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Original Article

In vivo anti-inflammatory activity of M116, one extract obtained from the marine bacterium *Bacillus amyloliquefaciens*

[Actividad anti-inflamatoria *in vivo* de M116, un extracto obtenido de la bacteria marina *Bacillus amyloliquefaciens*]

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Abstract

Context: Marine organisms are sources of compounds with anti-inflammatory activity, many of them derived from the secondary metabolites produced by microorganisms.

Aims: To evaluate the possible anti-inflammatory effect of M116, an extract obtained by fermentation from the CBM-116 strain of the marine bacterium *Bacillus amyloliquefaciens*, which was isolated from sediments of the southern coast of the Cuban shelf.

Methods: The oral single and repeated different doses of the CBM-116 were evaluated for their ability to ameliorate edema using two *in vivo* experimental inflammation models: croton oil-induced atrial acute edema and cotton pellets-induced chronic granuloma, both in male Balb/c mice. The systemic production of redox biomarkers after repeated doses in the chronic inflammation model was also tested.

Results: A single application of M116 (50-200 mg/kg, 10 mL/kg, p.o.) decreases croton oil-induced acute inflammation in a dose-dependent manner. Single and repeated doses of extract (100-400 mg/kg, p.o.) also were able to inhibit chronic inflammation during both, transudative and proliferative phases of the inflammatory process. This effect was associated with the systemic reduction of oxidative stress.

Conclusions: M116 showed anti-inflammatory activity in the context of acute and chronic inflammation associated with its antioxidant mechanisms, which suggest the potential of the marine bacterium *Bacillus amyloliquefaciens* as a source of new products with biomedical application.

Keywords: anti-inflammatory activity; *Bacillus amyloliquefaciens*; marine microorganisms; inflammation.

Resumen

Contexto: Los productos naturales de origen marino han mostrado ser fuentes de compuestos con actividad anti-inflamatoria, muchos de los cuales provienen de microorganismos productores de metabolitos secundarios.

Objetivos: Evaluar la posible actividad anti-inflamatoria del extracto acuoso M116 de la bacteria marina *Bacillus amyloliquefaciens*.

Métodos: El extracto fue obtenido por fermentación a partir de la cepa CBM-116, aislada de los sedimentos de la costa sur de la plataforma cubana. La actividad anti-inflamatoria de M116 se evaluó en dos modelos experimentales: edema auricular inducido por aceite de crotón y granuloma inducido por gránulos de algodón en ratones jóvenes Balb/c machos.

Resultados: M116 (50-200 mg/kg, 10 mL/kg, p.o.) mostró acción anti-inflamatoria dependiente de la dosis en el modelo de edema auricular inducido por aceite de crotón. El extracto (100-400 mg/kg, p.o.) fue capaz de inhibir a dosis única la inflamación en el modelo de granuloma inducido por gránulos de algodón y de prevenir a dosis repetidas su formación. Los efectos fueron significativos en ambas fases transudativa y proliferativa, pero más robustos al inhibir la infiltración granulocítica de la fase crónica en este modelo y asociados a la reducción del estrés oxidativo sistémico.

Conclusiones: Estos resultados constituyen evidencias experimentales que avalan el perfil anti-inflamatorio del extracto acuoso M116 en condiciones de inflamación aguda y crónica, en relación con sus mecanismos antioxidantes. Lo cual sugiere las potencialidades de la bacteria marina *Bacillus amyloliquefaciens* como una valiosa fuente de nuevos productos con aplicación biomédica.

Palabras Clave: actividad anti-inflamatoria; *Bacillus amyloliquefaciens*; microorganismos marinos.

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INTRODUCTION

Inflammation is a tissue process with a defensive purpose against physical, chemical or biological aggressions to the organism, which occurs both in a physiological and pathological context. In particular, abnormal, excessive and chronic inflammatory response is involved in the development of diseases (Franceschi and Campisi, 2014; Mills et al., 2019). Microorganisms or tissue damage-induced inflammatory response trigger the release of pathogen-associated molecular patterns and damage-associated molecular patterns, respectively, which are recognized by tissue-resident immune cells such as macrophages and dendritic cells through receptors namely pattern recognition receptors (Ghosh and Hayden, 2008). Once activated, these immune cells produce chemoattractant molecules, a process controlled by the nuclear factor (NF)-κB, which positively regulates the expression of a wide variety of genes involved in mammalian immune and inflammatory responses. Comprising enzymes, such as cyclooxygenase-2 (COX-2) and nitric oxide synthase, cytokines/chemokines and their modulators, cell adhesion molecules, complement factors, growth factors, immunoreceptors, among others (Liu et al., 2017). Cytokines and chemokines released by these immune cells, along with formyl-peptide released by dying cells, activate vascular endothelial cells and provide a gradient of signals that guide neutrophils to the inflamed tissue following a spatial, temporal, and hierarchic cascade of mediators in the context of sterile or non-sterile inflammation (Fattori et al., 2016; McDonald et al., 2010). In the inflammatory foci, neutrophils release reactive oxygen and nitrogen species (ROS and RNS), pro-inflammatory cytokines, and pro-inflammatory lipid mediators, which ultimately induce cardinal local and systemic signs and symptoms of inflammation (Patil et al., 2019). During the progression of inflammation, COX-2 increases the production of prostaglandin (PG) E_2, which contributes to neuronal sensitization leading to inflammatory pain (Ferreira and Nakamura, 1979). In addition, growing evidence implicates a critical role of nitroxidative stress caused by the presence of superoxide (SO; $O_2^{\bullet -}$), nitric oxide (NO), and subsequently peroxynitrite (PN; $ONOO^-$) in acute and chronic inflammation (Salvemini et al., 2011).

On the other hand, available steroidal and non-steroidal anti-inflammatory drugs utilized as the first line in the treatment of inflammatory diseases are associated with significant adverse effects, including gastrointestinal, liver, renal, cardiovascular, and endocrine damage as well as drug-drug interactions (Bindu et al., 2020; Ramamoorthy and Cidlowski, 2016). Most of these drugs are contraindicated in elderly patients, who often suffer from inflammatory chronic diseases associated with other comorbidities and polypharmacy (Franceschi and Campisi, 2014). Therefore, the search for safe natural compounds with lower adverse effects has been a promissory strategy in growing.

To date, there is a long list of active marine natural products on specific molecular targets involved in the pathophysiology of diseases (Williams and Andersen, 2020), among which compounds with anti-inflammatory activity stand out (Souza et al., 2020). Many of these substances derive from microbial cultures, producers of secondary metabolites (Engel et al., 2002; Fenical and Jensen, 2006; Xiong et al., 2013). Despite some controversy, the diversity of marine microorganisms includes bacteria, viruses, archaea, protists, and fungi (Engel et al., 2002). It has been recognized that compared with terrestrial microorganisms, marine bacteria and fungi can produce more natural secondary metabolites with novel structures and high activities in the extreme conditions of life (Blunt et al., 2013; Ibrar et al., 2020). In particular, anti-inflammatory peptides, polyketides, and other substances have been obtained from marine microorganisms, which include phenolic compounds that act on molecular inflammatory pathways (Karthikeyan et al., 2022; Kondratyuk et al., 2012; Li et al., 2021).

A screening carried out that evaluated the bioactive capacity of extracts obtained from strains of the Collection of Marine Bacteria at the Institute of Marine Sciences (ICIMAR), Havana, Cuba, identified one from *Bacillus amyloliquefaciens* bacteria (named M116) with potential anti-inflammatory properties. Phytochemical study showed that phenolic compounds are the main presents in the M116 extract, as well as, proteins and carbohydrates are in lower concentrations (unpublished data), chemical entities with recognized anti-inflammatory properties (da Silva et al., 2017; Estrada et al., 2011; Kumar et al., 2019; Shao et al., 2019) Recently, the anti-inflammatory effects of an oral supplementation of *Bacillus amyloliquefaciens* in dextran sulfate-induced colitis in mice have been demonstrated (Cao et al., 2019). Evidence points to the fact that this organism could be a valuable source of new anti-inflammatory agents. Here, the *in vivo* anti-inflammatory effects of the M116 extract obtained from *Bacillus amyloliquefaciens* were evaluated under acute and chronic inflammatory experimental conditions, as well as its possible mechanistic link to oxidative stress.

MATERIAL AND METHODS

Microorganism

Bacillus amyloliquefaciens CBM-116 strain was isolated from the sediments of the southern coast of the Cuban shelf, deposited in Marine Bacteria Collection (batch 2018-116), ICIMAR, Havana, Cuba, and authenticated by Dr. Eudalys Ortiz, head of the ICIMAR Microbiology Department.

M116 extract from *Bacillus amyloliquefaciens*

The dry extract M116 was obtained from the filtration process of the fermented broth of the CBM-116 strain through membranes with a diameter of 45 mm and a pore size of 0.22 µm and concentrated to dryness by rotary evaporation at a reduced pressure of 1 atm at 60°C. Immediately before its use, M116 was dissolved in distilled water for oral administration in experiments.

Animals

All experiments were performed following the European Regulations on Animal Protection (Directive 86/609), the Declaration of Helsinki and/or the Guide for the Care and Use of Laboratory Animals, as implemented by the United States National Institutes of Health (NIH Publication number 85–23, revised 1996). The protocols were authorized by the Institutional Animal Experimentation and Ethics Committee from the ICIMAR, Havana, Cuba (Number: CE-ICIMAR-011/2019). Male Balb/c mice (8-10 weeks, weighing 18-20 g) were obtained from the Center for Experimental Animals Production (Havana, Cuba). The animals were acclimatized in the laboratory for at least 7 d before each experiment. They were housed in a temperature-controlled environment at 22.0 ± 0.5°C, 40%–60% relative humidity, a 12-hour light/dark cycle (light on from 07:00 to 19:00), and with food and water available ad libitum. The experiments took place during the light period. The animals in each treatment group (n = 6-10 per group) were tested randomly.

Croton oil-induced acute ear edema model

Edema was induced by a single topical application of a fresh solution of croton oil 4% in acetone (20 µL) on the inner surface of the right ear in mice. The same volume of vehicle (acetone) was dropped on the inner surface of the left ear (control). The mice were sacrificed by cervical dislocation five hours after the dropping of croton oil, and the bilateral ears were cut off. The central part of the ear was punched out using a puncher with a 6 mm diameter and weighted using an analytical balance to later calculate the difference between the weight of the inflamed (right) ear and the control (left) ear (Tubaro et al., 1986). The anti-inflammatory activity was expressed through the percentage (%) of edema inhibition with respect to the negative control group.

Experimental protocol

To evaluate the ability of different oral doses of M116 to inhibit ear edema induced by the croton oil, animals were randomly distributed in six groups (n = 10 per group): negative control that received distilled water (vehicle), four M116 treated groups (10, 50, 100 and 200 mg/kg) and a positive control group that received indomethacin (10 mg/kg). All treatments were administered 1 hour before the induction of atrial edema.

Cotton pellets-induced granuloma model

Implantation of cotton pellets

Cotton pellets weighing 10 mg were cut from dental rolls (Johnson and Johnson No. l-6") and were sterilized by autoclaving. Prior to implantation, each pellet was treated with 0.2 ml of antibiotic solution (10,000 units/mL penicillin and 10,000 pg/mL streptomycin). After carrying out asepsis and antisepsis of the operatory region, cotton pellets were implanted into male mice under anesthesia with thiopental (70 mg/kg, i.p.) and diazepam (5 mg/mL, i.p.). A small 1 cm incision was made on the skin along the dorsal median line, and the dermis was decollated from the underlying cellular subcutaneous tissue by the insertion of a blunt-ended pair of scissors. Two pellets were implanted bilaterally on the skin of the scapular regions, one on each side of the incision, which was closed with a stainless-steel clip (Bailey et al., 1982). Animals were kept under controlled conditions for 7 days to induce chronic systemic inflammation (Gou et al., 2017).

Sampling and extraction of granulomas

Under a short exposure to anesthesia with diethyl ether until loss of the righting reflex (75 s), which to have an insignificant effect on cytochrome P450 enzymes which are involved in oxidative stress (Garrido-Suárez et al. 2018), blood samples were taken from ocular plexus. Then serum was separated by centrifugation at 1500 *g* for 15 min at 4°C and stored at -20°C until its analysis. Immediately, the animals were euthanized by cervical dislocation yet under anesthesia, and the cotton pellets and the accompanying granulomatous tissue were removed from the mice with a pair of forceps by reflecting the overlying skin and removing the granuloma together with a small amount of any capsular material present. Initial-

ly, the granuloma was weighed to determine the wet weight. Afterward, each granuloma was usually placed in a glass Petri dish for drying at 37°C for 24 h in an oven. It was then weighed again to determine the dry weight.

The Transudative Weight (TW) = granuloma wet weight - granuloma dry weight was calculated as a measure of liquid content in the transudative phase of the inflammation. The granuloma-associated tissue (GAT) = dry weight – cotton pellet weight was determined (Antonisamy et al., 2019; Patil et l., 2019).

Experimental protocol

Mice were randomly divided into 8 groups (n = 6-7 per group). The negative control group received distilled water (vehicle), and the treatment groups received oral doses of 1, 10, 50, 100, 200, and 400 mg/kg of M116. The positive control group received dexamethasone (3 mg/kg, i.p.). Two experimental sets were carried out to test different dosing schedules. (1) In order to evaluate the anti-inflammatory effect of oral single doses of M116. In this experiment, the extract was administered 1 h before the sacrifice at 7 days after surgery. (2) To evaluate the effect of repeated doses of M116, the rest of animals received a daily dose of M116 for 7 days. In this experiment, the last dose also was administrated 1 h before the sacrifice.

Redox biomarkers

Serum nitric oxide (NO) concentration was measured according to Sastry et al. (2002). Total nitrate and nitrite (NO_3/NO_2) levels were determined using the Griess method (Miranda et al., 2001). Hydroperoxides (HPO) were determined according to Nouroozzadeh et al. (1994), with minor modifications. For that, one part of $FeSO_4$ $(NH_4)_2SO_4$ × $6H_2O$ solution and 96% H_2SO_4 was mixed with 100 parts of xylenol orange and sorbitol. Absorbance was determined at 560 nm. HPO concentration was determined using a standard curve of 30% hydrogen peroxide. Advanced oxidation protein products (AOPPs) were quantified according to Witko-Sarsat, with modifications (Witko-Sarsat et al., 1996). The transformation of iodine to diatomic iodine was followed by the change in optical density at 340 nm. Results were expressed as µM of chloramine T, used as standard.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed, along with calculations of the median-effective dose (ED_{50}) using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, United States). For compliance with homogeneity of variance, the Shapiro-Wilk test was used. Data from the acute inflammation model was analyzed using the Mann-Whitney test. Inter-group statistically significant differences were tested using analysis of variance (ANOVA) followed by Dunnett's *posthoc test* in the chronic inflammation model. $P<0.05$ was considered statistically significant. In all experiments, the percentage (%) of edema inhibition was calculated as % = (edema weight or granuloma weight control – edema weight or granuloma weight treated) / edema weight or granuloma weight control × 100. Where the edema weight = right ear weight - left ear weight. The granuloma weight corresponds to TW and GAT, respectively.

RESULTS

M116 inhibits acute inflammation in a model of croton oil-induced atrial edema in mice

M116 (50-200 mg/kg) produced a significant dose-dependent inhibition of the croton oil-induced acute inflammation in the ear of the animals (Table 1). The extract exerts a maximum inhibition (60.43 ± 6.11%) of atrial edema at 200 mg/kg, 5 h after the application of the irritant agent. As expected, indomethacin (10 mg/kg), a control nonsteroidal anti-inflammatory drug (NSAID), shows a significant anti-inflammatory effect (83.28 ± 1.85%).

M116 inhibits chronic inflammation cotton pellet-induced granuloma in Balb/c mice

Compared with the vehicle control group, M116 significantly decreased the TW of granuloma ($p<0.001$) using only the repeated schedule in mice at a 50 mg/kg dose. However, at higher doses (100-400 mg/kg), the reduction of TW was significant ($p<0.01$, $p<0.001$, Fig. 1A) for both dosing schedules (single and repeated doses). Compared with the vehicle-treated controls, M116 starting from 50 to 400 mg/kg dose significantly decreased the GAT ($p<0.01$, $p<0.001$, Fig. 1B) in both dosing schedules. The anti-inflammatory effect of M116 was as effective as dexamethasone, with no statistical difference between groups. The construction of the dose-response curves and the determination of the median-effective dose (ED_{50}) (TW: ED_{50} = 68.6 ± 6.1 mg/kg) and (GAT: 56.2 ± 5.3 mg/kg) show that single oral administration of M116 produced a dose-dependent reduction of liquid content and cell infiltration respectively from the granuloma (Fig. 2A). Meanwhile, the administration of repeated doses of M116 exhibited the higher levels of inhibition of the granuloma formation displaying lower values (TW: ED_{50} = 17.2 ± 4.8 mg/kg) and (GAT: ED_{50} = 11.2 ± 6.3 mg/kg) indicating its long-term anti-inflammatory effect (Fig. 2B).

Table 1. Effect of oral doses of M116, extract from the marine bacterium *Bacillus amyloliquefaciens*, on atrial edema induced by croton oil in Balb/c mice.

Treatment	Dose (mg/kg)	Edema weight (g)	Inhibition (%)
Control		8.17 ± 0.46	-
M116	10	7.95 ± 0.08	2.74 ± 0.96
	50	5.12 ± 0.51**	37.36 ± 6.21
	100	3.99 ± 0.62**	51.16 ± 7.60
	200	3.23 ± 0.50***	60.43 ± 6.11
Indomethacin	10	1.37 ± 0.16***	83.28 ± 1.91

Data are presented as the mean ± standard error of the mean (SEM), n = 10 animals per group. Animals were orally administered with a single dose of M116, 1 hour before induction of atrial edema. Edema was measured 5 h after croton oil application. Edema weight = right ear weight - left ear weight. Edema inhibition (%) = (edema weight control – edema weight treated) / edema weight control × 100. **p<0.01, ***p<0.001 indicates statistical differences compared with the negative control, Mann Whitney test.

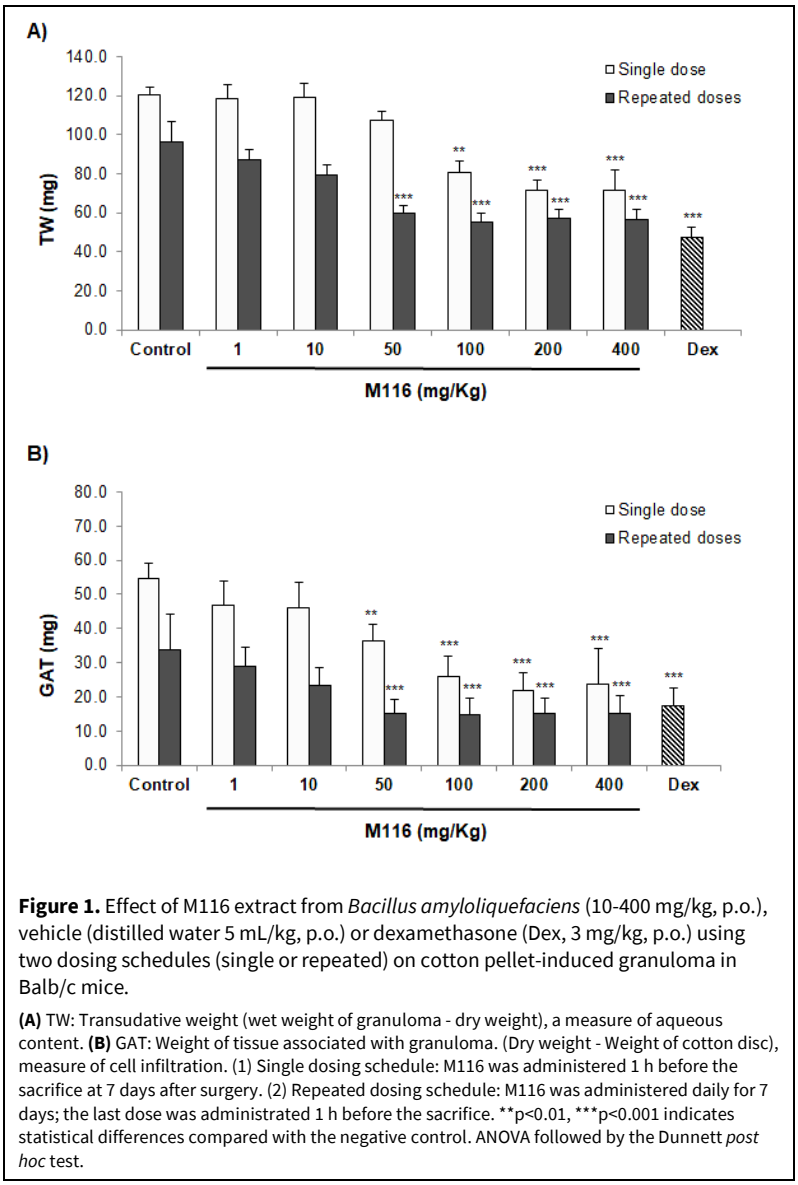

Figure 1. Effect of M116 extract from *Bacillus amyloliquefaciens* (10-400 mg/kg, p.o.), vehicle (distilled water 5 mL/kg, p.o.) or dexamethasone (Dex, 3 mg/kg, p.o.) using two dosing schedules (single or repeated) on cotton pellet-induced granuloma in Balb/c mice.

(A) TW: Transudative weight (wet weight of granuloma - dry weight), a measure of aqueous content. **(B)** GAT: Weight of tissue associated with granuloma. (Dry weight - Weight of cotton disc), measure of cell infiltration. (1) Single dosing schedule: M116 was administered 1 h before the sacrifice at 7 days after surgery. (2) Repeated dosing schedule: M116 was administered daily for 7 days; the last dose was administrated 1 h before the sacrifice. **p<0.01, ***p<0.001 indicates statistical differences compared with the negative control. ANOVA followed by the Dunnett *post hoc* test.

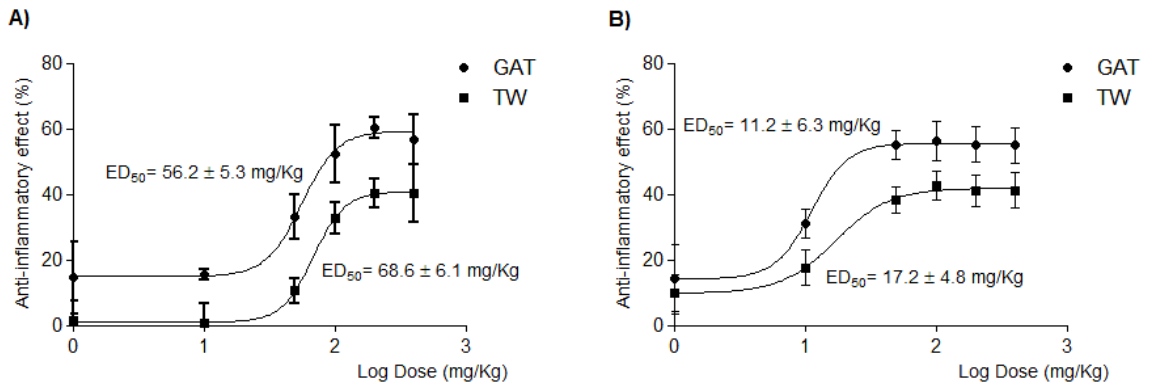

Figure 2. Dose-response curves of oral M116 extract on transudative weight (TW) and granuloma-associated tissue weight (GAT) in cotton pellet-induced chronic granuloma model in Balb/c mice.

(**A)** Single dosing schedule. (**B)** Repeated dosing schedule. ED_{50}, the medium effective dose was determined using GraphPrism 5 software. Values represent the mean ± SEM expressed as a function of the logarithm of the dose (mg/kg).

$$\% \text{ inhibition of inflammation } = \frac{(\text{Control} - \text{Treated})}{\text{Control}} \times 100$$

Table 2. Effect of repeated oral doses of M116 extract on systemic redox status related to cotton-pellets-induced granuloma in mice.

Treatment	Dose (mg/kg)	NO (µM/mgPr)	HPO (µM/mgPr)	AOPP (µM chloramine T/mgPr)
Vehicle (naive mice)		18.05 ± 2.12	48.45 ± 5.20	64.19 ± 4.56
Vehicle		5.4 ± 6.5[d]	150.3 ± 15.6[d]	121.1 ± 3.9[d]
M116	10	62.0 ± 11.1[d]	127.8 ± 15.6[d]	108.2 ± 6.4[d]
	100	61.0 ± 10.2[d]	81.6 ± 4.1[a,d]	80.9 ± 3.4[a,d]
	200	55.8 ± 7.6[d]	75.5 ± 4.9[a,c]	76.4 ± 5.4[a,c]
	400	30.0 ± 3.2[a]	64.2 ± 5.9[b]	72.4 ± 6.3[a]
Dexamethasone	3	26.0 ± 1.6[a]	63.3 ± 5.2[b]	36.1 ± 6.5[a,d]

The concentrations of NO, HPO, and AOPP were measured in serum blood samples from the ocular plexus before the euthanasia at 7 days after chronic inflammation induction. Each data point represents the mean ± standard error of the mean (SEM) of 6-7 animals, [a]p<0.01, [b]p<0.001, represent the statistical difference between treated groups and control group treated with vehicle, and [c]p<0.01, [d]p<0.001 represent the statistical difference between treated groups and naïve mice. Distilled water was used as the vehicle by the oral route. NO: nitric oxide, HPO: total hydroperoxides, AOPP: advanced oxidation protein product. One-way ANOVA, Dunnett *post hoc* test.

M116 inhibits systemic oxidative stress associated to cotton pellet-induced granuloma in Balb/c mice

The results summarized in Table 2 show that the serum production of nitrites as a surrogate marker of NO at 7 days after surgery was increased ($p<0.05$) in inflamed mice treated with a vehicle, compared with naive animals. In addition, lipid peroxidation measured as total HPO and a surrogate marker of protein damage (AOPP) were also increased ($p<0.05$) in inflamed mice with respect to naive ones. Compared with the negative control group, the animals treated with M116 at the highest dose evaluated (400 mg/kg) showed inhibition of NO concentration ($p<0.01$). Of note, M116 (100-400 mg/kg) also exerted a significant decrease in lipid and protein damage in a dose-dependent manner ($p<0.01$ and $p<0.001$, Table 2).

DISCUSSION

Oceans cover three-quarters of the earth's surface, and under them live the majority of the species on the planet. Evolution has permitted these organisms to develop unique metabolic and physiological capacities determined by the survival mechanisms (Donia and Hamann, 2003; Engel et al., 2002). One of them involves the production of substances exerting effects on other species, which has favored that the oceans have been explored to obtain novel bioactive molecules (Blunt et al., 2013; Mayer et al., 2017). In this way, its potential to obtain new anti-inflammatory drugs has been recognized (Fernando et al., 2016; Kondratyuk et al., 2012; Shin et al., 2016; Wei et al., 2013). This work supports the anti-inflammatory effects of a new extract obtained by fermentative route from the culture of *Bacillus amyloliquefaciens* bacterium

isolated from the sediments of the south coast of the Cuban platform.

It was found that a single oral dose ranging from 50 to 200 mg/kg of M116 was able to reduce acute inflammation induced by croton oil in the ear, a murine model of acute dermatitis. A significant decrease in edema weight (37.36%) and a reduction of the inflammation (nearly ~60%) at the superior dose evaluated are outcomes consistent with M116 anti-inflammatory effect. This model is one of the most widely used *in vivo* assays for screening novel anti-inflammatories (Lucca et al., 2018; Sindhu et al., 2017; Tubaro et al., 1986). It is particularly an appropriate model for the evaluation of these agents by a topical route. However, also provide significant reproducible results using the oral route (Antonisamy et al., 2019; Pinto et al., 2015). In congruence with the expected clinical route of administration to treat inflammatory diseases and the frequent oral intake of natural products, we performed these experiments using the same route (Butterweck and Nahrstedt, 2012). Croton oil topically applied triggers the typical phases of acute inflammation (vasodilation-erythema, extravasation-edema, and neutrophil infiltration with maximum inflammation) (Patil et al., 2019; Saraiva et al., 2011), and its pro-inflammatory mechanisms have been well characterized (Tubaro et al., 1986). 12-O-tetradecanoyl-phorbol-13-acetate (TPA), the main irritant contained in croton oil, activates protein kinase C, and the mitogen-activated protein kinases (MAPKs), which activates NF-κB and the activating protein-1(AP-1) (Garg et al., 2008). These factors modulate gene transcription of pro-inflammatory cytokines, including interleukin 1-β, tumor necrosis factor-α, and interleukin-6, cell-surface receptors as platelet-activating factor receptor 1, and other stress response genes such as phospholipase-A_2, (PLA_2) implicated in fatty acid metabolism. These signaling pathways induce mast cell degranulation releasing histamine and serotonin to facilitate vasodilation and vascular permeability (Pinto et al., 2015). The neutrophil migration during the inflammatory response results mainly from the release by resident cells of neutrophil chemoattractant factors, which induce the rolling and adhesion of neutrophils on endothelial cells, followed by their transmigration to the extravascular space (Dal Secco et al., 2006). In particular, the levels of eicosanoids synthesized by cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzymes are pivotal in the onset of acute inflammation induced by croton oil (Prasher et al., 2019; Saraiva et al., 2011). As several different inflammatory pathways are involved in this model, some inflammatory drugs with distinct mechanisms of action, such as NSAIDs and melatonin, may inhibit its progression (Barbosa et al., 2017; Pinto et al., 2015; Sangchart et al., 2021). Hence, the present results identify the significant anti-inflammatory activity of M116 in an integrated biological system evaluating its dose-response, but speculating on a possible mechanism of action is not possible. Nevertheless, metabolites from other species of the genus Bacillus have recognized anti-inflammatory effects attributed to their ability to inhibit LOX and COX enzyme activities (Abdel-Wahab et al., 2022). Thus, the findings of the present study, together with previous observations about the genus, suggest that M116 could inhibit acute inflammation through the modulation of these enzymes, which must be corroborated in further investigations.

On the other hand, M116 shows a significant interference in the instauration of transudate and proliferative elements of the granulomatous tissue formation, which constituted a hallmark of a chronic inflammatory process. Granuloma formed over a 7-day period is a tissue mass formed during the repair process of inflammation characterized by a capsule containing fibroblasts and mostly infiltrating mononuclear cells, angiogenesis, and exudation (Patil et al., 2019). Inflammatory chemoattractant factors such as cytokines, chemokines, complement system, and eicosanoids are involved in the formation of granuloma (Antonisamy et al., 2019; Bailey et al., 1982; Kamei et al., 2004). M116 decreases the levels of TW, suggesting its possible modulation of the vasodilation and vascular permeability implicated in plasma extravasation and edema formation from the early phase of the granuloma development. In addition, the decrease of GAT levels by M116 could also suggest the inhibition of the cellular infiltration into the granuloma that took place at a virtually constant rate over a 7-day period (Bailey et al., 1982). Consequently, M116 could reduce vascularized fibrogranular tissue formed during this late phase. Meanwhile, the dose-response curves showed that the inhibitory effects of M116 are superior in a repeated doses schedule. Interestingly, M116 was as effective as dexamethasone in decreasing GAT. Previous evidence suggests that steroids act during the early proliferative phase (0-3 days) of granuloma development mediated by neutrophils, whereas treatment on days 4-7 is ineffective in infiltrating mononuclear cells (Bailey et al., 1982). Steroids elicit their actions mainly by means of the classic glucocorticoid receptor (GR). Most of the anti-inflammatory effects of glucocorticoids appear to result from a negative regulatory mechanism of transrepression of NF-κB and AP-1 inhibition. However, rapid, non-genomic action through the cytosolic GR also liberates accessory proteins that participate in secondary signaling cascades, such as the inhibition of PLA_2 activity impairing the release of arachidonic acid (AA) and phosphorylation of annexin-A1 (Ramamoorthy and Cidlowski, 2016). Annexin-A1

inhibits neutrophils growth and blocks leukocyte migration across the inflamed endothelium, thereby decreasing exudation during granular tissue formation (Kadmiel and Cidlowski, 2013; Ramamoorthy and Cidlowski, 2016). A mode of action that should focus on testing for M116 in future works, considering that steroid compounds isolated from marine microorganisms with antimicrobial and cytotoxic bioactivities have been reported (Dos Santos Dias et al., 2019). Particularly, cholic acid derivatives have been recently isolated from the *Bacillus amyloliquefaciens* (Dobson et al., 2018).

Cytokines produced by various cell types in response to a variety of stimuli constitute a link between cellular injury and the development of local signs and symptoms (pain, redness, swelling) and systemic symptoms of inflammation, such as fever or sickness behaviors syndrome (Kelley and Kent, 2020). Along with this, oxidative stress is closely implicated in the onset and progression of inflammation since the free radicals cause damage that promotes the release of pro-inflammatory mediators, increases the activity and recruitment of cells of the immune system, supporting the inflammatory response (Biswas, 2016; Reuter et al., 2010). An imbalance between oxidant/antioxidant mechanisms has also been observed in models of inflammatory nociception (Salvemini et al., 2011). Here we observed that M116, from doses of 100 mg/kg, reduced lipid and protein damage in treated animals and NO concentration using higher doses. Reports of anti-inflammatory evaluation of another marine extract from *Turbinaria ornata* algae showed that the algae effects were associated with the inhibition of both phases of inflammation and the reduction of oxidative stress (Subash et al., 2016).

Phytochemical screening showed the presence of phenolic compounds in M116, structures, which inhibit croton oil-induced inflammation (Estrada et al., 2011). Phenolic compounds can interact with enzymes of AA metabolism, inhibit the histamine release, the cell migration, and protect the vascular endothelium, with decreased exudation (Kumar et al., 2019). Polysaccharides isolated from marine sources, such as, whose isolated of the brown algae *Turbinaria ornata* (Ananthi et al., 2010) and structures found in the extract exhibit anti-inflammatory activity. Thus, this family of compounds joined to the polyphenolics could be responsible for the anti-inflammatory effects observed for the M116 extract.

In particular, only one study has reported the anti-inflammatory effects of oral supplementation of *Bacillus amyloliquefaciens* in dextran sulfate-induced colitis in mice. The study reveals an improvement in colonic tissue morphology, a decrease in immunoglobulin levels, and the inhibition of pro-inflammatory cytokines in the treated animals (Cao et al., 2019). This report is consistent with the present results across M116 and supports these species' potential as a source of new anti-inflammatories.

CONCLUSION

M116 extract obtained from Cuban marine platform *B. amyloliquefaciens* CBM-116 strain has *in vivo* anti-inflammatory activity in acute and chronic conditions, associated with its antioxidant mechanism. Thus, this marine microorganism could be a promissory source for obtaining new anti-inflammatory molecules.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Hernández-Balmaseda I	López F	Cuellar C	Garrido-Suárez BB	Ortíz E	Fernández MD	Rodeiro I
Concepts or ideas	x						x
Design	x						x
Definition of intellectual content	x			x	x	x	x
Literature search	x	x		x		x	x
Experimental studies	x	x	x				
Data acquisition	x	x	x				
Data analysis	x	x	x				x
Statistical analysis	x	x	x				
Manuscript preparation	x	x		x	x	x	x
Manuscript editing	x	x		x			x
Manuscript review	x	x	x	x	x	x	x

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