A lectin from the green seaweed *Caulerpa cupressoides* reduces mechanical hyper-nociception and inflammation in the rat temporomandibular joint during zymosan-induced arthritis


**Abstract**
Seaweed lectins have been widely investigated as anti-nociceptive and anti-inflammatory agents. This study analyzed the anti-nociceptive and anti-inflammatory responses of a lectin from the green seaweed *Caulerpa cupressoides* (CcL) on zymosan-induced arthritis of the rat temporomandibular joint (TMJ). Rats received i.v. CcL 30 min prior to injection of zymosan (2 mg/art.) or 0.9% saline into the left TMJ. Mechanical hyper-nociception was measured by the electronic von Frey method at baseline and 4 h after zymosan injection. Animals were euthanized 6 h after zymosan injection and the synovial fluid was collected for leukocyte counting and myeloperoxidase activity assessment. Other animals were treated with ZnPP-IX (3 mg/kg; s.c.), a specific heme oxygenase-1 pathway inhibitor, and naloxone (10 μg/art.), a nonselective opioid receptor antagonist. TMJ tissues were excised to perform histopathological and immunohistochemistry analyses. CcL (0.1, 1 or 10 mg/kg) significantly reduced zymosan-induced hyper-nociception (81, 83 and 89.5%, respectively) and inflammation in the rat TMJ, based on histopathological and immunohistochemistry analyses, respectively. Therefore, CcL reduces TMJ hyper-nociception and inflammation with a mechanism that is partially dependent on TNF-α and IL-1β inhibition. CcL reveals a potentially valuable alternative tool for future studies of TMJ disorders.

**Keywords:**
Marine alga
Hemagglutinin
Mechanical hyper-nociception
Inflammatory arthropathies

**1. Introduction**
Temporomandibular joint (TMJ) disorders are a group of conditions that result in TMJ pain, which frequently limits talking, chewing, and other basic daily activities. Increased levels of pro-inflammatory cytokines (IL-1β and TNF-α) have been detected in patients with temporomandibular disorders (TMD), suggesting that these cytokines may play a role in the pathogenesis of synovitis and degenerative changes of the cartilaginous tissue and bone of the temporomandibular joint [5,6].

Heme oxygenase 1 (HO-1) plays an important role in the antioxidant defense system, and its induction would provide a negative feedback for cell activation and the production of inflammatory mediators, which could modulate, at least in part, the inflammatory pain process [7–9]. A role for the endogenous opioid system in the phenomenon of pain [10] and a role for the peripheral opioid receptors in TMJ anti-nociception, are well-demonstrated [11,12].
Non-steroidal anti-inflammatory drugs are largely used to ameliorate TMJ inflammation, although these drugs are associated with several adverse effects (mainly gastrointestinal, cardiovascular and kidney toxicity) [13,14]. Therefore, there is a great need for scientific studies that search for new bioactive compounds, such as those derived from plants, with therapeutic action and that confirmed efficacy and safety [15–18].

Seaweeds have lectins, which are proteins or glycoproteins of non-immune origin containing at least one non-catalytic domain that binds reversibly to specific mono- or oligosaccharides [19]. The biotechnological importance of these polymers has been described for various fields, including biochemistry [20], agriculture [21] and, more recently, they have been recognized as important tools to control nociception [22] and inflammation [23]. However, to the best of our knowledge [1,24], there are no studies about the use of lectins on experimental arthritis of the rat TMJ.

*Caulerpa compressoides* var. *lycopodium* C. Agardh is a species of green seaweed belonging to the family Caulerpaceae and is widely found along the coast of northeast Brazil. The isolated lectin from this algal species (CcL) was previously purified and characterized by physicochemical procedures that indicated a dimer (23–44 kDa) with hemagglutinating activity containing glycine, aspartic acid, glutamic acid, serine and a low content of basic amino acids. Furthermore, it has been reported in the literature that this lectin can be inhibited by acids, serine and a low content of basic amino acids. Furthermore, it has been reported in the literature that this lectin can be inhibited by the glycoprotein porcine stomach mucin, a glycoprotein with a terminal GalNAc residue, and fucose and galactose as internal residues [25].

Our group demonstrated CcL anti-nociceptive and anti-inflammatory effects in classical models of nociception and acute inflammation in vivo [26], as well as immunomodulatory properties on cell viability in vitro, inducing T helper 2 immune responses in mouse splenocytes and producing high levels of IL-10 and IL-6 [27]. Therefore, we analyzed the unexplored anti-nociceptive and anti-inflammatory efficacy of CcL in a model of zymosan-induced TMJ inflammatory hyper-nociception in the rat. Additionally, we investigated the putative involvement of IL-1β, TNF-α, HO-1 and the endogenous opioid system in CcL efficacy.

2. Materials and methods

2.1. Animals

Male *Wistar* rats (180–240 g) from the Animal Care Unit of the Federal University of Ceará in Fortaleza, Brazil, were used throughout the experiments. They were housed in a temperature-controlled room with free access to water and food on a 12/12 h light/dark cycle. For each experiment, groups of six animals were segregated and handled separately. All procedures and animal treatments were carried out at ambient temperature (20–22 °C), and special care was taken to avoid environmental disturbances that might influence animal responses. This study was conducted in accordance with the guidelines set forth by the U.S. Department of Health and Human Services and with the approval of the Ethics Committee of the Federal University of Ceará, Fortaleza, Brazil (CEPA no. 59/13).

2.2. Drugs and reagents

The following drugs and reagents were used: zymosan, Zinc Protoporphrina-IX (ZnPp-IX), indomethacin, DEAE-cellulose, o-dianisidine dihydrochloride, potassium phosphate monobasic, and potassium phosphate dibasic which were purchased from Sigma (St. Louis, MO, USA); hematoxilyn, eosin and ethylenediaminetetraacetic acid (EDTA) were purchased from VETEC Química Farm, LTDA, SP (Brazil). The reagents and the lectin (CcL) were solubilized in 0.9% sterile NaCl (saline) or ultrapure water. All chemicals were of analytical grade.

2.3. Marine alga

The green seaweed *C. compressoides* var. *lycopodium* (Caulerpaceae, Bryopsidales) was collected from the Atlantic coast of Brazil (Pacheco Beach, Caucaia, Ceará). After collection, the material was cleaned of epiphytes, washed with distilled water and stored at −20 °C until use. A voucher specimen (no. 4977) was deposited in the Herbarium Prisco Bezerra in the Department of Biological Sciences, Federal University of Ceará, Brazil.

2.4. Erythrocytes

Blood samples were obtained from healthy adult New Zealand albino rabbits and maintained in the Animal Care Unit of the Federal University of Ceará, Fortaleza, Brazil.

2.5. Isolation of *C. compressoides* lectin (*CcL*)

The CcL was obtained from the fresh algal tissue macerated in the presence of liquid nitrogen [25], with some modifications that were proposed by Vanderlei et al. [26]. Extraction of CcL was performed with Tris–HCl 25 mM, pH 7.5, (TB) at a ratio of 1:4 (w/v, alga:TB). After constant stirring for 4 h, the homogenate was filtered through nylon cloth and then centrifuged (8000 × g, 30 min, 4 °C). The precipitate was discarded and the resulting supernatant (crude extract) was subjected to ion-exchange chromatography on a DEAE-cellulose column (1.0 × 27.5 cm), equilibrated and eluted with TB. After elution of the unbound proteins and pigments, the adsorbed proteins were eluted from the gel using TB containing 0.5 M NaCl. The fraction containing hemagglutinating activity was pooled, dialyzed and freeze-dried.

2.6. Experimental protocols

2.6.1. Induction of TMJ arthritis

This method was based on Chaves et al. [1]. Thirty minutes before injection with zymosan, rats were pretreated (0.1 ml/100 g body weight) with CcL (0.1, 1 or 10 mg/kg) by intravenous (i.v.) injection or 0.9% sterile saline. Control animals (sham) received the same volume of sterile saline. Rats were briefly anesthetized with inhaled isoflurane and received an intra-articular (i.art.) injection of zymosan (2 mg, 40 μl total volume) dissolved in sterile saline into the left TMJ using a 30-gauge needle and a 1 ml syringe. Sham animals received saline i.art.

Before the zymosan or saline injection, the TMJ skin region was carefully shaved, the postero-inferior border of the zygomatic arch was palpated, and the needle was inserted inferior to this point and advanced in a medial and anterior direction until the needle made contact with the condyle. This contact was verified by moving the mandible, and the puncture of the needle into the joint space was confirmed by the loss of resistance. The animals were euthanized under anesthesia at 6 h after zymosan-induced arthritis and inflammatory parameters (total cell count and myeloperoxidase assay activity) were evaluated.

2.6.2. Evaluation of mechanical hyper-nociception

Mechanical hyper-nociception in the rat TMJ was evaluated by measuring the threshold intensity of force that, when applied to the TMJ region, would elicit a reflex response in the animal (e.g., head withdrawal). The measurements consisted of a continuous force applied to the TMJ area, performed by an examiner unaware of the treatments, and using a digital device (Insight, Ribeirão Preto, SP, Brazil) that consisted of a rigid filament linked to an electronic device that measured the response threshold in grams (g) when the filament was applied to the surface of the tested region. The facial areas to be tested around the TMJ were carefully shaved, and the animals were placed into individual plastic cages 45 min before the beginning of the tests. The animals were submitted to conditioning sessions, consisting of head withdrawal threshold measurements, in the testing room for
4 consecutive days under controlled temperatures (20–22 °C) and low illumination intensity [28]. On the fifth day, the basal force threshold value was recorded (in triplicate) before injection of CcL (0.1, 1 or 10 mg/kg, i.v.), zymosan (2 mg/art., 35 μl), sterile saline (0.9% NaCl, w/v, i.art.) or indomethacin (5 mg/kg body weight, s.c.) and was recorded again after 4 h.

2.6.3. Collection of the TMJ synovial fluid and cell counting
Six hours after zymosan injection (i.art.), the rats were euthanized under anesthesia and exsanguinated. The superficial tissues were dissected, and the TMJ cavity was washed to collect the synovial fluid by a pumping-and-aspiration technique using 0.05 ml of EDTA in neutral buffered PBS. This procedure was repeated twice. The total number of white cells in the synovial lavage fluid was counted using a Neubauer chamber [1].

2.6.4. Determination of myeloperoxidase (MPO) activity
MPO is an enzyme found primarily in azurophilic granules within neutrophils and has been used extensively as a biochemical marker of granulocyte infiltration into various tissues. The extent of neutrophil accumulation in the TMJ synovial fluid was measured using an MPO activity assay as previously described [29]. Briefly, MPO activity was determined by measuring the change in absorbance at 450 nm using o-dianisidine dihydrochloride and 1% hydrogen peroxide. A unit of MPO activity was defined as the activity that required the conversion of 1 μmol of hydrogen peroxide to water within 1 min at 22 °C. The results are reported as MPO units/joint.

2.6.5. Histopathological analysis
Six hours after zymosan-induced arthritis, the rats were euthanized and the TMJ was excised. The specimens were fixed in 10% neutral buffered formalin for 24 h, demineralized in 10% EDTA (7% formic acid), embedded in paraffin, and sectioned along the long axis of the TMJ. Sections of 5 μm, which included the condyle, articular cartilage, articular disk, synovial membrane and periarticular tissue, were evaluated under light microscopy. For the specimens processed for routine hematoxylin–eosin (H & E) staining, histological analysis was used to assign a graded 0–4 score based on the cell influx into the synovial membrane, the periarticular tissue and the muscoskeletal tissue [1].

2.6.6. Evaluation of possible involvement of the opioid system
To assess the possible participation of the opioid system in the anti-nociceptive response to CcL, rats were pre-treated with naloxone (10 μg/art., 15 μl), 35 min before the injection with zymosan (2 mg/art., 35 μl). Five minutes later, the animals received the CcL (10 mg/kg, i.v.), morphine (1000 μg, 0.1 ml, s.c.) or 0.9% sterile saline. The mechanical hyper-nociception responses to these treatments were recorded after 4 h of zymosan (2 mg/art., 35 μl) or sterile saline injected i.art. into the left TMJ of the rats. When two drugs were injected in the same TMJ, the total volume of injection was 50 μl [30].

2.6.7. Evaluation of possible involvement of the HO-1 pathway in nociceptive and inflammatory responses
This assay was based on Freitas et al. [31], Vanderlei et al. [9] and Grangeiro et al. [8]. Animals (n = 6 per group) were treated with ZnPP-iX (3 mg/kg, s.c.), followed by administration of CcL (10 mg/kg, i.v.) 30 min later. Zymosan (2 mg/art., 40 μl) was injected after 1 h. Mechanical hyper-nociception and cell number from the TMJ synovial lavage fluid were measured after 4 and 6 h following the stimulus, respectively. MPO activity assessment was also performed to confirm the data. The results were expressed as in Sections 2.6.2, 2.6.3 and 2.6.4.

2.6.8. Immunohistochemistry for IL-1β, TNF-α and HO-1
Immunohistochemistry for IL-1β, TNF-α and HO-1 were performed using the streptavidin–biotin (Labeled Streptavidin Biotin — LSAB) method in formalin-fixed, paraffin-embedded tissue sections (4 μm thick), mounted on glass slides previously prepared with an organosilane-based adhesive (3-aminopropyltriethoxysilane). Briefly, it consisted of the following steps: the sections went through 2 baths in xylol, each lasting 10 min. After this, they were immersed in three passages of absolute alcohol, then washed in running water, and immediately passed through distilled water.

After antigen retrieval, endogenous peroxidase was blocked (15 min) with 3% (v/v) hydrogen peroxide, and the sections were washed in phosphate-buffered saline (PBS). Sections were incubated overnight (4 °C) with a primary rabbit anti-TNF-α, IL-1β and HO-1 antibodies (ab13243, ABCAM®, England, UK), at a dilution of 1:200, and afterwards washed with a phosphate buffered saline solution, PBS (phosphate buffered saline).

The slides were incubated with a secondary antibody LSAB Kit (DAKO®, Carpinteria, CA, EUA) for 10 min at ambient temperature. The slides were then incubated with a biotinylated goat anti-rabbit antibody diluted 1:400 in PBS-BSA. After washing, the slides were incubated with an avidin–biotin–horseradish peroxidase conjugate (Strep ABC complex, VECTASTAIN ABC Reagent and peroxidase substrate solution) for 30 min according to the VECTASTAIN protocol. IL-1β and TNF-α were visualized with the chromogen 3,3-diaminobenzidine (DAB). Negative control sections were processed simultaneously as described above but with the first antibody being replaced by 5% PBS-BSA. None of the negative controls showed IL-1β, TNF-α or HO-1 immunoreactivity. Counter-staining was performed with hematoxylin, and afterwards the specimens were dehydrated in alcohol and diaphanized in xylol. Positive values were assigned to all cells that exhibited brown staining in the cytoplasm, irrespective of the staining intensity.

2.6.9. Statistical analysis
The data are presented as the mean ± standard errors (S.E.M.) for six animals per group. Differences between the means were compared using a one-way ANOVA followed by the Bonferroni test. In the histopathological analysis of the TMJ, the Kruskal–Wallis test was used, followed by Dunn’s test to compare medians. Values of p < 0.05 were considered to be statistically significant.

3. Results

3.1. CcL reduces mechanical hyper-nociception, leukocyte cell count and MPO activity on zymosan-induced TMJ acute arthritis in rats
The i.art. injection of zymosan (2 mg/art., 40 μl) elicited (p < 0.001) a mechanical hyper-nociception response (58.4 ± 6.4 g) compared with the sham group (118.8 ± 4 g) at 4 h after the inflammatory stimulus, as measured by a clear decrease in the mechanical threshold for head withdrawal. The i.v. treatment of rats with CcL (0.1, 1 or 10 mg/kg), administered 30 min prior to zymosan into the rat left TMJ, caused a reduction in mechanical hyper-nociception at all tested doses by 81% (107.3 ± 4.4 g), 83% (108.5 ± 5 g) and 89.5% (112.5 ± 3.4 g), respectively, (p < 0.001), as measured at 4 h after zymosan injection. Indomethacin (5 mg/kg, s.c.) also produced an anti-nociceptive effect of 69% (100 ± 8.5 g, p < 0.01) (Fig. 1A).

Additionally, the i.art. injection of zymosan into the rat left TMJ resulted in a significant increase in the number of polymorphonuclear cells (19,500 ± 241 cells/mm³) from the TMJ synovial fluid at 6 h, compared to the sham group (31 ± 6 cells/mm³). Pretreatment of rats with CcL (0.1, 1 or 10 mg/kg, i.v.) decreased (p < 0.001), in a dose-dependent manner, the leukocyte cell count from the TMJ synovial lavage fluid by 77.3% (4450 ± 808.5 cells/mm³), 80.7% (3750 ± 1233 cells/mm³) and 98.5% (295.5 ± 156 cells/mm³), respectively, in comparison with the zymosan group (Fig. 1B). Indomethacin resulted in 90.7% (1800 ± 638 cells/mm³) inhibition (p < 0.0001) of the leukocyte number in the synovial fluid. The MPO activity also decreased in the TMJ synovial lavage fluid at all doses tested by 63% (29.5 ± 9 U/joint fluid), 92% (10 ± 4.5 U/joint fluid) and 98% (2.5 ± 1 U/joint fluid), respectively.
(p < 0.05) (Fig. 1C). Animals pretreated with indomethacin (5 mg/kg, s.c.) also showed anti-inflammatory effects (55.5%, 53.3 ± 2 cells/mm³, p < 0.05).

3.2. CcL reverses tissue alteration in the zymosan-inflamed TMJ as assessed by H & E staining

Table 1 shows the scores attributed to TMJ histopathological analysis and compares the values between the sham and zymosan groups. Groups of animals were treated with zymosan (2 mg/art., 40 μL) and CcL (10 mg/kg, i.v.). The sham group received injection (i.art.) of saline in the same volume. TMJ tissues were fixed, sectioned and stained with H & E. A significant (p < 0.05) increase in the inflammatory parameters was observed in the zymosan group.

Six hours after zymosan-induced TMJ arthritis, an inflammatory cell influx was observed in the synovial membrane, the periarticular tissue and the skeletal muscle tissue, compared to the sham group. The cell type was predominantly neutrophils, which are known to characterize acute inflammation. Edema was also observed in the synovium. Pretreatment with CcL (10 mg/kg, i.v.) reduced the cellular infiltrate and edema in the synovial membrane, periarticular tissue (p < 0.01) and skeletal muscle tissue (p < 0.05), when compared to the zymosan group. The inflammatory process was considered as moderate to severe. These data can be seen in the TMJ photomicrographs (Fig. 2).

3.3. Naloxone does not block the anti-nociceptive effect of CcL

Rat pretreatment with naloxone (10 μg/art., 15 μL), 5 min before CcL (10 mg/kg, i.v.), did not interfere the antinociceptive response of this compound on zymosan-induced TMJ acute arthritis in rats. By contrast, at this dose, naloxone blocked the antinociceptive action of morphine (1000 μg, 0.1 ml, s.c.) in this model (Fig. 3).

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cell influx in the synovial membrane</th>
<th>Cell influx in the periarticular tissue</th>
<th>Cell influx in the muscular tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>Zy</td>
<td>4 (3–4)*</td>
<td>4 (3–4)*</td>
<td>2 (2–3)*</td>
</tr>
<tr>
<td>CcL</td>
<td>0 (0–0)***</td>
<td>1 (1–1)***</td>
<td>0 (0–2)***</td>
</tr>
</tbody>
</table>

0 — uninjured; 1 — low injury; 2 — mild injury; 3 — moderate injury; 4 — severe injury.
* p < 0.05 versus sham group (Kruskal–Wallis, Dunn’s).
** p < 0.05 indicate significant difference in relation to Zy group (Kruskal–Wallis, Dunn’s).
*** p < 0.01 indicate significant difference in relation to Zy group (Kruskal–Wallis, Dunn’s).
3.4. CcL does not have anti-nociceptive and anti-inflammatory actions through the HO-1 pathway

To investigate the possible role of the HO-1 pathway in the anti-nociceptive and anti-inflammatory effects of CcL, the rats were pretreated (s.c.) with ZnPP-IX (3 mg/kg), which is a specific HO-1 inhibitor, resulting in the inhibition of this pathway. It was observed that the anti-nociceptive and anti-inflammatory effects of the CcL (10 mg/kg, i.v.) on the zymosan-induced arthritis in the rat TMJ model were not inhibited in the presence of ZnPP-IX (Fig. 4A, B), a result that was further confirmed by MPO activity measurement and histopathological analysis (Fig. 4C).

3.5. CcL decreases IL-1β and TNF-α abundance in zymosan-induced TMJ arthritis in rats

Immunohistochemical analysis showed an increase in TNF-α, IL-1β and HO-1 expression, which was characterized by brown-colored cells in the arthritic TMJ of rats injected with zymosan (i.art.), when compared with the sham group (Figs. 6, 7, 8). The sham control group showed expression of TNF-α, IL-1β and HO-1 only in synovial cells. The negative control group sections were composed of arthritic TMJs of rats injected with zymosan that were not treated with anti-TNF-α, anti-IL-1β or anti-HO-1 antibodies.

A significant decrease in TNF-α expression was observed in the CcL-treated group compared with the zymosan group because the immunohistochemical analysis did not reveal expression of this pro-inflammatory cytokine in the synovial membrane or in the neutrophils. These profiles suggest an immunocellular response by CcL during the acute phase. For IL-1β, there was an intense staining in the synovial cells and neutrophils in the zymosan group, and this staining was reduced in the CcL group, similar to the level of the sham controls. This basal expression of IL-1β and TNF-α in the sham group may be due to
an injury caused by i.art. injection, nonetheless, the zymosan group showed intense staining for both cytokines from the synovial cells and neutrophils (Figs. 6, 7).

Finally, immunohistochemical analysis showed intense expression of HO-1 in synovial cells and neutrophils from the synovial membrane of rats pretreated with zymosan, and expression of HO-1 in synovial cells in the CcL group was similar to that of the zymosan group. In addition, a basal constitutive level of HO-1 in the synovial cells was noted in the sham group (Fig. 8).

4. Discussion

Previous studies evaluating biopolymers derived from seaweeds have suggested lectins as research agents that can allow researchers to comprehensively investigate events related to both nociception and inflammation in animal models [22,23,26,32]. In this work, we demonstrated for the first time the anti-nociceptive and anti-inflammatory efficacy of C. cupressoides lectin (CcL) in a model of zymosan-induced TMJ arthritis in rats. Furthermore, we also evaluated the putative involvement of leukocyte influx, cytokine expression (IL-1β and TNF-α), the endogenous opioid system and HO-1 pathways in CcL efficacy.

Experimental animal models of TMJ inflammatory hyper-nociception and arthritis have been proposed for the study of painful inflammatory conditions. Some authors have suggested inducing TMJ arthritis surgically [33] or mechanically [34], using systemic [35,36], submandibular [37], or intra-articular (i.art) injections [38]. Recently, our group set up a new model of zymosan-induced arthritis in the rat TMJ [1]. In this model, zymosan, a polysaccharide from yeast cell walls, elicits both hyper-nociception and inflammatory markers including an increase in vascular permeability, edema and cell migration associated with severe synovitis [39,3].

Table 2

<table>
<thead>
<tr>
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<th>Cell influx in the muscular tissue</th>
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</thead>
<tbody>
<tr>
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<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>Zy</td>
<td>4 (3–4)†</td>
<td>4 (3–4)†</td>
<td>2 (2–3)†</td>
</tr>
<tr>
<td>ZnPP-IX</td>
<td>3 (1–3)</td>
<td>4 (2–4)†</td>
<td>2 (1–3)†</td>
</tr>
<tr>
<td>CcL</td>
<td>0 (0–0)**</td>
<td>1 (1–1)†**</td>
<td>0 (0–2)**</td>
</tr>
<tr>
<td>ZnPP-IX + CcL</td>
<td>1 (1–1)†**</td>
<td>1 (1–1)†**</td>
<td>2 (0–2)</td>
</tr>
</tbody>
</table>

0 — uninjured; 1 — low injury; 2 — mild injury; 3 — moderate injury; and 4 — severe injury.

† p < 0.05 versus sham group (Kruskal–Wallis, Dunn’s).

** p < 0.05 versus Zy group (Kruskal–Wallis, Dunn’s).
Using zymosan-induced arthritis in the rat TMJ, some authors have recently suggested electroacupuncture as an important therapeutic strategy for treating TMJ arthritis [24]. Although it was demonstrated that some products derived from seaweeds, such as sulfated polysaccharides, inhibit the zymosan-induced inflammatory response in rats [17,40], there is no evidence for an effect of lectins.

In the present study, i.art. injection with 2 mg of zymosan decreased the mechanical nociceptive thresholds, which in turn was increased by CcL treatment. Regarding the inflammatory parameters, CcL administration decreased leukocyte number, which was certified by a decrease in MPO activity in the synovial lavage. Furthermore, histopathological analysis from rat TMJs treated with CcL showed a correlated decrease in both leukocyte influx and MPO activity in the synovial membrane. These data are in accordance with our previous results showing that CcL has anti-nociceptive and anti-inflammatory functions in classical models of nociception in mice and acute inflammation in rats [26]. Additionally, in the present study, histopathological analyses of immunohistochemical stains showed a decrease in both IL-1β and TNF-α expression in the synovial cells and neutrophils of the zymosan-induced TMJ inflammatory hyper-nociception.

Cytokines are produced by various cell types in response to a variety of stimuli and constitute a link between cellular injury or recognition of non-self and the development of signs and symptoms of inflammation [41,42]. Intra-plantar injection of both TNF-α and IL-1β induced hyper-nociception in mice [43]. Furthermore, writhing responses to zymosan and acetic acid were shown to be mediated by TNF-α and IL-1β [44]. Moreover, in mechanical (von Frey filaments) and thermal (hot plate) hyper-nociception induced by complete Freund’s adjuvant, there was a sequential release of TNF-α and IL-1β [45,46].

It was also revealed that TNF-α activates IL-1β, leading to the induction of connective tissue disorders in chronic inflammatory conditions [47], and TNF-α was addressed as a biochemical marker of pain and clinical outcome in temporomandibular joints [48,49]. The resolution of the inflammatory response during arthritis might be attributed to the inhibition of pro-inflammatory cytokines, such as IL-1β and TNF-α. These two factors are assumed to be the most important cytokines in TMJ disorders [5,47]. In fact, it seems that these cytokines induce
the production of metalloproteinases that irreversibly degrade the extracellular matrix components [5], including articular cartilage, as well as causing bone destruction and cell proliferation [50]. Taken together, these experiments strongly suggest that the release of cytokines constitutes a link between the TMJ injuries and the release of primary hyper-nociceptive mediators. This concept allows us to understand why the inhibition of cytokines causes analgesia [51].

According to Gondim et al. [24], electroacupuncture inhibited zymosan-induced TMJ inflammatory hyper-nociception, as well as neutrophil migration, vascular permeability, and TNF-α levels in synovial

Fig. 6. Immunohistochemistry analysis for TNF-α of zymosan-induced TMJ arthritis in rats. (A) Negative control sections (absence of anti- TNF-α antibody) from arthritic rats (400×). (B) The synovial membrane from the sham group showed light expression of TNF-α. (C) The synovial membrane from the zymosan group (2 mg/art.) with an intense TNF-α reaction (400×). (D) CCL (10 mg/kg, i.v.) showing no TNF-α reaction in the synovial membrane after zymosan-induced arthritis (400×). Black arrows indicate synoviocytes.

Fig. 7. Immunohistochemistry analysis for IL-1β of zymosan-induced TMJ arthritis in rats. (A) Negative control sections (absence of the anti- IL-1β antibody) from arthritic rats (400×). (B) The synovial membrane of the sham group revealed light expression of IL-1β (400×). (C) The synovial membrane of the zymosan group (2 mg/art.) with an intense IL-1β reaction (400×). (D) CCL (10 mg/kg, i.v.) showing no expression of IL-1β in the synovial membrane after zymosan-induced arthritis (400×). Black arrows indicate synoviocytes.
In conclusion, we demonstrated the anti-nociceptive and anti-inflammatory efficacy of CcL in a model of zymosan-induced TMJ inflammatory hyper-nociception in rats. Additionally, our results strongly suggest that CcL efficacy involves IL-1β and TNF-α inhibition. Given the well-demonstrated anti-nociceptive and anti-inflammatory efficacy of CcL, the design of novel compounds is highly encouraged with the hope of defining new pharmacological targets for the treatment of inflammatory TMJ pain.

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