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Beyond inflammation: Centrally mediated antinociceptive properties of *Spirulina platensis* LEB-18 biomass via the opioid system

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ABSTRACT

The management of pain is a world health issue. Available painkillers induce undesired side effects and are sometimes inefficient. *Spirulina* biomass has promoted anti-inflammatory activity in preclinical and clinical trials. This work characterizes the antinociceptive properties of *Spirulina platensis* LEB-18 biomass (SP-LEB18) and their mechanisms of action. In the CFA model in mice, SP-LEB18 reduced paw edema and mechanical allodynia, confirming its anti-inflammatory action and showing its antinociceptive activity. Cytokines levels were evaluated by ELISA; SP-LEB18 promoted an increase of IL-10 levels and a reduction of TNF-α and IL-1β levels. SP-LEB18 promoted centrally mediated antinociception, as indicated by the tail flick test. When the same set of experiments was conducted with IL-10 knockout mice, the antinociception was still detected in the tail flick test, but not in the CFA model. Pretreatment with naloxone abolished the effect of SP-LEB18, demonstrating a pure antinociceptive action of *Spirulina* biomass via the opioid system.

1. Introduction

Pain is an important physiological alert against harmful threats. It allows the conscious perception of damaging stimuli and triggers a quick systemic response to avoid injuries on behalf of our physical integrity (Dinakar & Stillman, 2016, Sneddon, 2018). Even though pain is essential for self-preservation and survival, in some cases it overpasses the boundaries of its biological role, becoming abnormally intense or long-lasting (Basbaum, Bautista, Scherrer, & Julius, 2009). When this happens, pain itself must be dealt with as a pathologic entity, demanding a proper pharmacological treatment. Regardless of the amount of clinically available analgesic drugs, there is still a gap in the therapeutic management of pain. Nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids are the two drug classes most commonly used as painkillers (Cashman, 1996; Becker, Bair, Picchioni, Starrels, & Frank, 2018). Both classes induce undesired side effects that limit their use in the long term (Fitzgerald, 2003; Boom et al., 2012; Dahan, Overdyk, Smith, Aarts, & Niesters, 2013), which stimulates the search for analgesic drugs with highest safety and efficacy.

Natural products have been used for curative purposes since the beginnings of humankind (Calixto, Scheidt, Otuki, & Santos, 2001; Newman & Cragg, 2016). Knowledge on medicinal plants has evolved since their use in ancient rituals, now allowing the industrial-scale production of plant-derived drugs (Rishton, 2008; Atanasov et al., 2015; Newman & Cragg, 2016). Nevertheless, many other living organisms are rich sources of bioactive compounds still unexplored. The surface of Earth is 70% covered by water, the habitat of thousands of life forms (Montaser & Luesch, 2011). The unknown vastness of biomolecules produced by aquatic organisms may offer novel chemical structures and potential candidates for the development of new analgesic drugs (Kong, Jiang, & Zhang, 2010; Montaser & Luesch, 2011).

Spirulina is a genus of cyanobacteria mostly found in tropical and subtropical waters. Its biomass is a rich source of proteins, lipids, carbohydrates, carotenoids, phenolic and other active compounds, which makes it highly nutritional (Deng & Chow, 2010; Hosseini, Khosravi-Darani & Mozafariet, 2013; Lau, Matsui, & Abdullah, 2015; Serban et al., 2016; Finamore, Palmery, Bensehaila, & Peluso, 2017). For this reason, *Spirulina* biomass-based products have been used for a few

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decades as dietary supplements. However, besides the nutritional gains, these products promote many other well-known benefits to human health. Studies mainly focused on *Spirulina platensis*, *S. maxima*, and *S. fusiformis* have led to the discovery of many biological effects promoted by their biomass and isolated compounds. These effects include diabetes and hyperlipidemia control, antimicrobial, anticancer, antioxidant, immunomodulatory, and anti-inflammatory activities, allowing a potentially wide therapeutic use of *Spirulina* biomass (Deng & Chow, 2010; Hosseini et al., 2013; Hernández-Lepe, Wall-Medrano, Juárez-Oropeza, Ramos-Jiménez, & Hernández-Torres, 2015; Martínez-Galero et al., 2016; Serban et al., 2016; Wu, Miron, Klímová, Wan & Kuča, 2016).

Such multiplicity of combined actions results in a highly efficient control of the inflammatory process. *Spirulina* biomass reduces oxidative stress, the expression of inflammatory mediators such as cytokines, as well as the activity of inflammatory enzymes (Chen et al., 2012; Hwang, Chen, & Chan, 2013; Xia et al., 2016). Its anti-inflammatory action has been demonstrated in many experimental models (Remirez, González, Merino, Rodriguez, & Ancheta, 2002; Rasool, Sabina, & Lavanya, 2006; Joventino et al., 2012; Somchit et al., 2014) and clinical trials have shown that it can be safely used to modulate cytokines and oxidative stress in humans (Mao, Water & Gershwin, 2005; Park et al., 2008).

Anti-inflammatory drugs often display associated analgesic properties. Even though the anti-inflammatory actions mediated by *Spirulina* biomass are widely studied, little is known about its effects on nociception. Based on its recognized biological actions, *Spirulina* biomass might potentially be used as a therapy in painful conditions. Therefore, the present work aimed to investigate the antinociceptive effects of *S. platensis* LEB-18 biomass along with its mechanisms of action.

2. Material and methods

2.1. Animals

Most experiments were conducted using male Swiss mice. In the mechanism of action studies, male wild-type (WT) and IL-10 knockout (KO) C57BL/6 mice were used. Mice (25–30 g) were obtained from the Animal Facilities of the Institute Gonçalo Moniz (FIOCRUZ; Salvador, Brazil) and housed in temperature-controlled rooms (22 ± 2 °C), with access to food and water *ad libitum*, under a 12:12 h light–dark cycle of artificial light. Animal care and handling procedures were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH, 8023) and were approved by the Ethics Committee for Animal Experimentation of FIOCRUZ (reference number L-IGM025/2017).

2.2. Spirulina platensis LEB-18 dried biomass

Spirulina platensis LEB-18 dried biomass (SP-LEB18) was obtained as previously described by De-Jesus et al. (2018). Briefly, *S. platensis* LEB-18 was obtained from the Mangueira Lagoon (Santa Vitória do Palmar, RS, Brazil) and cultivated in an open raceway pond with 240 L of capacity containing Zarrouk medium under constant agitation. After thirty days of cultivation, the batch was centrifuged at 15,000*g* for 15 min and the pellet was lyophilized.

2.3. Complete Freund's Adjuvant (CFA)-induced inflammation

The antinociceptive effect of SP-LEB18 was initially investigated in the CFA-induced inflammatory pain model. CFA is a solution containing *Mycobacterium tuberculosis* (1 mg/mL) in 85% paraffin oil and 15% mannide monooleate oil. Mice were orally treated with SP-LEB18 (50 – 400 mg/kg, v.o.) or vehicle in the same volume (saline, 200 μL, v.o.) and after forty minutes received a subcutaneous intraplantar injection of CFA (20 μL, i.pl.) in the right hind paw (Opretzka et al., 2019). Dexamethasone, by intraperitoneal route (2 mg/kg, i.p.), was used as a reference drug. Following this protocol, three parameters were evaluated as described in the next sections: paw edema, mechanical nociceptive threshold, and local cytokines levels.

2.4. The plethysmometer test for edema quantification

Local edema formation was quantified by the measurement of the paw volume using a plethysmometer apparatus (Ugo Basile; Comerio, Italy), immediately before (baseline) and at different times after CFA inoculation (Nascimento et al., 2016). The magnitude of paw edema was calculated as the increase of paw volume in mm^3 compared to baseline.

2.5. The von Frey test for mechanical nociceptive threshold determination

Considering that the reduction of the nociceptive threshold is one of the main manifestations of inflammatory pain, the nociceptive threshold to mechanical stimuli was measured with von Frey filaments (Stoelting; Chicago, IL, USA). Thresholds were evaluated daily three days before the experimental day (baseline) and at different time points after the CFA inoculation. Mice were placed in acrylic cages upon a wired grid floor for fifteen minutes to acclimate to their surroundings. The inoculated hind paw was then touched with a series of filaments with logarithmically incremental stiffness (0.008–4 g) until they were slightly flexed. A positive response was characterized by the abrupt removal of the touched paw. A tilted mirror placed beneath the grid provided a clear view of the mice's hind paws. The mechanical nociceptive threshold was calculated by the up-and-down method described by Dixon (1965). Values represent the filament weight (g) to which mice respond in 50% of presentations.

2.6. Quantification of cytokine levels by ELISA

Mice were euthanized with anesthetic overdose and skin tissues were removed from the inoculated hind paws three hours after CFA injection. Tissue proteins were extracted from 100 mg tissue/mL phosphate buffered saline (PBS) to which 0.4 M NaCl, 0.05% Tween 20 and protease inhibitors (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KI aprotinin A/100 ml) were added. The samples were centrifuged for 10 min at 3000 g and the supernatant was frozen at −80 °C for later quantification. Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-10 (IL-10) levels were determined using commercially available immunoassay ELISA kits for mice (R&D System; Minneapolis, MN, USA), according to the manufacturer's instructions. The results were expressed as picograms of cytokine per milligram of protein (Lauria et al., 2018)

2.7. Tail flick test

The tail flick test, a well-stablished assay to central analgesics, was next performed. Before the experiments, mice were kept inside restrainer tubes during thirty minutes per day for three days to ensure ambiance. Mice received SP-LEB18 (100–400 mg/kg, v.o.), vehicle (saline), or morphine (5 mg/kg, i.p.; reference drug) and the latency times were determined. To perform the assay, mice were gently placed inside the restrainer tubes and the tips of their tails (2 cm) were submerged into heated water (48 ± 0.2 °C). Immediately, a stopwatch was activated and the time until mice took out their tails from the water was measured. A cut-off time of ten seconds was stablished to avoid tissue damage (Santos et al., 2012). For the antagonism assay, mice were pretreated with the opioid receptor antagonist naloxone (5 mg/kg, i.p.) and then received SP-LEB18 (200 mg/kg, v.o.) or morphine (5 mg/kg, i.p.). The latency times were then determined, as described above.

2.8. The rota-rod test for assessment of motor impairment

The rota-rod apparatus (Insight; Ribeirão Preto, SP, Brazil) consists of a bar that rotates at a constant speed of 8 rpm and mice need to walk forwards to avoid falling from it. The animals were selected previously by eliminating those mice that did not remain on the bar for two consecutive periods of 120 s. Mice received SP-LEB18 (400 mg/kg, v.o.), vehicle (saline), or the reference drug diazepam (10 mg/kg, i.p.). Forty minutes later, mice were challenged to remain on the rotarod for three consecutive periods of 120 s, and the results were expressed as the average time they resist in the bar without falling (Lima et al., 2014).

2.9. Statistical analysis

Data was presented as means ± standard deviation (SD) of measurements made on six mice per group. Comparisons between three or more treatments were made using one-way ANOVA with Tukey's post-hoc test, or for repeated measures, two-way ANOVA with Bonferroni's post-hoc test. Data were analyzed using GraphPad Prism 6 computer software (GraphPad, San Diego, CA, USA). Statistical differences were considered to be significant at $p < 0.05$.

3. Results

3.1. Antinociceptive effect of SP-LEB18 in inflammatory pain model

Once the anti-inflammatory properties of SP have been previously described, the antinociceptive effect of SP-LEB18 was initially evaluated in an inflammatory pain model. With this aim, SP-LEB18 was tested in CFA-induced mechanical allodynia, considering that allodynia is a frequent clinical manifestation of inflammatory pain (Fig. 1). Vehicle-treated mice intraplantarly injected with CFA had a drastic fall in their mechanical nociceptive thresholds, characterizing the state of inflammatory allodynia. The oral pretreatment with SP-LEB18 at the dose of 100 mg/kg partially prevented the CFA-induced allodynia after three ($p < 0.05$) and six ($p < 0.05$) hours. At the doses of 200 and 400 mg/kg, this effect was observed earlier, starting in the first hour post injection and lasted to the sixth ($p < 0.05$) and eighth ($p < 0.05$) hours, respectively. The SP-LEB18-induced antinociception was dose-dependent and 200 mg/kg was the maximum effective dose. Surprisingly, at the doses of 200 ($p < 0.05$) or 400 mg/kg ($p < 0.05$), SP-LEB18 was more efficacious than dexamethasone and the mice's thresholds were very close to their baselines after three and six hours.

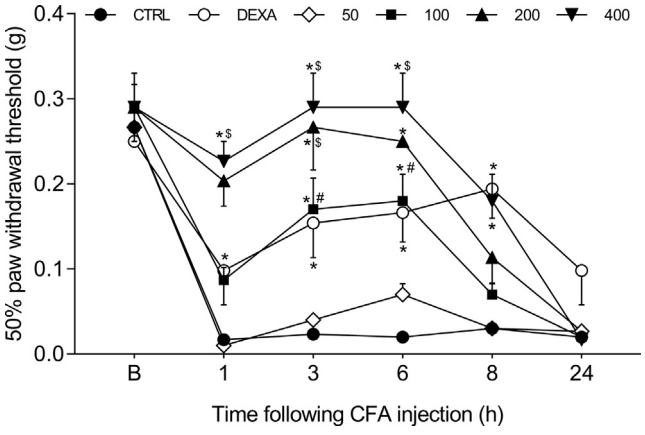

Fig. 1. Antinociceptive effect of SP-LEB18 in inflammatory pain model. Mice were orally treated with SP-LEB18 (50–400 mg/kg) or vehicle (saline, CTRL) one hour before intraplantar injection of CFA. Dexamethasone (DEXA, 2 mg/kg, i.p.) was used as reference drug. The mechanical nociceptive threshold (axis of ordinates) was evaluated at different times after the injection of CFA (axis of abscissas) and is represented as the filament weight (g) in which the animal responded in 50% of the presentations. The baseline (B) represents the mean threshold before the day of the experiment. Data are expressed as means ± SD (n = 6). *p < 0.05 compared with CTRL group. $^{\$}$p < 0.05 compared with DEXA group. $^{\#}$p < 0.05 compared with SP-LEB18 400 mg/kg group. Two-way ANOVA followed by Bonferroni's test.

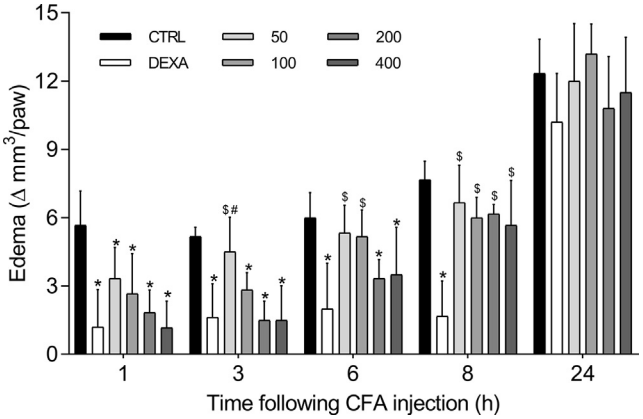

Fig. 2. Influence of SP-LEB18 on CFA-induced edema. Mice were orally treated with SP-LEB18 (50–400 mg/kg) or vehicle (saline, CTRL) one hour before intraplantar injection of CFA. Dexamethasone (DEXA, 2 mg/kg, i.p.) was used as a reference drug. The paw edema (axis of ordinates) was evaluated at different times after the injection of CFA (axis of abscissas) and is represented as the increase in the paw volume in mm^3. Data are expressed as means ± SD (n = 6). *p < 0.05 compared with CTRL group. $^{\$}$p < 0.05 compared with DEXA group. $^{\#}$p < 0.05 compared with SP-LEB18 400 mg/kg group. Two-way ANOVA followed by Bonferroni's test.

3.2. SP-LEB18 displays antiedematogenic activity

The anti-inflammatory action of SP-LEB18 was confirmed in the CFA-induced paw edema model (Fig. 2). Intraplantar injection of CFA elicited persistent paw edema in mice, which was monitored from one to twenty-four hours following the injection. Whereas the control group exhibited a gradual and consistent increase in paw volume as a result of the inflammatory process, edema formation was partially prevented in mice treated with SP-LEB18 in all tested doses. The antiedematogenic effect of SP-LEB18 lasted for up to one hour at the dose of 50 mg/kg ($p < 0.05$), three hours at the dose of 100 mg/kg ($p < 0.05$) and six hours at the doses of 200 ($p < 0.05$) or 400 mg/kg ($p < 0.05$). A dose-dependence was observed, and no difference was found between the doses of 200 and 400 mg/kg, indicating that 200 mg/kg is the maximum effective dose. The efficacy of SP-LEB18 was similar to that of dexamethasone, which was used as a reference drug, but the effect was shorter-lasting.

3.3. SP-LEB18 modulates the balance between anti- and proinflammatory cytokines

Considering the relevance of cytokines in inflammatory pain, the effect of SP-LEB18 on the local production of the proinflammatory cytokines TNF-α and IL-1β along with the anti-inflammatory cytokine IL-10 was evaluated during CFA-induced inflammation (Fig. 3). CFA-injected mice treated with saline had an increase in the production of TNF-α (Fig. 3A; $p < 0.05$) and IL-1β (Fig. 3B; $p < 0.05$) compared to the naive group, without alterations in IL-10 levels (Fig. 3C). The overexpression of both proinflammatory cytokines was prevented by the pretreatment with SP-LEB18 at the dose of 200 mg/kg (Fig. 3A and B; $p < 0.05$). Moreover, SP-LEB18 increased the production of IL-10 (Fig. 3C; $p < 0.05$) compared to the control group. The effects of SP-LEB18 were similar to those of dexamethasone.

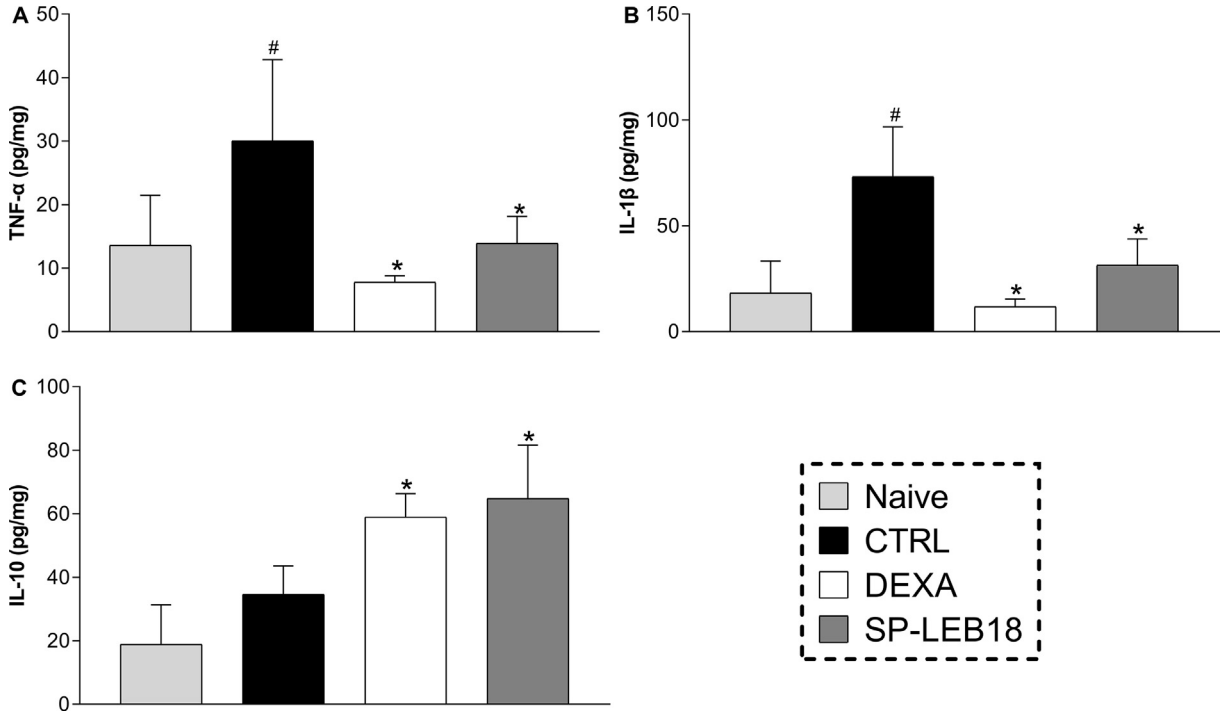

Fig. 3. Influence of SP-LEB18 on the balance between pro- and anti-inflammatory cytokines during CFA-induced inflammation. Mice were orally treated with SP-LEB18 (200 mg/kg) or vehicle (saline, CTRL) three hours before the intraplantar injection of CFA. Dexamethasone (DEXA, 2 mg/kg, i.p.) was used as a reference drug. Naive group consists of mice that did not receive any experimental manipulation. Skin tissue samples were collected for (A) TNF-α, (B) IL-1β, and (C) IL-10 determination by ELISA. The results are represented as picograms of cytokine per milligram of protein (axis of ordinates) for each experimental group. Data are expressed as means ± SD (n = 6). *p < 0.05 compared with CTRL group. #p < 0.05 compared with Naive group. One-way ANOVA followed by Tukey's test.

3.4. SP-LEB18 induces antinociception by centrally mediated mechanisms

Because the antinociceptive effect of SP-LEB18 had been more pronounced and long-lasting than its antiedematogenic action, it was possible that this biomass had an intrinsic analgesic property not directly linked to the control of inflammation. Aiming to investigate a pure analgesic action of SP-LEB18, mice were submitted to the tail flick test (Fig. 4), which is capable of detecting centrally mediated antinociception. While the latency time of control mice remained stable throughout the experimental period, the groups that had been treated with SP-LEB18 at all tested doses showed a dose-dependent increase in latency time ($p < 0.05$). The doses of 200 and 400 mg/kg were as efficacious as morphine and the antinociceptive effect of SP-LEB18 was longer-lasting than that induced by the gold standard analgesic.

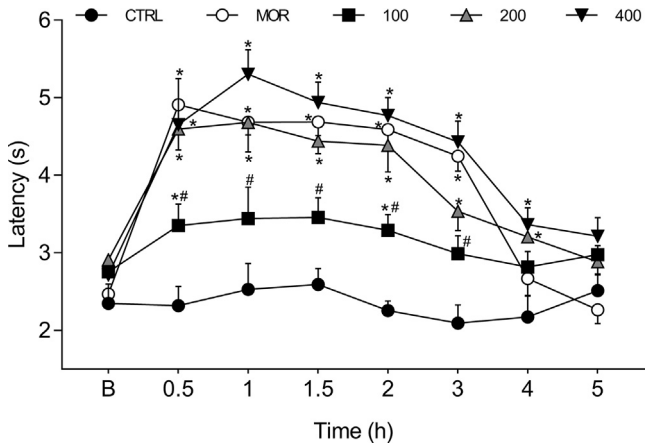

Fig. 4. Antinociceptive effect of SP-LEB18 in the tail flick test. Mice were orally treated with SP-LEB18 (100–400 mg/kg) or vehicle (saline, CTRL) thirty minutes before the test. Morphine (MOR, 5 mg/kg, i.p.) was used as reference drug. The latency time (s) until mice took off their tails from the thermal stimulus (axis of ordinates) was evaluated at different times (axis of abscissas). The baseline (B) represents the mean latency time before the day of the experiment. Data are expressed as means ± SD (n = 6). *p < 0.05 compared with CTRL group. #p < 0.05 compared with SP-LEB18 400 mg/kg group. Two-way ANOVA followed by Bonferroni's test.

3.5. Motor function is preserved in mice treated with SP-LEB18

Since the behaviors evaluated in pain models depend on the integrity of mice's motor capacity, it must be assured that SP-LEB18 had no influence on motor function. With this aim, mice treated with SP-LEB18 were submitted to the rotarod performance test (data not shown). SP-LEB18 at the dose of 400 mg/kg did not reduce the time mice spent on the rolling bars when compared to control group. In contrast, the permanence time was diminished by diazepam (10 mg/kg), used as reference drug for inducing movement impairment ($p < 0.05$).

3.6. IL-10 plays a key role in the anti-inflammatory action promoted by SP-LEB18

Once data from the tail flick test suggested that SP-LEB18 possesses intrinsic analgesic properties, further investigations were conducted to clarify its mechanisms of action. Because SP-LEB18 had increased the local production of IL-10, a cytokine with relevant analgesic properties, the contribution of IL-10 to its antinociceptive effects was evaluated. For this purpose, SP-LEB18 was tested in IL-10 KO mice in the CFA-induced inflammation model. SP-LEB18 at 200 mg/kg reduced both the mechanical allodynia (Fig. 5A; $p < 0.05$) and the paw edema (Fig. 5B; $p < 0.05$) in WT mice. However, SP-LEB18 was ineffective on IL-10 KO mice, in which both paw edema and mechanical allodynia developed similarly to the vehicle-treated mice. These results showed that the antinociceptive and anti-edematogenic effects of SP-LEB18 during

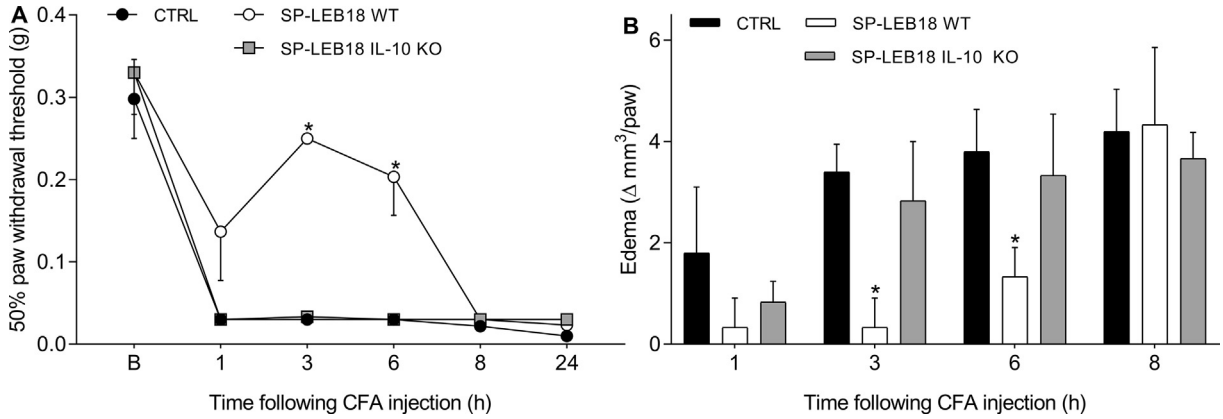

Fig. 5. Effect of SP-LEB18 on IL-10 KO mice during CFA-induced inflammation. Wild-type (WT) or IL-10 knockout (IL-10 KO) mice were orally treated with SP-LEB18 (200 mg/kg) or vehicle (saline, CTRL) one hour before the intraplantar injection of CFA. Panel A: The mechanical nociceptive threshold (axis of ordinates) was evaluated at different times after the injection of CFA (axis of abscissas) and is represented as the filament weight (g) in which the animal responded in 50% of the presentations. The baseline (B) represents the mean threshold before the day of the experiment. Panel B: The paw edema (axis of ordinates) was evaluated at different times after the injection of CFA (axis of abscissas) and is represented as the increase in the paw volume in mm3. Data are expressed as means ± SD (n = 6). *p < 0.05 compared with CTRL group. Two-way ANOVA followed by Bonferroni's test.

inflammatory conditions are mediated by IL-10.

3.7. IL-10 does not mediate the intrinsic antinociceptive effect of SP-LEB18

In order to distinguish the anti-inflammatory mechanisms from the mechanisms of intrinsic analgesia, the effects of SP-LEB18 in IL-10 KO mice were also investigated in the tail flick test (Fig. 6). In this assay no difference in SP-LEB18 response (200 mg/kg) was found between mutant and WT mice. Both IL-10 KO ($p < 0.05$) and WT ($p < 0.05$) mice showed a consistent antinociceptive profile that lasted up to three hours after treatment. Therefore, IL-10 does not contribute to the centrally mediated component of SP-LEB18-induced antinociception.

3.8. Opioid signaling pathways are activated by SP-LEB18 to promote antinociception

Since a centrally mediated antinociception had been suggested by the results of the tail flick test (Figs. 4 and 6), the contribution of opioid pathways to the intrinsic antinociceptive effect of SP-LEB18 was

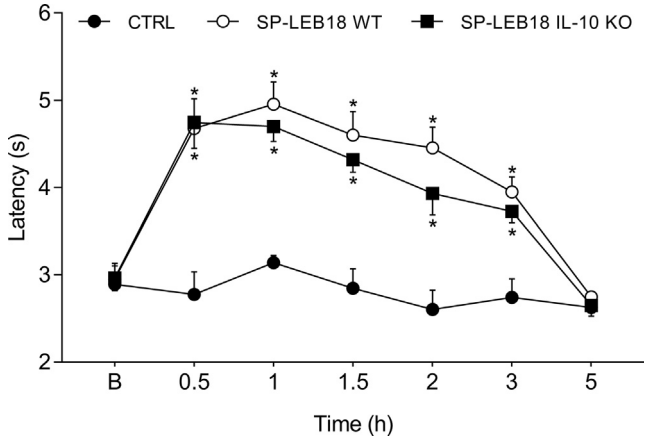

Fig. 6. Effect of SP-LEB18 on IL10 KO mice in the tail flick test. Wild-type (WT) or IL-10 knockout (IL-10 KO) mice were orally treated with SP-LEB18 (200 mg/kg) or vehicle (saline, CTRL) thirty minutes before the test. The latency time (s) until mice took off their tails from the thermal stimulus (axis of ordinates) was evaluated at different times (axis of abscissas). The baseline (B) represents the mean latency time before the day of the experiment. Data are expressed as means ± SD (n = 6). *p < 0.05 compared with CTRL group. Two-way ANOVA followed by Bonferroni's test.

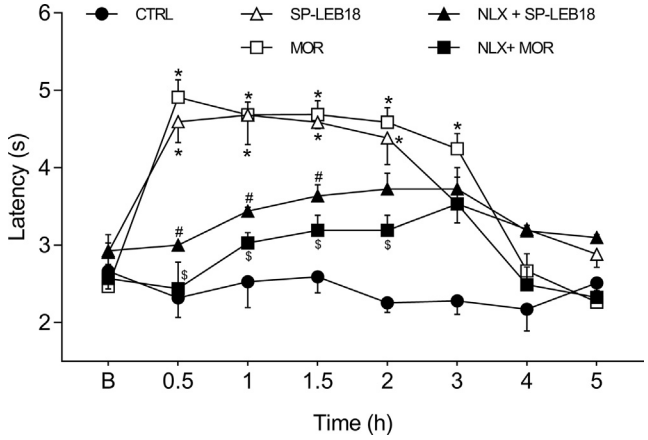

Fig. 7. The pharmacological blockage of opioid receptors reduces the antinociception induced by SP-LEB18. Mice were pretreated with saline or naloxone (NLX, 5 mg/kg) by intraperitoneal route and after forty minutes received SP-LEB18 (200 mg/kg, v.o.), morphine (MOR, 5 mg/kg, i.p.), or vehicle (saline, CTRL). The latency time (s) until mice took off their tails from the thermal stimulus (axis of ordinates) was evaluated at different times (axis of abscissas). The baseline (B) represents the mean latency time before the day of the experiment. Data are expressed as means ± SD (n = 6). *p < 0.05 compared with CTRL group. #p < 0.05 compared with SP-LEB18 group. $p < 0.05 compared with MOR group. Two-way ANOVA followed by Bonferroni's test.

evaluated. To test this hypothesis, the pharmacological blockage of opioid receptors was performed using the non-selective opioid antagonist naloxone. The antinociception promoted by SP-LEB18 (200 mg/kg) in the tail flick test was reduced by naloxone (5 mg/kg) (Fig. 7; $p < 0.05$), suggesting that opioid receptors are activated through direct or indirect mechanisms by SP-LEB18. As expected, the antinociceptive effect of morphine (5 mg/kg) in the tail flick test was prevented by naloxone ($p < 0.05$).

4. Discussion

Spirulina platensis and its biotechnological uses have been studied for more than a half century. The anti-inflammatory properties of *Spirulina* biomass and derived products are well described in the literature, being associated with antinociceptive effects in inflammatory models of pain (Shih, Cheng, Wong, Kuo, & Chou, 2009). Nonetheless,

the biological actions of *Spirulina* biomass are not fully explored and novel uses for it are still being discovered. The results reported herein provide the first evidence of an intrinsic antinociceptive action of SP-LEB18 by oral route. In addition to its anti-inflammatory effects, which were dependent on IL-10, SP-LEB18 promoted centrally mediated antinociception modulated by the opioid system. Therefore, SP-LEB18 induced antinociception independently of its anti-inflammatory effects.

Based on the anti-inflammatory properties described for *Spirulina* biomass, the antinociceptive effect of SP-LEB18 was firstly evaluated on the CFA-induced inflammation model. CFA promotes the release of several inflammatory mediators, such as bradykinin, prostaglandin E_2 (PGE_2), nitric oxide (NO), and cytokines (Woolf, Allchorne, Safieh-Garabedian, & Poole, 1997; Patel, Beaino, Anderson, & Janjic, 2015). These inflammatory mediators induce long-lasting edema and inflammatory pain, which is characterized by phenomena such as hyperalgesia, allodynia, and spontaneous nociception (Klaumann, Wouk, & Sillas, 2008; Barros et al., 2010). Here we showed that the treatment with SP-LEB18 effectively reduced both paw edema and mechanical allodynia induced by CFA in mice.

The antiedematogenic activity of *Spirulina* biomass has been previously described using the models of carrageenan-induced paw edema (Joventino et al., 2012), PGE_2-induced paw edema (Somchit et al., 2014), and UVB-induced ear edema (Yogianti et al., 2014). Similarly, the antinociceptive effect of *Spirulina*-based products has been described in the carrageenan-induced inflammation (Shih et al., 2009) and in the formalin test (Joventino et al., 2012). Importantly, the daily consumption of *S. platensis* biomass induces analgesic effect in patients with chronic pain presenting inflammatory component (Jensen et al., 2016).

Spirulina-induced antinociception has been attributed to its anti-inflammatory properties by decreasing inflammatory mediators such as NO, PGE_2, and TNF-α (Shih et al., 2009). Corroborating this hypothesis, in the present study the oral treatment with SP-LEB18 induced antinociception in the CFA model parallel to the reduction of the local levels of the proinflammatory cytokines TNF-α and IL-1β. *Spirulina*-mediated reduction of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, has also been observed by other authors, as shown in different inflammatory models, e.g. carrageenan-induced paw edema in rats (Joventino et al., 2012), CFA-induced arthritis (Ali, Barakat & Hassanet, 2015), and ulcerative colitis (Abdel-Daim, Farouk, Madkour & Azabet, 2015). TNF-α and IL-1β are key inflammatory cytokines released in the very early events following tissue injury (Oliveira, Sakata, Issy, Gerola, & Salomão, 2011). They have the ability to amplify inflammation by triggering the release of other mediators, such as prostanoids, kinins, and amines, thereby leading to the formation of the "inflammatory soup" and, consequently, the development of inflammatory hyperalgesia (Ferreira, Lorenzetti, Bristow, & Poole, 1988; Cunha, Poole, Lorenzetti, & Ferreira, 1992; Woolf et al., 1997; Vranken, 2012). Besides the well-known indirect actions of TNF-α and IL-1β in the genesis and maintenance of inflammatory pain, it has been proposed that these cytokines might as well trigger action potentials through the direct activation of the nociceptors during inflammatory conditions (Cook, Christensen, Tewari, McMahon, & Hamilton, 2018).

On the other hand, IL-10 is a major anti-inflammatory cytokine whose actions antagonize those of TNF-α and IL-1β (Poole, Cunha, Selkirk, Lorenzetti, & Ferreira, 1995; Cunha, Poole, Lorenzetti, Veiga, & Ferreira, 1999). Anti- and pro-inflammatory cytokines modulate each other's actions in a delicate balance that ultimately defines the magnitude of inflammation and ensures that it is self-limited (Poole et al., 1995; Cunha et al., 1999; Zhang & An, 2007). Remarkably, along with the downregulation of TNF-α and IL-1β, the increase of IL-10 levels promoted by SP-LEB18 was demonstrated in the present study. Corroborating this result, *Spirulina* biomass ameliorates lead acetate-induced inflammation in rats by increasing IL-10 levels (Khalil et al., 2018). This cytokine inhibits hyperalgesic responses (Poole et al., 1995), reverts experimentally-induced neuropathic pain (Milligan et al., 2005; Bao et al., 2014), and induces antinociceptive effect in many inflammatory painful syndromes.

Even though the ability of *Spirulina* biomass in modulating the expression of inflammatory mediators had been previously reported, there was no direct evidence supporting a mechanistic hypothesis for *Spirulina*-mediated attenuation of inflammatory pain. Considering the well-stablished role of IL-10 in modulating pain, it is possible to hypothesize that SP-LEB 18-induced antinociception results from increased IL-10. This hypothesis was here evaluated using knockout mice as a biotechnological tool. Importantly, both antiedematogenic and antinociceptive effects of SP-LEB18 during CFA-induced inflammation were abolished in IL-10 KO mice. The results reported herein strongly suggest that the probable mechanism of action of SP-LEB18 under inflammatory conditions is upregulating IL-10, which in turn reduces the local levels of the pronociceptive cytokines TNF-α and IL-1β.

Considering that SP-LEB18 has an intricate chemical composition (De-Jesus et al., 2018), it is possible that more than one of its constituents could contribute to its antinociceptive properties, meaning that multiple unrelated pathways might be involved. Interesting, in CFA-inoculated mice, the antinociceptive effect of SP-LEB18 persisted up to 8 h, at which point the antiedematogenic effect was no longer observed. This longer-lasting antinociception may reflect an intrinsic analgesic property of SP-LEB18, regardless of its anti-inflammatory effects. This hypothesis was corroborated by the results of the tail flick test, which allows the detection of centrally mediated analgesia. The tail flick test was originally described by D'Amour and Smith (1941), who have shown that opioid-treated rats present an increased latency time of the twitch reflex elicited by radiant heat applied to their tails. The tail flick has shown to be a spinal reflex that is inhibited by opioid drugs (Irwin, Houde, Bennett, Hendershot, & Seevers, 1951) and this test has been extensively used in the screening of drugs that promote central analgesia. In the present study, SP-LEB18 induced antinociception longer-lasting and as efficacious as morphine in the tail flick. These data provide the first experimental evidence of the centrally mediated antinociception induced by SP-LEB18. Importantly, in the tail flick test SP-LEB18 induced antinociceptive effect in wild type and IL-10 KO mice with similar efficacy. These results suggest that SP-LEB18 has an antinociceptive action centrally mediated and independent of IL-10, meaning that there should be a second mechanism of analgesia involved.

Because opioids are the major class of central analgesics, the influence of the opioid system on the antinociceptive effect of SP-LEB18 was next evaluated. The effect of pharmacological blockade of opioid receptors was evaluated in the tail flick test by using the non-selective opioid antagonist naloxone. The response of SP-LEB18-treated mice had a profile similar to the one exhibited by morphine-treated mice in the tail flick test; both responses were inhibited by naloxone, showing the involvement of opioid pathways in the antinociceptive effect of SP-LEB18. Drugs that modulate the opioid system are the most clinically relevant therapeutic agents currently used in severe painful conditions. Agonists of opioid receptors have been shown to occur in a variety of natural sources, such as plants (Gama et al., 2013), snake venoms (Santos et al., 2012), and even sea organisms (Johnson et al., 2017). This remarkable ubiquity of morphine-like substances reinforces the therapeutic potential of natural products in the pharmacological control of pain.

Phycocyanins and β-carotene stand out as potential analgesic substances found in *Spirulina* biomass because they have shown consistent anti-inflammatory properties under several conditions (Bai et al., 2015; Chen et al., 2012; Hwang et al., 2013; Shalaby & Shanab, 2013; Mallikarjun-Gouda, Udaya-Sankar, Sarada, & Ravishankar, 2015; Teng et al., 2016; Xia et al., 2016). However, further research is needed to determine whether those compounds are relevant for the intrinsic antinociceptive effect of SP-LEB18.

5. Conclusions

In the present work, SP-LEB18 displayed a dose-dependent anti-nociceptive action in experimental models of pain without altering the motor function of mice. Two distinct mechanisms were associated with this pharmacological effect. The first one is mediated by the over-expression of IL-10 and the following reduction of the pronociceptive cytokines TNF-α and IL-1β, therefore diminishing inflammatory pain. The second mechanism involves a pure analgesic activity of SP-LEB18 rather than a consequence of its anti-inflammatory properties. This action is centrally mediated and relies on the contribution of the opioid system. To the best of our knowledge, this is the first demonstration of the intrinsic analgesic property of *Spirulina* biomass.

S. platensis LEB18 can be cultivated in artificial lakes that simulate its natural habitat, making possible the production of huge amounts of biomass in a short period of time with a low-cost technology. Besides, *Spirulina* biomass-based products are already consumed in different regions of the world (Gantar & Svir£ev, 2008; Lu, Xiang, & Wen, 2011; Vardaka, Kormas, Katsiapi, Genitsaris, & Moustaka-Gouni, 2016; Furmaniak et al., 2017). Their use as dietary supplements is considered safe and is approved by the Food and Drug Administration (FDA). The double mechanism of action of SP-LEB18 described herein, i.e. increasing IL-10 levels and activating the opioid system, allows its application in different modalities of painful disorders. Such features would represent significant advantages for the clinical use of a SP-LEB18-based analgesic drug. However, there are many unknown *Spirulina* metabolites and potential impurities that could cause harm, so more research is needed to generate safe functional products. In addition, it is important to bear in mind the limitations of using an animal model to predict pharmacological properties in human, considering the pharmacokinetic and pharmacodynamic differences between species. Therefore, the analgesic properties of SP-LEB18 still need to be investigated in a clinical setting. Pharmacological and chemical studies with the aim of elucidate the mechanism(s) responsible for the anti-nociceptive action and to identify the active principles present in the SP-LEB18 are continuing.

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Ethical statement

Animal care and handling procedures were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH, 8023) and were approved by the Ethics Committee for Animal Experimentation of FIOCRUZ (reference number L-IGM025/2017).

CRediT authorship contribution statement

Dourivaldo Silva Santos: Data curation, Investigation. **Pedro Santana Sales Lauria:** Formal analysis, Writing - original draft. **Afrânio Ferreira Evangelista:** Investigation. **Francine Johansson Azeredo:** Investigation, Writing - review & editing. **Jorge Alberto Vieira Costa:** Resources, Methodology. **Milena Botelho Pereira Soares:** Resources, Writing - review & editing. **Janice Izabel Druzian:** Funding acquisition, Resources, Writing - review & editing. **Cristiane Flora Villarreal:** Conceptualization, Writing - original draft, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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