea*. The red algae or *Rhodophyceae* are major algae groups in bioactive compounds due to their potential pharmacological properties like antimicrobial, antioxidant, anticancer, analgesic, and anti-inflammatory effects.[1,3] Bioactive compounds with free radical scavenging potential have been able to harness any reactive oxygen species.[4] Endothelial dysfunction,[5] lung disease,[6] gastrointestinal disorder,[7] and atherosclerosis[4] can permute inflammatory reactions due to oxidative stress. Some marine algae metabolites, which hold antioxidant effects, are known to have anti-inflammatory properties as well.[8-10]

The inflammation can be regarded as a set of complicated processes including defensive responses of organisms to antigenic stimulation or injuries.[11,12] The inflammation, especially the chronic type, is also a common complication of many diseases and leads to weakness of the immune system. Furthermore, it is the pathophysiological response of healthy tissues to injured tissues that leads to the local accumulation of plasma proteins and blood cells. Anti-inflammatory agents could treat pain by inhibiting the production of prostaglandins through blocking the enzyme cyclooxygenase (COX).[13,14]

Pain is a protective mechanism that can be the result of an acute or a chronic lesion.[14] Pain can be categorized as either acute or

chronic. Acute pain involves a warning mechanism in tissue damages and may be caused by many events or circumstances. While chronic pain is an independent and complex entity that seriously affects patients' life quality and thus is considered an illness.[15,16] Prostaglandins made by COX-2 can cause pain by binding to receptors associated with G proteins and increasing cAMP level in cells.[17,18] The pain relievers and anti-inflammatory drugs are classified into opioid analgesics (e.g., enkephalin, endorphin, morphine, and methadone) and non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin and acetaminophen. Moreover, some NSAIDs have so much side effects that often lead to drug discontinuation. Increased risk of digestive, cardiovascular, and renal diseases and drug-related morbidity are regarded as important symptoms of long-term use of these drugs.[15] Accordingly, finding new natural products with anti-inflammatory effects without any side effects is an important issue. In this regard, during the past decades, herbal drugs have been the subject of interest because of their pharmacological effects.[18,19]

Laurencia caspica (red macro algae) is an important seaweed that grows in the southern coast of the Caspian Sea.[19] The phytochemistry of Laurencia species has been attributed to the large amounts of various halogenated secondary metabolites such as diterpenes, halogenated sesquiterpenes, and acetogenins produced by these genera.[20] Some studies have demonstrated that the secondary metabolites from the red alga have potential effects such as anti-inflammatory, anti-cancer, and analgesic activities.[20-22] The anti-inflammatory activity of red algae, *L. glandulifera* and *L. obtuse*, in previous studies have been reported.[23,24] As there is no report on the anti-inflammatory activity of *L. caspica* to the best of our knowledge, the objective of current work was to evaluate the effect of methanolic extract of *L. caspica* on pain and inflammation in mice.

Materials and Methods

Chemicals

Glacial acetic acid-xylene was purchased from Sigma-Aldrich (Germany). Formalin was obtained from Merck Co. (Darmstadt, Germany). Aspirin used as the reference was also purchased from Iran Daru Co., Tehran, Iran.

Sample Collection and Extract Preparation

Laurencia caspica, the red algae samples, were collected from Ramsar beaches, Caspian Sea, Mazandran, Iran, in May 2015, and transferred within a plastic bag to the Biotechnology Laboratory of Islamic Azad University. The samples were identified according to a study by Sterrer.[25] First, the algae were washed thoroughly to be completely free of sand and epiphytic organisms and were immersed in distilled water to remove salts. Then, the specimens were spread over a clean and sterile cloth in shadow for three days to dry. In the next step, the samples were completely powdered by grinding using a blender (Waring, USA).

Dried red alga powder (100 g) was dissolved and soaked in 500 mL of methanol for 10 hours at room temperature.[15] The extract was concentrated to a suitable volume using rotary evaporator (Model: WG-EV311-V-PLUS, USA).

Total Phenolic Content Assessment

Total flavonoid content was measured using Folin-Ciocalteu reagent according to a modified method of Ainsworth.[26] The methanolic extract of red algae (0.5 mL) was mixed with 2 mL of the Folin-Ciocalteu reagent. Then the sodium carbonate solution, 7.5% w/v (4 mL), was added. After incubation at room temperature for 30 minutes, the absorbance of the solution was measured at 765 nm using UV-visible spectrophotometer (Jenwey, UK). The total phenolic content was calculated from the linear equation of a standard curve determined with gallic acid ($Y = 0.008X + 0.0727$, $R2 = 0.9967$). The result was estimated as mg/g gallic acid equivalent of dry extract.

Animals

Male Swiss mice (20-25 g) applied in this work were provided by the Animal House of Pasteur Institute, Karaj, Iran, and preserved in proper hygienic conditions at the Animal House of Islamic Azad University, Lahijan Branch, Iran. The animals were acclimatized for one week to the experimental conditions in ventilated plastic cages at 22°C with a relative humidity of 45%-50%, under a 12-hour light/dark cycle. Animals were fed with laboratory chow and water ad libitum. To do the experiment, animals were randomly assigned into 6 groups and deprived of food and water.

Acute Toxicity Study

For acute toxicity, Swiss mice were categorized into different groups (n = 6), and intraperitoneally administered with different doses (25, 200, and 2000 mg/kg) of *L. caspica* extract. The animals were monitored for 72 hours for any toxic signs and mortality.[27]

Drug Administration

The animals were categorized into six groups (n = 8). Group I received normal saline. Group II, served as positive control, received dexamethasone (15 mg/kg) for formalin and xylene tests. Morphine (10 mg/kg) was injected for the induction of paw edema. Group III, group IV, group V, and group VI received 15, 30, 60, 120 mg/kg of the methanolic extract of *L. caspica*, as the treatment groups, respectively.

Formalin Test

The formalin paw assay was carried out as reported by Hunskaar et al.[28] The animals in each test group acclimatized for 30 minutes before the experiment and then intraperitoneally received extract at doses of 15, 30,

60, and 120 mg/kg. Paw edema was induced in mice by injection of 25 µL formalin 5% into the plantar side of right hind paw 30 minutes after injection of methanolic extract, dexamethasone, and normal saline. The time spent for licking and biting the injected paw was taken as behavioral response to pain and was recorded for each group. After formalin injection, responses were observed for 5 minutes and 20-30 minutes, respectively.

Xylene-Induced Ear Edema Test in Mice

The xylene test was performed using the procedure described by Shang et al.[29] The ear edema was induced by application of xylene (30 µL/ear) on the right ear. Animals receiving different doses of methanolic extract (15, 30, 60, and 120 mg/kg i.p.), either received 10 mg/kg morphine as a positive control or normal saline (10 mL) as a negative control. Fifteen minutes after injection, mouse ear edema was induced by the topical application of 30 µL/ear of xylene. Two hours after xylene daubing, the mice were euthanized. Two ear punches were taken from each mouse and weighed. The edema was indicated by a rise in the weight of right ear punch compared to that of the left ear.

Hot Plate Test

This test was done according to a reported assay.[30] The hot plate apparatus was set at 54 ± 0.1°C and the mice were put on the hot surface. The pain response latency was measured based on the time elapsed between placement and licking of their hind paws. After recording the baseline reaction (cut off = 45 seconds), the animals were intraperitoneally (i.p.) administered with normal saline (10 mL/kg) as the negative control, morphine (8 mg/kg) as the positive control, and *L. caspica* extract at 4 doses (15, 30, 60, and 120 mg/kg). The reaction time to hot plate was observed at 0, 15, 30, 45, and 60 minutes after injection. The results are summarized in Table 1 and the maximum possible effect (MPE) percentage was measured using the following formula to find the time with maximum nociceptive effect:

MPE (%) = (Test latency- baseline latency) × 100/ (cut off-baseline latency)

Acetic Acid-Induced Writhing Test

The acetic-induced writhing test was conducted according to the method reported by Aoki et al.[30] Experimental groups were administered with 15, 30, 60, and 120 mg/kg of methanolic extracts of *L. caspica*. In addition, morphine (10 mg/kg) and normal saline (10 mL/kg) were administered to the positive and negative control groups, respectively. The animals were administered with an intraperitoneal injection of 0.1 mL/10 g body weight acetic acid (0.7%). The abdominal constrictions during 30 minutes after acetic acid injection were measured. Animals pretreated with morphine (5 mg/kg, p.o.) were used as positive control. To evaluate the possible involvement of the opioid system in the antinociceptive activity of the algae extract, naloxone (4 mg/kg, i.p.) was injected 15 minutes before the administration of the extract (120 mg/kg, p.o.) or morphine (5 mg/kg, p.o.) to each group of animals. The frequency of constrictions for each concentration versus the normal saline control was applied for estimating the analgesic activity. The amount of inhibition of writhing was calculated using the following equation:

$$\text{Inhibition } (\%) = [(\text{mean writhing}_{\text{control}} - \text{mean writhing}_{\text{treated}}) / \text{mean writhing}_{\text{control}}] \times 100$$

Statistical Analysis

All data were represented as the mean ± SEM (n = 8 per group). Statistical analyses were performed by SPSS software version 19.0, and the results were analyzed by one-way analysis of variance (ANOVA) followed by the post hoc analysis using Tukey's test. A value of $P < 0.05$ was taken as the level of significance.

Table 1. Effects of Intraperitoneal Injection of Methanolic Extract of *Laurencia caspica* on Acute and Chronic Pain Revealed by Formalin Test

Drug (mg/kg)		Nociceptive Phase	Licking Time (s)	Inhibition of Nociception (%)
Control (normal saline)		Acute	74.65 ± 2.127	-
		Chronic	216.54 ± 32.14	-
Morphine (5)		Acute	28.05 ± 1.582[a]	79.45
		Chronic	36.40 ± 5.201[c]	86.86
Indomethacin (10)		Acute	74.40 ± 6.005	0.61
		Chronic	4.65 ± 3.107[c]	0.52
Methanolic extract (mg/kg)	15	Acute	71.02 ± 1.085	0.61
		Chronic	135.71 ± 2.423[a]	0.52
	30	Acute	65.46 ± 2.54	17.86
		Chronic	87.15 ± 4.314[b]	7.34
	60	Acute	50.61 ± 3.22[a]	62.93
		Chronic	42.11 ± 1.173[c]	54.55
	120	Acute	33.04 ± 1.14[a]	77.26
		Chronic	25.63 ± 1.388[c]	83.53

Results have been shown as mean ± SEM in seconds (n = 7).
[a] $P < 0.05$, [b] $P < 0.001$; [c] $P < 0.0001$ compared to the control group.

Results

Total flavonoid content of the methanolic extract was calculated to be about 0.0537 mg of quercetin/g extract. The latencies after injection (baseline latency) were analyzed by the ANOVA; no significant difference was seen between the test and control groups. No mortality was observed after 72 hours in animals administered with different concentrations of extract. Moreover, no toxicity signs were observed in treated groups at 25 and 200 mg/kg. But, the sedative effect was shown at a dose of 2000 mg/kg.

Formalin Test

The test revealed that the maximum dosage (120 mg/kg) of methanolic extract in both phases (acute and chronic) can mitigate pain significantly ($P<0.05$). As can be seen, there was no difference regarding morphine response between two phases (79.45% and 86.86% in acute and chronic, respectively). Moreover, all dosages of the methanolic extract of *L. caspica* inhibited pain at the acute phase compared to the control.

Xylene Test

The anti-inflammatory effect of intraperitoneal injection of the methanolic extract on xylene-induced ear edema is illustrated in Table 2. The xylene-induced ear edema decreased significantly in the treatment group compared to the control group. A dose-dependent manner was observed in inhibition of the edema. Hence, the maximum inhibition was measured at a dose of 120 mg/kg, which was significantly different from the other doses of methanolic extract and control ($P<0.05$).

Hot Plate Test

After injection, the latencies were analyzed though no significant difference was observed between the treatment and control groups. The maximum efficacy was determined at 15 mg/kg of methanolic extract after 30 minutes. Response latency in different groups has been summarized in Table 3. Considering MPE, the most effective dose was observed at 30 minutes after extract injection (Figure 1).

Table 2. The Effect of Intraperitoneal Administration of Methanolic Extract of *Laurencia caspica* on Xylene-Induced Ear Edema in Mice

Treatment	Dose (mg/kg)	Weight Gain of Ear (mg)	Percentage of Inhibition (%)
Control	Normal saline	15.2 ± 0.35	
L. caspica extract	15	13.2 ± 0.24	13
	30	12.6 ± 0.15[a]	17
	60	11.1 ± 0.38[a]	27
	120	6.87 ± 0.15[b]	54
Dexamethasone 15	Dexamethasone 15	6.46 ± 0.28[b]	57

[a] $P<0.05$; [b] $P<0.01$ compared to negative control.

Acetic Acid-Induced Writhing Test

The writhing test was used for the evaluation of antinociceptive activity of methanolic extract of *L. caspica*. The pain induced by acetic acid in mice was reduced in all groups treated with methanolic extract.

The frequency of writhing was significantly different in all treatment groups in comparison with the control group ($P<0.05$). The concentration of 120 mg/kg was indicated in the least writhing number (9.8 ± 1.44) with 70.8% inhibition. The results have been presented in Table 4. Morphine (5 mg/kg) as the reference drug showed significant protective effects ($P<0.001$). Prior administration of naloxone significantly increased the number of abdominal writhing in both morphine ($P<0.001$) and the extract ($P<0.01$) treated mice. The dose-dependent manner was observed in the reduction of pain induced by acetic acid. Among all tested groups, the dose of 120 mg/kg had a significant impact on pain relief compared to the control and the reference drug (naloxone) ($P<0.05$).

Discussion

Intensive investigations have been directed on the treatment of pain. Plant extracts and their isolated natural compounds have been greatly applied for their pain reducing properties. During the last decade, the pharmaceutical market, based on phyto-therapeutics, has grown dramatically duo to increasing demand around the world. Recently, natural marine products have attracted a great attention as they have provided new perspectives for novel pharmaceutics, which are unlike any found on the ground.[31]

The methanolic extract of *L. caspica* demonstrated the antinociceptive activity in acute and chronic phases, where the greatest analgesic effect in both phases was attributed to dose 120 mg/kg. Furthermore, the analgesic impact was confirmed in a dose-dependent manner in the formalin and writhing tests. In xylene test, it was observed

Table 3. Response Latency Observed by Hot Plate Test in Mice Groups (n = 7)

Treatment (mg/kg)	Latency (s)				
	0	15	30	45	60
Control	8.64 ± 1.50	10.20 ± 2.30	10.6 ± 7.30	9.3 ± 2.44	7.9 ± 2.50
Morphine	9.32 ± 1.80	13.45 ± 3.60[a]	15.73 ± 4.30[b]	14.99 ± 2.60[b]	13.3 ± 4.00[a]
15	8.42 ± 1.49	12.53 ± 3.27[a]	17.33 ± 4.46[b]	12.92 ± 3.80[a]	9.41 ± 2.60
30	8.73 ± 2.65	13.16 ± 1.14[a]	13.36 ± 1.11[a]	13.04 ± 2.17[a]	9.8 ± 1.27
60	8.95 ± 2.56	13.16 ± 1.62[a]	13.30 ± 1.15[a]	13.01 ± 2.17[a]	11.3 ± 2.56
120	9.14 ± 1.14	13.20 ± 2.42[a]	13.44 ± 3.21[a]	13.22 ± 3.48[a]	12.6 ± 2.72

[a] $P<0.05$; [b] $P<0.01$ compared to negative control.

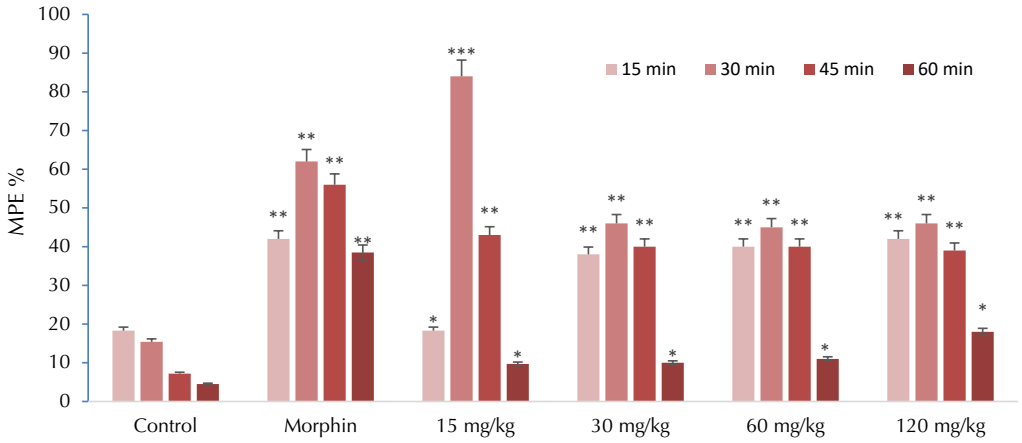

Figure 1. The Maximum Possible Effect (%MPE) of Acute Pain Inhibition at Different Time Points in Hot Plate Test (n = 7). * $P < 0.05$; ** $P < 0.01$ compared to negative control.

that all treatment doses had an anti-inflammatory effect, with the highest impact seen at the maximum dose of extract (i.e., 120 mg/kg). It was proposed that the anti-inflammatory property of methanolic extract can be due to the inhibition of enzymes such as COX and lipoxygenase, which importantly act in arachidonic acid metabolism.[32,33] Lactonic and phenolic compounds, flavonoids, fatty compounds, triterpenes, steroids, reduced carbohydrates, and other sugars have been the main ingredients of red algae, *Dichotomaria obtusata*.[15] Among phytochemical compounds, flavonoids are natural polyphenol compounds that can inhibit nitric oxide (NO) synthase enzyme. Flavonoids are able to inhibit N-methyl D-aspartate receptors and reduce the intracellular calcium level. Consequently, the calcium-dependent activation of NO synthase and phospholipase A2 is diminished. In addition, flavonoids could inhibit the activity of COX so that the production of prostaglandin E from arachidonic acid is prevented. Regarding the fact that prostaglandins play a role in inflammation, it is suggested that the red algae flavonoids exert anti-inflammatory effects.[33,34]

It is proposed that the terpenoids isolated from red algae *L. dendroidea* inhibit cell proliferation, inflammation, and metastasis.[1] The anti-inflammatory effects of *L. glandulifera* are explained by the presence of neorogioltriol, which is a tricyclic brominated diterpenoid.[23] This compound influences acute and chronic phases of inflammation via the effect on signaling pathways including nuclear factor-κB (NF-κB).[35] Studies on red algae have demonstrated that terpenoids from red algae *L. obtuse* have anti-inflammatory properties.[24] The anti-inflammatory effects of red algae *L. obtuse* are different from those of C15 acetogenins (12Z)-cis-maneonene-D and (12E)-cis-maneonene-E. During inflammatory responses, the significant role of these components in mediating apoptosis of neutrophils has been proved.[24,36]

Table 4. Effect of Methanolic Extract of *Laurencia caspica* on Acetic Acid-Induced Abdominal Writhing Test in Mice

Treatment	Dose (mg/kg)	Number of writhing
Control	Normal saline	31.6.00 ± 1.07
L. caspica extract	15	21.4 ± 3.70[a]
	30	17.5 ± 2.24[a]
	60	9.2 ± 2.13[a]
	120	9.8 ± 1.44[a]
Morphine	5	19.32 ± 1.71[b]
Morphine/naloxone	5 / 4	34.12 ± 2.84[c]
Extract/naloxone	120 / 4	23.65 ± 2.73[a]

Results have been indicated as mean ± SEM; (n = 7).
[a] $P < 0.001$ compared to the control group; [b] $P < 0.001$ compared to the group receiving morphine; [c] $P < 0.01$ compared to the group receiving extract (120 mg/kg).

The methanolic extract of *L. caspica* significantly reduced the licking episodes in the injected paw in a dose-dependent manner. This result indicated that the antinociceptive property of the algae extract may contribute to the treatment of chronic pain.[31] Writhing test is another test model that was used for screening antinociceptive action. In this test, abdominal constriction is induced by acetic acid by inducing endogenous mediators and stimulating the pain nerve endings.[37-39] The methanolic extract represented significant inhibition on acetic acid-induced writhing response. The hot plate test is used for central antinociceptive effect on animal models like mice.[40] In this study, both the methanolic extract and morphine increased pain response latency, compared with the control group.

Pre-exposure to naloxone eliminated the analgesic effect of red algae extract and morphine, showing that the analgesic action of the extract may be dependent on endogenous opioid systems.[41] The methanolic extract of *L. caspica* also demonstrated good potential in attenuating nociception observed using acetic acid, however the potential was less than that of morphine with naloxone. Furthermore, the methanolic extract of *L. caspica* reduced formalin-triggered inflammation maximally at the dose of 120 mg/kg. The results were comparable with indomethacin, a nonselective COX inhibitor.[42,43] The potential anti-inflammatory effects of phytoconstituents

like oleanonic acid in the red algae have been shown in experimental models related with the activation of 5-lipoxygenase.[44] The anti-inflammatory and analgesic activities of methanolic extract of *L. caspica* may partly be ascribed to the presence of bioactive compounds.

Conclusion

To conclude, the methanolic extract of *L. caspica* revealed significant antinociceptive activity in laboratory animals. The observed effects could be attributed to the presence of phytoconstituents like triterpenoid and flavonoid compounds. It is recommended that further studies are conducted to discover novel anti-inflammatory and anti-nociceptive compounds from active methanolic extract of *L. caspica*.

Ethical Approval

All animal experiments were done according to the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). All experiments were approved by the Animal Care and Ethics Committee of Islamic Azad University and approved with the code IR.IAU. RASHT.REC.

Conflict of Interest Disclosure

The authors declare no conflict of interests.

Authors' Contribution

KK: Supervision, concept, and design and manuscript writing; MR and SK: Data collection and analysis.

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