Lectin from *Abelmoschus esculentus* reduces zymosan-induced temporomandibular joint inflammatory hypernociception in rats via heme oxygenase-1 pathway integrity and tnf-α and il-1β suppression

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**A B S T R A C T**

Temporomandibular joint (TMJ) disorders show inflammatory components, heavily impacting on quality of life. *Abelmoschus esculentus* is largely cultivated in Northeastern Brazil for medicinal purposes, having it shown anti-inflammatory activity. We evaluated A. esculentus lectin (AEL) efficacy in reducing zymosan-induced temporomandibular joint inflammatory hypernociception in rats along with the mechanism of action through which it exerts anti-inflammatory activity. Animals were pre-treated with AEL (0.01, 0.1 or 1 mg/kg) before zymosan (Zy) injection in the TMJ to determine anti-inflammatory activity. It promoted HO-1 overexpression whilst decreased TNF-α and IL-1β expression in the TMJ tissue. These findings suggest that AEL efficacy depends on TNF-α/IL-1β inhibition and HO-1 pathway integrity.

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1. Introduction

Temporomandibular joint (TMJ) disorders are a group of conditions that result in TMJ pain, limiting basic daily activities with high levels of inflammatory pain-related disability [1]. Experimental models that allow the study of the mechanisms underlying these conditions are of great clinical relevance. In this regard, we developed a rat model of TMJ inflammation using intra-articular injection of the pro-inflammatory agent zymosan [2].

The temporomandibular joint inflammation is the result of the release of pro-inflammatory cytokines, in particular tumor necrosis
factor-alpha (TNF-α) and interleukins. Increased levels of pro-inflammatory cytokines have been detected in patients with temporomandibular disorders (TMD), suggesting that these cytokines may play a role in the pathogenesis of synovitis. Besides, over the last few years numerous studies have led to the appreciation of heme oxygenase-1 role in this process (HO-1) [1,3].

HO-1 is induced in a variety of cells including endothelial cells, monocytes/macrophages, neutrophils and fibroblasts by heme, endotoxins, cytokines, nitric oxide and other mediators produced during inflammatory responses [4–6], and its induction would provide a negative feedback for cell activation and the production of inflammatory mediators, beyond its role in the antioxidant defense system [7,8]. In inflammatory and immune conditions, high levels of mediators with the potential to induce HO-1 are produced, and the expression of this protein could be part of an adaptive mechanism for limiting cytotoxicity [9].

In order to develop potential tools for novel therapies to ameliorate inflammatory pain, there has been a continuous increase in the use of natural products, which have encouraged scientific studies to search for new substances with therapeutic action and to confirm the efficacy of medicines derived from plants. In recent years there has been growing interest in alternative therapies and the use of natural products [10–12].

The Abelmoschus esculentus (Malvaceae) species is originated from Africa and is spread across a number of tropical countries, including Northeastern Brazil. This plant has been used to treat various disorders, amongst which inflammatory conditions [13,14]. Some recent studies have demonstrated anti-inflammatory and anti-inflammatory activity of the A. esculentus lectin (AEL) [15]. Thus, the present study aimed to investigate the unexplored anti-nociceptive and anti-inflammatory efficacy of AEL in the rat model of zymosan-induced TMJ inflammatory hypernociception. Additionally, considering our previous findings showing that the inhibition of HO-1 pathway is associated with increased inflammatory response [16], we investigated whether AEL efficacy depends on the integrity of the HO-1 pathway. Additionally, we investigated the putative involvement of TNF-α, IL-1β, and nitric oxide (NO) in AEL efficacy.

2. Materials and methods

2.1. Source material

A. esculentus seeds were collected in the municipality of Conde, Paraíba, Brazil for botanical identification. Professor Rita Balthazar de Lima (Department of Botany, Federal University of Paraíba – UFPB, Brazil) identified species of the Malvaceae family to which A. esculentus species belong. The specimen was deposited in the UFPB herbarium under the identification number of 41,386. Lectin purification was performed in BioGeR (Laboratory of Biochemical Genetics and Radiobiology).

2.2. Extraction of lectin

Seeds were grounded to powder and its lipids removed with n-hexane. To obtain the protein extract, the powder was placed in added in Tris-HCl buffer pH 7.4 with 0.1 M NaCl 0.15 M for 3 h and then centrifuged at 5,000 rpm at 4 °C for 20 min. The resulting precipitate was discarded, and the supernatant was subjected to ammonium sulfate precipitation, obtaining a lectin fraction within the range of 30–60% saturation. The lectin fraction was dialyzed exhaustively against water, lyophilized, and then isolated by ion exchange chromatography on DEAE-Sephacel equilibrated with bissacetic sodium phosphate 0.025 M pH 7.4. Lectin elution was prepared using the gradient of bissacetic sodium phosphate 0.025 M and NaCl pH 7.4 1 M. Elution was monitored by spectrophotometer at a wavelength of 280 nm, being it dialyzed against water, frozen and lyophilized. Furthermore, this lectin under study is endotoxin free which ultimately mean it does not exert toxic effects on animals under investigation.

2.3. Animals

Male Wistar rats (160–220 g) (n = 6) were housed in standard plastic cages with food and water available ad libitum. They were maintained in a temperature-controlled room (23 ± 2 °C) with a 12/12-hour light-dark cycle. All experiments were designed to minimise animal suffering and to use the minimum number of animals required to achieve a valid statistical evaluation. The experimental groups in this investigation consisted of 6 animals in each group in a total of 13 groups as following: Group 1: Sham, Group 2: Zymosan, Group 3: Zymosan + Indomethacin, Group 4: Zymosan + AEL (0.01 mg/kg), Group 5: Zymosan + AEL (0.1 mg/kg), Group 6: Zymosan + AEL (1 mg/kg), Group 7: Sham + Evans Blue, Group 8: Zymosan + Evans Blue, Group 9: AEL (1 mg/kg) + Zymosan + Evans Blue, Group 10: Zymosan + ZnPP-IX, Group 11: Zymosan + ZnPP-IX + AEL (1 mg/kg), Group 12: Zymosan + aminoguanidine, Group 13: Zymosan + aminoguanidine + AEL (1 mg/kg). The animal supplier for this study was the Central Animal House of the Federal University of Ceará and the experimental protocol was conducted in accordance with the Institutional Animal Care and with the approval of the Ethics Committee of the Federal University of Ceará, Fortaleza, Brazil (CEPA no. 74/2013).

2.4. TMJ inflammatory hypernociception induction

Rats (n = 60) were briefly anaesthetised with inhaled isoflurane and received an intra-articular (i.art.) injection of 2 mg zymosan (40 μL total volume) dissolved in sterile saline into the left TMJ using a 30-gauge needle and 0.5 mL syringe. Sham animals received only saline i.art. Before zymosan or saline injections, 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 the moving of the mandible, and the puncture of the needle into the joint space was confirmed by the loss of resistance. Gentle aspiration ruled out intravascular placement, after which the specified volume of zymosan or saline was injected.

As previously shown by our group [2], during the time course of zymosan TMJ inflammatory hypernociception development, it is maximal at 4 h of arthritis whereas cell influx peaks at 6 h. Based on these results we used these time points to assess both nociceptive (head withdrawal threshold) and inflammatory parameters (total cell counting and myeloperoxidase assay).

2.5. Evaluation of inflammatory hypernociception

Inflammatory hypernociception in the TMJ was evaluated by measuring the threshold of force intensity that needed to be applied to the TMJ region of rats (n = 60) until the occurrence of a reflex response of the animal (e.g., head withdrawal). The measurements were performed by an examiner unaware of the treatments and used a digital device (Insight, Ribeirão Preto, SP, Brazil) that consisted of a rigid filament linked in 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 put into individual plastic cages 45 min before the beginning of the tests. The animals were submitted to a conditioning session of head withdrawal threshold measurements in the testing room for 4 consecutive days under controlled temperatures (23 ± 2 °C) and low illumination. On the fifth day, the basal force threshold value was recorded (in triplicate) before the intra articular injections of either zymosan or vehicle and after 4 h.
2.6. Synovial lavage collection and cell counting

Six hours (6 h) after zymosan-injections the rats (n = 60) were sacrificed under anesthesia and exsanguinated. The superficial tissues were dissected, and the TMJ cavity was washed to collect the synovial lavage 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 was counted using a Neubauer chamber.

2.7. Myeloperoxidase activity analysis

Myeloperoxidase (MPO) is an enzyme found primarily in the azurophilic granules of the neutrophils and has been used extensively as a biochemical marker of granulocyte infiltration into various tissues. In our study, the MPO assay was conducted on the collected synovial lavage of rats (n = 60) at 6 h after zymosan injection. Briefly, the synovial lavage was centrifuged at 4500 rpm for 12 min at 4 °C. MPO activity was assayed by measuring the change in absorbance at 450 nm using o-dianisidine dihydrochloride and 1% hydrogen peroxide. The results are reported as the MPO units/joint fluid. A unit of MPO activity was defined as the conversion of 1 μmol of hydrogen peroxide to water in 1 min at 22 °C.

2.8. Histopathological analysis

After sacrifice, 6 h after zymosan-induced TMJ inflammatory hypernociception, the TMJ of rats (n = 42) was excised. The specimens were fixed in 10% neutral buffered formalin for 24 h, deamineralized in 10% EDTA, embedded in paraffin, and sectioned along the long axis of the TMJ. Sections of 5 μm, which included the condyle, articular cartilage, articular disc, synovial membrane, periarticular tissue, and the skeletal muscle tissue, were evaluated under light microscopy. For the specimens processed for routine hematoxylin–eosin (H&E) staining, histological analysis considered a 0–4 score grade based on the cell influx in the synovial membrane (SM).

2.9. Immunohistochemistry for TNF-α, IL-1β and HO-1

Immunohistochemistry analysis of the TMJ of rats (n = 18) for IL-1β, TNF-α, and HO-1 was 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, or 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®, Carpenteria, CA, EUA) for 10 min at ambient temperature. The slides were then incubated with a biotinylated goat anti-rabbit antibody dilute 1:400 in PBS-BSA. After washing, the slides were incubated with an avidin–biotin–horseradish peroxidase conjugate (Strept AB complex, VECTASTAIN ABC Reagent and peroxidase substrate solution) for 30 min according to the VECTASTAIN protocol. TNF-α, IL-1β, or HO-1 were visualized with the chromogen 3,3-diaminobenzidine (DAB) (DAKO®, Carpenteria, CA, EUA). 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 TNF-α, IL-1β, or HO-1 immunoreactivity. Counter-staining was performed with hematoxylin, and afterwards the specimens were dehydrated in alcohol and diaphonized in xylol. Positive values were assigned to all cells that exhibited brown staining in the cytoplasm, irrespective of the staining intensity.

2.10. TMJ periarticular tissue and trigeminal ganglion TNF-α and IL-1β ELISA assays

TNF-α and IL-1β concentrations were determined in the TMJ periarticular tissue and trigeminal ganglion 6 h after zymosan injection in rats (n = 12) that received 1 mg/kg AEL or vehicle (0.9% sterile saline). TMJ periarticular tissue and trigeminal ganglion were removed and stored at −80 °C. The material was homogenized in a solution of RIPA Lysis Buff System (Santa Cruz Biotechnology, USA), and the supernatant was used to determine the cytokine levels were quantified by the following kits: TNF-α–Rat TNF-α/IFNα/IL1β Quantikine ELISA Kit (R&D Systems, catalog number RTA00) and IL-1β–Rat IL-1 beta/IL-1F2 Quantikine ELISA Kit (R&D Systems, catalog number DY501) by enzyme-linked immunosorbent assay (ELISA). All assays were carried out according to the manufacturer’s instructions. Briefly, microtiter plates were coated overnight at room temperature (20–23 °C) with an antibody capture against rat (4.0 μg/mL) or IL-1β (0.8 μg/mL). The plate was blocked by adding of Reagent Diluent to each well, incubated at room temperature for a minimum of 1 h. After blocking the plate, the samples and standard at various dilutions were added and incubated at room temperature for 2 h. The plate was washed three times with buffer and of the Detection Antibody, diluted in Reagent Diluent with NGS 2% (350 ng/mL) was added (100 μL/ well). After further incubation at room temperature for 2 h, the plate was washed and Streptavidin-HRP was added. The colour reagent (H2O2 and Tetramethylbenzidine; 100 μL/well) was added 15 min later and the plate was incubated in the dark at room temperature for 15–20 min. The enzyme reaction was stopped with H2SO4, and absorbance was measured at 450 nm. TNF-α and IL-1β concentrations were expressed as pg/mL.

2.11. Evans blue extravasation measurement

In another sequence of experiments, AEL (1 mg/kg) was administered to rats (n = 18) 30 min prior to zymosan. 30 min before euthanasia, Evans Blue 2.5% (5 mg/kg, i.v.) was administered to assess plasma extravasation. Immediately after the extraction, the periarticular tissue was weighed and placed in 2 mL of formaldehyde overnight. The supernatant (100 μL) was extracted, and the absorbance at 630 nm was determined in spectrophotometer. The concentration was determined by comparison to a standard curve of known amounts of Evans blue dye in the extraction solution, which was assessed within the same assay. The amount of Evans blue dye (μg) was then calculated per mg of exudates.

2.12. Pharmacological modulation

Thirty minutes before injection with zymosan, rats were pre-treated (0.1 mL/100 g body weight) with AEL (0.01, 0.1 or 1 mg/kg) by intravenous (i.v.) injection or 0.9% sterile saline. Animals (sham group) received the same volume of 0.9% sterile saline. A positive control group of rats was pre-treated with indomethacin (5 mg/kg, s.c.) 1 h before zymosan injection. The animals (n = 36) were euthanized under anaesthesia at 6 h after zymosan-induced inflammatory hypernociception and inflammatory parameters (total cell count and myeloperoxidase assay activity) were evaluated.

To analyse the possible effect of HO-1 and NO pathway on anti-nociceptive and anti-inflammatory efficacy of A. esculentus lectin in the model of zymosan-induced TMJ inflammatory hypernociception in the rat, another series of experiments were performed. Animals (n = 24) were pre-treated with ZnPP-IX (3 mg/kg, s.c.), a specific HO-1 inhibitor, or with aminoguanidine (30 mg/kg, i.p.), a selective inhibitor of nitric oxide synthase (iNOS), alone or followed by an injection (i.v.) of AEL (1 mg/kg) 30 min later. After 30 min, zymosan (2 mg) was injected
(i.art.) and inflammatory hypernociception and inflammatory parameters (total cell count and myeloperoxidase assay activity) were evaluated as described above 4 h after zymosan injection.

2.13. Statistical analysis

The data are presented as the means ± S.E.M. or medians, when appropriate. Differences between means were compared using one-way ANOVA followed by the Bonferroni test. The Kruskal-Wallis test followed by Dunn’s test was used to compare medians. A probability value of \( p < 0.05 \) indicated significant differences.

3. Results

3.1. AEL reduces mechanical hypernociception, leukocyte cell count, MPO activity and Evans blue dye extravasation measurement on zymosan-induced TMJ mechanical inflammatory hypernociception in rats

Zymosan injection (2 mg/i.art., 40 μL) elicited \( (p < 0.05) \) a mechanical inflammatory hypernociception response (43.8 ± 2.2 g) compared with the sham group (122.0 ± 3.8 g) 4 h after the inflammatory stimuli has been applied, as measured by a prominent decrease in the mechanical threshold. Treatment with AEL (0.01, 0.1 or 1 mg/kg; i.v.) 30 min prior zymosan (i.art.) reduced mechanical hypernociception at all tested doses by (56.7 ± 1.1 g), (69.1 ± 2.1 g) and (81 ± 1.7 g), respectively, \( (p < 0.05) \) 4 h after zymosan injection. Indomethacin (5 mg/kg, s.c.) also produced antinociceptive effect (94.1 ± 10.9 g, \( p < 0.05 \)) (Fig. 1A).

Additionally, zymosan injection (i.art.) resulted in a significant increase in the number of polymorphonuclear cells (41,967 ± 3563 mm\(^3\)) in the TMJ synovial lavage after 6 h, compared with the sham group (615.8 ± 66.2 cells/mm\(^3\)). Pretreatment with AEL (0.01, 0.1 or 1 mg/kg, i.v.) decreased \( (p < 0.05) \) the leukocyte cell count in the TMJ synovial lavage by (7083 ± 3584 cells/mm\(^3\)), (2413 ± 354.2 cells/mm\(^3\)) and (1183 ± 219.3 cells/mm\(^3\)), respectively, in comparison with the zymosan group (Fig. 1B). Indomethacin administration resulted in inhibition \( (p < 0.05) \) of the leukocyte number (1610 ± 490.6 cells/mm\(^3\)) in the synovial lavage. MPO activity also decreased in the TMJ synovial lavage at all doses tested (35.1 ± 12.4 U/joint fluid), (22.0 ± 8.1 U/joint fluid) and (22.1 ± 10.7 U/joint fluid), respectively in comparison with the zymosan group (127.5 ± 27.6 U/joint fluid) (Fig. 1C).

These changes were accompanied by plasma extravasation that occurred in the TMJ during 6 h, being this parameter determined by Evans blue dye extravasation. The zymosan injection (i.art.) resulted

![Graphs A, B, C, D](#)
in a significant increase in Evans blue dye extravasation measurement (166.3 ± 6.7 μg/mg) in comparison with the sham group (136.9 ± 1.0 μg/mg). Pre-treatment with AEL 1 mg/kg, i.v. decreased (p < 0.05) Evans Blue dye extravasation (131.7 ± 3.1 μg/mg) compared with the zymosan group (Fig. 1D).

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

Six hours after zymosan-inducing TMJ inflammatory hypernociception, inflammatory cell influx was observed in the synovial membrane (SM) (Fig. 2B) compared with the sham group in which this parameter was absent (Fig. 2A). The cell types were predominantly neutrophils, which characterizes acute inflammation. Oedema was also observed in the synovium (Fig. 2B). Table 1 shows the scores attributed to TMJ histopathological analysis, comparing the sham, zymosan and AEL groups.

A significant (p < 0.05) increase in the inflammatory parameters was observed in the zymosan group compared with the sham group. Only AEL (1 mg/kg) reduced the inflammatory parameters with a lower inflammatory cell influx in the SM. In the AEL (1 mg/kg) TMJ photomicrography, it is observed the reduction of inflammatory cells and oedema in the SM (Fig. 2C).

3.3. AEL decreases TNF-α and IL-1β levels of TMJ tissue and trigeminal ganglion in zymosan-induced TMJ inflammatory hypernociception in rats

The zymosan injection (i.art.) resulted in a significant increase in TNF-α and IL-1β levels TMJ tissue (Fig. 3A and C) and trigeminal ganglion (Fig. 3B and D) compared with sham group. AEL (1 mg/kg) also reduced TNF-α and IL-1β levels in both TMJ tissue (2.52 ± 0.07) (3.11 ± 0.49) and trigeminal ganglion (2.52 ± 0.09) (1.55 ± 0.34), respectively, when compared with the zymosan group (7.94 ± 0.49, 11.01 ± 0.83 and 7.12 ± 0.40, 4.72 ± 0.47, respectively).

3.4. Effect of zinc protoporphyrin IX (ZnPP IX), a specific HO-1 inhibitor, on the AEL efficacy in the zymosan-induced TMJ inflammatory hypernociception

To investigate the role of HO-1 activity in the anti-inflammatory effect of AEL (1 mg/kg), animals were pretreated with ZnPP-IX (3 mg/kg; s.c.), a specific HO-1 inhibitor. The effects of AEL on the zymosan-induced TMJ inflammatory hypernociception (Fig. 4A), polymorphonuclear cells count (Fig. 4B), and MPO activity in the TMJ synovial lavage (Fig. 4C) were not observed in the presence of ZnPP-IX (3 mg/kg). Further, the effects of AEL (1 mg/kg) on the TMJ histopathological analysis (H&E) were not observed in the presence of ZnPP-IX (3 mg/kg) (Table 1) (Fig. 2D).

3.5. Effects of aminoguanidine, a selective inhibitor of nitric oxide synthase (iNOS), on the AEL efficacy on the zymosan-induced TMJ inflammatory hypernociception

To investigate the possible role of the NO in the antinociceptive and anti-inflammatory effects of AEL, rats were pre-treated with aminoguanidine (30 mg/kg; i.p.), a selective inhibitor of nitric oxide synthase (iNOS). It was observed that the AEL (1 mg/kg, i.v.) anti-nociceptive and anti-inflammatory effects on the zymosan-induced inflammatory hypernociception in the rat TMJ model were not inhibited in the presence of aminoguanidine, a result that was further confirmed by inflammatory hypernociception, cell counting and MPO activity measurement (Fig. 5A, B and C).

![Fig. 2. Histopathological analysis of TMJs: (A) sham group(400×); (B) zymosan group (400×) showing inflammatory cell influx in the synovial membrane; (C) AEL (1 mg/kg) group (400×) showing lower inflammatory cell influx in the synovial membrane, in detail an 1000×-magnification; and (D) ZnPP-IX (3 mg/kg) + AEL (1 mg/kg) + zymosan group (400×). ZnPP-IX reverted the effects of AEL (1 mg/kg) on the TMJ histopathological analysis (H&E) in the synovial membrane, in detail an 1000×-magnification. (SM): synovial membrane, (H&E) Hematoxylin and eosin staining. Black arrows indicate neutrophils.]
3.6. Immunohistochemical analysis

Immunohistochemical analysis for inflammatory cytokines expression showed increased TNF-α expression by inflammatory cells and synoviocytes (Fig. 6C), which was characterized by brown-coloured cells in the zymosan-injected rat TMJ compared with sham group (Fig. 6B), which exhibited only a light expression of TNF-α on endothelial cells, fibroblasts, and macrophages. IL-1β was, too, expressed on the same cell types of the TNF-α screened group, being characteristic of such expression the already described brown-coloured pattern of the zymosan-injected rat TMJ (Fig. 7C) when compared with the sham group (Fig. 7B), which in turns exhibited only a light expression of IL-1β only on connective tissue.

HO-1 immunohistochemical analysis showed a light immunostaining on synoviocytes, inflammatory cells and fibroblasts, which was characterized by brown-coloured cells in zymosan-injected rat TMJ (Fig. 8C), similar to sham group (Fig. 8B), which exhibited only a light expression of HO-1. The negative control group sections were composed of TMJ of rats injected with zymosan (i.art.), which did not receive the primary antibodies anti-TNF-α, anti-IL-1β or anti-HO-1 antibodies. None of the negative controls showed immunoreactivity (Figs. 6A, 7A and 8A).

A significant decrease in TNF-α and IL-1β expression was observed in the AEL-treated group compared with the zymosan group since the immunohistochemical analysis did not reveal significant expression of this pro-inflammatory cytokine in the synovial membrane or in the neutrophils. These profiles suggest an immunocellular response by AEL during the acute phase. Besides, the staining was reduced in the AEL group similar to the level of the sham controls (Figs. 6D and 7D).

Finally, immunohistochemical analysis of HO-1 expression showed an increase in its expression, characterized by brown-coloured cells in

<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>
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<td>0 (0–0)</td>
<td>0 (0–0)</td>
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<tr>
<td>Zymosan (Zy)</td>
<td>3 (1–4)*</td>
<td>4 (2–4)</td>
<td>2.5 (2–3)</td>
</tr>
<tr>
<td>Zy + Indomethacin</td>
<td>0 (0–1)*</td>
<td>0 (0–1)*</td>
<td>0 (0–1)*</td>
</tr>
<tr>
<td>Zy + AEL (0.01 mg/kg)</td>
<td>1 (0–2)</td>
<td>3 (2–3)</td>
<td>2 (1–3)</td>
</tr>
<tr>
<td>Zy + AEL (0.1 mg/kg)</td>
<td>2 (1–3)</td>
<td>3 (1–4)</td>
<td>2 (1–3)</td>
</tr>
<tr>
<td>Zy + AEL (1 mg/kg)</td>
<td>0.5 (0–1)*</td>
<td>2.5 (1–4)</td>
<td>1.5 (0–3)</td>
</tr>
<tr>
<td>Zy + ZnPP-DX + AEL (1 mg/kg)</td>
<td>3 (2–4)*</td>
<td>4 (3–4)</td>
<td>2 (2–3)</td>
</tr>
</tbody>
</table>

Semi-quantitative analysis was done by evaluating the following histopathological criteria: inflammatory cell influx in the synovial membrane (SM), inflammatory cell influx into the periarticular tissue and inflammatory cell influx in skeletal muscle tissue. Assigning scores ranged from 0 to 4: 0-absent; 1-low; 2-mild; 3-moderate; 4-severe. Data represent median and range of 6 TMJs per group (Kruskal-Wallis, Dunn’s).

* p < 0.05 compared with group Zy.
* p < 0.05 compared with group AEL (1 mg/kg).

Fig. 3: Effects of Abemoschus esculentus lectin on TNF-α and IL-1β levels in the TMJ periarticular tissue (A) and (C) and in the trigeminal ganglion (B) and (D) in zymosan-induced TMJ inflammatory hypernociception. The data represent the mean ± SEM (n = 6). # p < 0.05 indicates a significant difference from the sham group, *p < 0.05 indicates a significant difference from the zymosan group (ANOVA, Bonferroni).
the synovial membrane of the zymosan-induced TMJ inflammatory hypernociception treated with AEL (1 mg/kg) (Fig 8D).

4. Discussion

We demonstrated the antinociceptive and anti-inflammatory efficacy of a lectin from *A. esculentus* in the zymosan-induced rat TMJ inflammatory hypernociception. These effects depended in part on the integrity of the HO-1 pathway as well as on the TNF-α and IL-1β levels, as there was a reduction of these cytokines concentration in the TMJ tissue and in the trigeminal ganglion. However, it did not exert its actions through the NO pathway. Sabitha et al. [17] and Kumar et al. [18] showed the safety of AEL in acute and chronic toxicity tests, not being recorded deleterious mortality of animals after high dose administration, suggesting the safety of AEL administration [18]. To our knowledge this is the first demonstration that a plant-derived lectin is able to exert antinociceptive and anti-inflammatory effects on the TMJ pain, especially on zymosan-induced TMJ hypernociception in rats. In this study, zymosan-injection (Lart.) diminished head withdrawal threshold, being it partially reverted by AEL treatment.

Regarding inflammatory parameters, AEL administration decreased MPO activity in the synovial lavage, and Evans blue dye extravasation in synovial exudates compared with the zymosan group. Further, the TMJ histopathological analysis of AEL-treated groups correlated with the decrease in leukocyte influx and MPO activity, since this treatment reduced the inflammatory parameters to a normal status with a lower inflammatory cell influx in the synovial membrane. These data are in accordance with our previous results showing that AEL possesses antinociceptive and anti-inflammatory functions in classical models of nociception in mice and acute inflammation in rats [15]. To confirm the AEL antinociceptive and anti-inflammatory effects, our results demonstrate that pretreatment with AEL was capable of reducing TNF-α and IL-1β levels in the TMJ tissue. Confirming these findings, the immunohistochemical analyses showed a significant decrease in TNF-α and IL-1β expression in synovial and inflammatory cells in the AEL-treated groups.

Cytokines are signaling proteins and glycoproteins involved in cellular communication [19-20], orchestrating the joint tissue degradation in osteoarthritis [21,22]. TNF-α has an enormously detrimental effect on bone and cartilage and animal models have shown that induction of IL-1β expression in the rat TMJ leads to the pathology development and increased nociception [23]. Moreover, a positive correlation was found between cytokines present in the synovial fluid and osteoarthritis, being suggested that IL-1β and TNF-α can affect the treatment outcome of patients with osteoarthritis [24].

Our results suggest that AEL efficacy in zymosan-induced TMJ inflammatory hyper-nociception might be related to TNF-α and IL-1β inhibition. As shown by Rivaranor et al., 2014 [11], who demonstrated the anti-nociceptive and anti-inflammatory efficacy of a lectin from the green seaweed *Caulerpa cupressoides* (CcL) in a model of zymosan-induced TMJ inflammatory hypernociception in rats, our study strongly suggest that the efficacy of AEL, similarly to CcL, involves inhibition of TNF-α and IL-1β production. Others authors have demonstrated anti-inflammatory effects of lectins, showing dependence on TNF-α and IL-1β inhibition in classical models of nociception and acute inflammation in vivo [25-29].
The resolution of the inflammatory response during inflammatory hyper-nociception might be attributed to the inhibition of pro-inflammatory cytokines, such as TNF-α and IL-1β. These two factors are assumed to be the most important cytokines in TMJ disorders [30]. In fact, it seems that these cytokines induce the production of metalloproteinases that irreversibly degrade the extracellular matrix components [31], including articular cartilage, as well as causing bone destruction and cell proliferation [32]. These studies strongly suggest that the release of cytokines constitutes a link between the TMJ injuries and the release of primary hypernociceptive mediators. This concept allows us to understand why the inhibition of cytokines causes analgesia [33].

AEL was also able to reduce TNF-α and IL-1β levels in the trigeminal ganglion, suggesting antinociceptive and anti-inflammatory activity involving the peripheral nervous system. It is already known that the perception of pain in the orofacial region involves peripheral and central mechanisms [34]. The trigeminal ganglion transmits impulses to the brainstem at the bridge region, making synapse with second-order neurons in the trigeminal nucleus [35,36]. In this process, nociceptors are sensitized by pro-inflammatory mediators such as prostaglandins (PGE2), serotonin, substance P, tumor necrosis factor (TNF), and interleukins [37]. It is relevant to note that result TMJ pain is the result of inflammatory episodes involving inflammatory the mentioned mediators [38], and it is worth noting that TNF-α and IL-1β are considered the principal cytokines in the pathogenesis of inflammatory hyperalgesia [39].

Further, to elucidate possible AEL mechanism of action, pharmacological modulation was performed using ZnPP-IX, an specific inhibitor of HO-1. After pretreating animals with it, the anti-nociceptive and anti-inflammatory efficacy of AEL was not observed, suggesting that HO-1 activity is involved in the AEL inhibitory effects [7,32,40]. We demonstrated that ZnPP-IX treatment potentiated the effect of acetic acid by increasing the number of writhes [16]. Some of our recent studies demonstrated the involvement of HO-1 pathway in anti-inflammatory and antinociceptive effects of natural products in the TMJ inflammatory hypernociception [11,12].

In fact, the immunohistochemical analyses showed an increase in HO-1 expression by synovial and connective tissue cells in the AEL-treated groups. HO-1 is induced by oxidative or nitrosative stress, cytokines and other mediators produced during inflammatory processes, likely as part of a defense system in cells exposed to stress to provide a negative feedback for cell activation and the production of mediators, which could modulate the inflammatory response. Hemeoxygenase (HO) is the rate-limiting enzyme that catalyzes the degradation of heme to liberate carbon monoxide (CO), biliverdin (BVD) and free iron in mammalian cells [7]. Over the last few years, numerous studies have demonstrated that HO-1 expression and the concomitant production of its metabolites, CO and BVD, have anti-inflammatory consequences [41–43]. Considering this, we demonstrated that the HO/BVD/CO pathway plays antinociceptive effects during acetic acid-evoked nociception [16].

In fact, heme-induced HO-1 was reported to result in a reduction of cell migration, exudation and pro-inflammatory mediators release in a zymosan-induced air pouch inflammation model [32]. There is evidence...
Fig. 6. Immunohistochemistry analysis for TNF-α in zymosan-induced rat TMJ inflammatory hypernociception. (A) Negative control sections (absence of anti-TNF-α antibody) from zymosan groups (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) AEL (1 mg/kg, i.v.) showed light expression of TNF-α in the synovial membrane after zymosan-induced inflammatory hypernociception (400×). Black arrows indicate synoviocytes.

Fig. 7. Immunohistochemistry analysis for IL-1β in zymosan-induced rat TMJ inflammatory hypernociception. (A) Negative control sections (absence of the anti-IL-1β antibody) from zymosan group (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) AEL (1 mg/kg, i.v.) showed light expression of IL-1β in the synovial membrane after zymosan-induced inflammatory hypernociception (400×). Black arrows indicate synoviocytes.
that CO stimulates soluble guanylate cyclase (sGC) activity and increases the cellular levels of cyclic GMP [44,45]. Ferreira et al. [33] have provided experimental support to suggest that elevated levels of cyclic GMP are associated with inhibition of nociceptor hypersensitivity. In this regard, our research group demonstrated increased antinociceptive response produced by the combination of agents that increase intracellular cyclic GMP concentrations [46].

To elucidate other possible mechanism of action of AEL, aminoguanidine, a specific inhibitor of inducible NO synthase, was administered, being followed by the AEL administration (1 mg/kg, i.v.), which did not reduce its effectiveness, suggesting that the NO pathway integrity is not required for AEL mechanism of action. Albeit the role of NO in the inflammatory pain appears to be an expected finding, the involvement of this molecule still needs further elucidation, probably due to its dual effect, behaving as a pro or anti-inflammatory agent, depending upon the route of administration of donors or inhibitors of its synthesis associated with the inflammatory stimulus in use [47].

5. Conclusions

We demonstrated the anti-nociceptive and anti-inflammatory efficacy of AEL in the zymosan-induced TMJ inflammatory hypernociception in rats. Additionally, our results strongly suggest that AEL efficacy partially depends on the HO-1 pathway integrity and it involves TNF-α and IL-1β inhibition. Considering the demonstrated anti-nociceptive and anti-inflammatory efficacy of AEL, designing novel therapeutics is relevant to define new perspectives for the inflammatory TMJ pain management.

Conflict of interest

The authors declare no conflict of interests regarding the publication of this article.

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