



Jacareubin inhibits FcεRI-induced extracellular calcium entry and production of reactive oxygen species required for anaphylactic degranulation of mast cells

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ABSTRACT

Mast cells (MCs) are important effectors in allergic reactions since they produce a number of pre-formed and *de novo* synthesized pro-inflammatory compounds in response to the high affinity IgE receptor (FcεRI) crosslinking. IgE/Antigen-dependent degranulation and cytokine synthesis in MCs have been recognized as relevant pharmacological targets for the control of deleterious inflammatory reactions. Despite the relevance of allergic diseases worldwide, efficient pharmacological control of mast cell degranulation has been elusive. In this work, the xanthone jacareubin was isolated from the heartwood of the tropical tree *Calophyllum brasiliense*, and its tridimensional structure was determined for the first time by X-ray diffraction. Also, its effects on the main activation parameters of bone marrow-derived mast cells (BMMCs) were evaluated. Jacareubin inhibited IgE/Ag-induced degranulation in a dose-response manner with an IC₅₀ = 46 nM. It also blocked extracellular calcium influx triggered by IgE/Ag complexes and by the SERCA ATPase inhibitor thapsigargin (Thap). Inhibition of calcium entry correlated with a blockage on the reactive oxygen species (ROS) accumulation. Antioxidant capacity of jacareubin was higher than the showed by α-tocopherol and caffeic acid, but similar to trolox. Jacareubin shown inhibitory actions on xanthine oxidase, but not on NADPH oxidase (NOX) activities. *In vivo*, jacareubin inhibited passive anaphylactic reactions and TPA-induced edema in mice. Our data demonstrate that jacareubin is a potent natural compound able to inhibit anaphylactic degranulation in mast cells by blunting FcεRI-induced calcium flux needed for secretion of granule content, and suggest that xanthenes could be efficient anti-oxidant, antiallergic, and antiinflammatory molecules.

1. Introduction

Allergies constitute an important public health problem in the world, around 40% of people have some type of allergy manifested as allergic rhinitis (10–30% adults), food allergy (8% children), animal allergy (15% cats and 10% dogs [1,2]), although that an effective treatment for those conditions has been elusive [3]. Current treatments are directed to palliate the symptoms caused by the inflammatory state that occurs in the development of the allergic episode [4], but little progress has been made in preventing the inflammatory condition

derived of the production of pro-inflammatory mediators. Evidence indicates that allergies are positively associated with the development of multiple chronic-degenerative diseases, such as atopy [5], chronic-obstructive pulmonary disease (COPD) [6], chronic asthma and some types of cancer [7,8].

Mast cells (MCs) are the main cell type responsible for the acute inflammatory reaction that occurs during an episode of type I hypersensitivity [9]. Allergens (antigens) present in the environment are recognized by IgE antibodies that are bound to the high affinity IgE receptor (FcεRI) present in the membrane of the MCs [10,11]. This

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leads to the activation of a complex signaling cascade that induce the rapid exocytosis of pre-formed granule compounds (anaphylactic degranulation), synthesis of lipid-derived mediators and long-term production of cytokines with pro-inflammatory activity. After FcεRI crosslinking, the protein tyrosine kinases Lyn and Fyn become activated and mediate the phosphorylation of the β chain of the FcεRI receptor. This creates docking sites for the protein kinase Syk, which associates to the phosphorylated beta chain and is activated by autophosphorylation and Lyn-dependent phosphorylation [12]. Activated Syk results in the phosphorylation of the adapter proteins GAB2 and LAT that disseminate the activation signal by binding different downstream effectors. Phosphorylated GAB2 mediates the activation of the phosphatidylinositol 3 kinase (PI3K), which promotes the membrane accumulation of PIP₃ and the initiation of cytoskeletal re-arrangements for granule secretion, whereas phosphorylation of LAT leads to the activation of phospholipase C (PLCγ) that catalyzes the production of diacylglycerol (DAG) and phosphatidylinositol-3-phosphate (IP₃) for the release of calcium from intracellular stores. Emptiness of intracellular calcium stores lead to the aggregation of the calcium sensor STIM1 in the endoplasmic reticulum and its association of Orai and TRPC calcium channels in plasma membrane to replenishment of intracellular calcium stores in what is called the store operated calcium entry (SOCE) [13–16]. FcεRI crosslinking also causes a rapid increase on the concentration of reactive oxygen species (ROS) produced by NADPH oxidase [17–19] by a mechanism not well defined yet.

The complexity of the FcεRI signaling system and the lack of detailed knowledge regarding the mechanisms that induce selective mediator production in mast cells, have complicated the development of effective therapeutics for allergic diseases and for inflammatory reactions that depend on mast cells. However, natural polyphenol compounds have been emerged as an important group of chemical agents with the capacity of mast cell inhibition [20–24]. Among the polyphenols isolated from plants with anti-allergic activity recently studied are: A) Flavonoids, including Luteolin and its analogues [25–28], quercetin [25,29], epigallocatechin-3-O-gallate [30]; B) Coumarins such as scoparone, thunberginols A and B [31,32]; C) Sesquiterpene derivatives such deacetyeupaserrin [33]; D) Xanthenes, that are present in mangosteen and mango fruits show potent anti-inflammatory activity in murine mast cells, being nujiangexanthone A, followed by α-, γ-, and β-mangostins and finally mangiferin. Most of these compounds inhibit Syk protein [34–38].

Jacareubin and other xanthenes are found in the heartwood of the American tropical tree *Calophyllum brasiliense* (Clusiaceae) where it has been isolated in good yields. Jacareubin has fungistatic and bacteriostatic activity [39,40], and good cytotoxicity activity against KB and HeLa cell lines *in vitro* [41] moderate inhibitory activity of gastric H⁺, K⁺-ATPase [42]. In this work we tested the potential antiallergic effect of the jacareubin in a widely-used cellular model of mast cell function (bone marrow-derived mast cells, BMMCs) and also analyzed its effects on *in vivo* models of inflammation. In both systems, jacareubin showed a potent inhibitory activity on MC degranulation and inflammation.

2. Materials and methods

2.1. Reagents

Anti-dinitrophenyl monoclonal immunoglobulin type E (IgE), 2',7'-dichlorofluorescein diacetate (DCF-DA), dinitrophenyl-human serum albumin (DNP-HSA), Evans blue dye, hydrogen peroxide (H₂O₂), quercetin, 12-O-tetradecanoylphorbol-13-acetate (TPA), hexadecyltrimethyl-ammonium bromide (HTAB), N,N-dimethylformamide (DMF), Igepal, phosphate-buffered saline (PBS), α-tocopherol, caffeic acid, 3,3',5,5'-tetramethyl-benzidine (TMB), dihydroethidium (DHE), Trizma base, HCl, triton X-100, EDTA, NaF, EGTA, phenylmethanesulfonyl fluoride, deoxyribonucleic acid, single stranded from salmon testes, nicotinamide adenine dinucleotide (NADPH), HEPES,

diphenyleneiodonium chloride (DPI), nitroterazolium blue (NBT), Na₂CO₃, quercetin, and indomethacin were purchased to Sigma-Aldrich (St. Louis, MO, USA), antioxidant trolox by Calbiochem (San Diego, CA, USA), FURA-2AM by Thermo Fisher Scientific (Waltham, MA, USA), ketamine and xilazine by PISA agropecuaria (Hgo, México), all organic solvents were purchased with J. T. Baker (Center Valley, PA, USA) and jacareubin was purified that described below.

2.2. Collection of plant material and purification of jacareubin

Calophyllum brasiliense Cambess (Clusiaceae) tree was collected by J.I. Calzada from the Selva Lancandona, Chiapas, Mexico (Bárceñas, 1995 [43]). Identification was done by the collector, a voucher (JIC-3116), and wood sample (00011-XALw) are deposited in the Herbarium and Xylarium “Dr. Faustino Miranda”, both from the Instituto de Ecología, A.C., at Xalapa, Veracruz, México.

2.3. Purification and solubilization of Jacareubin

A wood board of *C. brasiliense* kept at room temperature was processed with an edger to obtain drillings. The heartwood shavings (711.5 g) were extracted with methanol at room temperature and the solvent was removed by distillation with a rotary evaporator in vacuum. The dried extract 74 g was subjected to column chromatography silica gel 60, 1 Kg (Merck; Darmstadt, Germany) eluting with hexane–ethyl acetate (7:3). Analysis of fractions was performed by thin layer chromatography using silica gel 60, layer plates G/UV254, 0.20 mm (Macherey-Nagel; Düren, Germany), plates were observed under ultraviolet light 254, 366 nm, and after spraying them with a solution of 1% ceric sulfate in 1 N sulfuric acid.

Fractions 138–227 afforded a mixture of three xanthenes (9.85 g), a 6 g sample was subjected to column chromatography (1 m high and 5 cm diameter) using 120 g of silica gel 70/230 (Macherey-Nagel; Düren, Germany) to purify the jacareubin with an initial mobile phase of hexane and increasing polarity with ethyl acetate. Fractions eluted with solvent mixture 87:13 afforded jacareubin after recrystallization with acetone.

Jacareubin was obtained as yellow cubic and rectangular crystalline prisms of 1–5 mm long, melting point 260–270 °C with Fisher Johns Apparatus (Scorpion Scientific; Illinois, USA), reported 256–257 °C with decomposition. Nuclear Magnetic Resonance Spectra were recorded with a Bruker AVANCE III HD 700 MHz (Cambridge Isotope Laboratories, MA, USA). ¹H NMR (700 MHz, (CD₃)₂CO) δ 13.55 (1H, s, 1"-OH), 8.94 (2H, s, 5" and 6"-OH), 7.62 (1H, d, 8-H), 6.99 (1H, d, 1'-H), 6.68 (1H, d, 7-H), 6.33 (1H, s, 4-H), 5.73 (1H, d, 2'-H), 1.47 (6H, s, 4'-CH₃, 5'-CH₃) and ¹³C NMR (175 MHz, (CD₃)₂CO) δ 181.30 (C-9), 161.08 (C-1), 158.67 (C-3), 157.85 (C-4a), 152.25 (C-6), 146.87 (C-5a), 133.23 (C-5), 128.65 (C-2'), 117.47 (C-8), 115.79 (C-7), 114.71 (C-8a), 113.83 (C-1'), 105.21 (C-2), 103.55 (C-9a), 95.52 (C-4), 78.96 (C-3') and 28.48 (C-4' and C-5'). Mass spectra (DART) *m/z* (%): 327 M⁺ + 1 (100); 326 (7) M⁺, recorded in a AccuTOF JMS-T100LC (Tokyo, Japan) using ionization mode Direct Analysis in Real Time.

The spectral purity of the tested jacareubin was determined by High Performance Liquid Chromatography (HPLC) coupled to UV–Vis Photo Diode Array Detector, or to Mass Spectrometer Bruker Esquire 6000 (Bruker; Billerica, MA, USA). In both cases a peak with retention time 15.6–16 min was observed. HPLC was performed with an Agilent 1200 Series Binary SL (Agilent; Santa Clara, CA, USA) using a C-18 Silica Gel column 3.5 μm 100 × 2.1 mm Eclipse Plus (Agilent; Santa Clara, CA, USA), mobile phase: CH₃CN/H₂O (7:3) with CH₃COOH 0.1%. Detector: Waters 2996 UV–Vis Photo Diode Array (Waters; Mildford, MA, USA). Detector: Mass Spectrometer Bruker Esquire 6000 (Bruker; Billerica, MA, USA).

2.4. Identification of the three-dimensional structure of jacareubin by X-ray diffraction

A yellow prism of jacareubin, was selected and mounted on glass fiber, then placed on a Bruker D8 venture diffractometer (Bruker; Billerica, MA, USA) with Cu-target micro-focus X-ray source ($\lambda = 1.54178 \text{ \AA}$). The detector was placed at 50 mm from the crystal. Frames were collected with a scan width of 0.3° in the ω scan and the exposure time of 10 sec/frame at 150 K. Frames were integrated with the Bruker SAINT software package (Bruker; Billerica, MA, USA) using a narrow-frame integration algorithm. Systematic absences and intensity statistics were used in monoclinic space group. The structure was solved using Patterson methods using SHELXS-2014/7 program [44]. The remaining atoms were located via a few cycles of least squares refinements and difference Fourier maps. Hydrogen atoms were input at calculated positions, and allowed to ride on the atoms to which they are attached. Thermal parameters were refined for hydrogen atoms on the phenyl groups using a $U_{eq} = 1.2 \text{ \AA}^2$ and a $U_{eq} = 1.5 \text{ \AA}^2$ for methyl groups to precedent atom in all cases. For all complexes, the final cycle of refinement was carried out on all non-zero data using SHELXL-2014/7 [44]. Absorption correction was applied using SADABS program. Supplementary data for jacareubin has been deposited at the Cambridge Crystallographic Data Centre. Copies of this information are available free of charge upon request from The Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336033, email: deposit@ccdc.ac.uk or internet address <http://www.ccd.cam.ac.uk>) quoting the deposition number CCDC 1845875.

2.5. Animals

C57BL/6J male mice (25–30 g body weight), stock number 000664, were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). Mice were kept in sterile conditions (12-h dark/light cycles) with free access to water and food at the Unit for Production and Experimentation of Laboratory Animals (UPEAL) from Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional (Cinvestav) in Mexico City. All animal procedures were approved by the Cinvestav Institutional Ethics Committee (protocol 074-13).

2.6. BMMC isolation and culture

BMMCs were produced from C57BL/6J mice, as described [45]. Briefly, bone marrow was extracted from the tibias and femurs of mice and cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) medium supplemented with IL-3 (10 ng/ml; PeproTech, Rocky Hill, NJ, USA) and 10% FBS, 100 UI/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-ME, and 13 nonessential amino acids (Invitrogen, Carlsbad, CA, USA). Cultures were maintained for 4 week, and media were changed every 5–7 days. Subsequently, BMMC differentiation was assessed by detecting the expression of the Fc ϵ RI receptor on plasma membrane by flow cytometry and evaluating the release of β -hexosaminidase after IgE/Ag stimulation [46]. Only cultures 98% positive for Fc ϵ RI receptor expression and showing a dose response of β -hexosaminidase release after IgE/Ag addition were used in the study. For each experiment, BMMCs were sensitized with 100 ng/mL a monoclonal anti-DNP IgE (clone SPE-7; Sigma-Aldrich, St. Louis, MO, USA) for 24 h at 37°C . Routinely, after sensitization, cells were collected and resuspended in fresh culture medium or Tyrode's BSA buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2 , 5.6 mM glucose, 0.5 g/l BSA, and 20 mM HEPES pH 7.4) for cytotoxicity and calcium experiments. For ROS production experiments, sensitized cells were washed and resuspended in Tyrodés Buffer without BSA.

2.7. Solubilization of jacareubin for experiments with isolated cells

For *in vitro* testing of the effects of jacareubin on mast cells, the

compound was dissolved in 0.1% ethanol which was used as vehicle in all experiments.

2.8. Cytotoxicity assay

We used the colorimetric assay for the quantification of cell death and cell lysis Cytotoxicity Detection Kit (LDH, Roche Cat. No. 11 644 793 001 (Roche; Mannheim, Germany), following the supplier's instructions we used the BMMC Cell-free culture supernatant pretreated for 15 min with six concentrations of jacareubin from 300 pM to 30 μM .

2.9. Determination of β -hexosaminidase activity

MC degranulation was tested by measuring the activity of β -hexosaminidase in cell supernatants as described [47]. Briefly, two million of IgE-sensitized cells were centrifuged at $500\times g$ during 5 min and suspended in 1 mL Tyrode's/BSA buffer. Independent groups of cells were treated with vehicle, 0.003, 0.03, 0.3, 3, or 30 μM jacareubin for 15 min and then stimulated with antigen (1, 3, 9, or 27 ng/mL DNP-HSA) during 30 min at 37°C . After this treatment, cells were placed on ice for 2 min and centrifuged to $12,000\times g$ for 10 min at 4°C . Sixty microliters of supernatant (Triton-treated cell pellet) were placed in a 96-well plate containing 40 μL of 1 mM p-nitrophenyl-N-acetyl- β -D-glucosaminide (P-NAG) (Sigma-Aldrich, St. Louis, MO, USA), and incubated for one hour at 37°C before the addition of 120 μL of "stop" solution (Na_2CO_3 0.1 M/ Na_2HCO_3 0.1 M). β -hexosaminidase release was quantified by spectrophotometry in a microplate reader (Tecan Sunrise; Männedorf, Switzerland) at 405 nm, as described [48].

2.10. Determination of intracellular calcium concentration ($[\text{Ca}^{2+}]_i$)

Intracellular calcium concentrations $[\text{Ca}^{2+}]_i$ were measured in IgE-sensitized BMMCs subjected to distinct treatments. To do this, ten million cells were collected and suspended in Tyrode's/BSA buffer in the presence of 5 μM Fura 2-AM for 30 min at 37°C to allowing the loading of the cells. Fura 2-AM-loaded BMMCs were then suspended in 2 mL Tyrode's/BSA buffer and placed in the cuvette. Fluorescence at 510 nm after excitation at 340 nm was determined in intervals of 1.16 s using a spectrofluorometer (Fluoromax 3 Jobin Yvon, Horiba, Japan). Calcium concentration was calculated using the equation and parameters described by Grynkiewicz [49]. In a standard experiment, basal fluorescence was recorded during 100 s prior to the addition of the antigen DNP-HSA (27 ng/mL) or Thapsigargin (1 μM). In some protocols, we used BMMCs suspended in Ca^{2+} -free Tyrode's/BSA buffer and then added 1.8 mM CaCl_2 (final concentration) at 200 s to determine extracellular calcium entry.

2.11. ROS production and antioxidant activity

Two million of sensitized BMMC were collected and resuspended in 1 mL buffer Tyrode's without BSA in 1.5 mL flat top eppendorf tubes for each condition. Pre-incubation with vehicle, jacareubin or the antioxidant Trolox was performed for 15 min at 37°C and then DCF-DA 10 μM was added for 15 min, after cells were stimulated with antigen (27 ng/mL of DNP-HSA) for another 15 min. Once finished the reaction, the tubes were centrifuged at $750\times g$ at 4°C for 5 min, the supernatant was discarded and 300 μL of Igepal 0.1% at 37°C were added, pipetting vigorously to break the cell pellet. Tubes were then centrifuged at $12,000\times g$ for 5 min at 4°C and 200 μL of the supernatant were placed in a 96-well plate. Fluorescence was determined by exciting at 488 nm and detecting emission at 565 nm in a BioTek Microplate luminometer, model FLx800 (BioTek; Winooski, VT, USA) for 1 h, taking readings every 15 min (modified method) [29], values shown are the average of the five measurements made.

2.12. NADPH oxidase activity

NADPH oxidase activity was assessed by the fluorogenic oxidation of DHE to ethidium (Eth) by superoxide [50]. In brief, 1×10^6 BMMCs were resuspended by pipetting in 40 μ L ice-cold lysis buffer (20 mM tris HCl pH 7.4, 1% triton X-100, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM, EGTA and 1 mM phenylmethanesulfonyl fluoride) and subjected to three freeze/thaw cycles using liquid nitrogen and ice, respectively. Lysates were incubated with DHE (0.02 mmol/L), salmon testes DNA (0.5 mg/mL) and NADPH (0.2 mmol/L) in HEPES buffer (25 mmol/L HEPES, and 1 mmol/L EDTA) in presence or absence of 0.3, 3 and 30 μ M of Jacareubin. The NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI; 0.1 mmol/L), was used to confirm that NADPH oxidase was the source of superoxide. Eth-DNA fluorescence was measured at an excitation of 480 nm and emission of 610 nm for 2.5 h at 37 °C using a fluorescence microplate reader (Synergy HT multi-mode reader, Biotek, Winooski, VT, USA).

2.13. Xanthine oxidase activity

Oxidation of DHE by superoxide produced by xanthine oxidase (0.1 mU) was tested in a master mix containing xanthine (0.122 mM), EDTA (0.122 mM), DNA (0.5 mg/mL), nitrotriazolium blue (NBT, 30.6 μ M), Na_2CO_3 (49 mM) [51]. Eth-DNA fluorescence was measured at an excitation of 480 nm and emission of 610 nm for 2.5 h at 37 °C using a fluorescence microplate reader (Synergy HT multi-mode reader, Biotek, Winooski, VT, USA). When needed, jacareubin resuspended in ethanol was added immediately before the addition of the enzyme. Vehicle (ethanol) *per se* did not modify the activity of the enzyme.

2.14. Passive cutaneous anaphylaxis model (PCA)

Each mouse was anesthetized with 17 μ L ketamine and 1.1 μ L of xilazine dissolved in 160 μ L of saline solution by intraperitoneal (i.p.) injection. For IgE sensitization, 5 μ L 100 ng of monoclonal anti-DNP IgE were administered in the left ear and 5 μ L saline were injected in the right ear (control ear). Twenty four hours after sensitization, animals were challenged with 100 μ L 200 μ g of DNP-HSA antigen dissolved in 1% Evans Blue dye, administered through the tail vein [28,52]. Vehicle or different doses of jacareubin were administered intraperitoneally to each mouse 2 h before challenge with antigen. Once the mouse was exposed to the antigen, the mice were allowed to rest with free access to food and water for 30 min. Mice were sacrificed using a CO_2 chamber, and similar portions of the ears of each mouse were cut, weighed and incubated for 12 h at 65 °C with 300 μ L of N, N Dimethylformamide to extract all Evans blue dye from the tissue of the ear. Two hundred microliters of each sample was taken and read in a spectrophotometer VersaMax ROM v.2.14 (Molecular Devices; San Jose, CA, USA) at 620 nm.

2.15. TPA-induced edema model in mice ear

The TPA-induced ear edema assay in mice Swiss Webster (Charles River, Wilmington, MA, USA), was performed as previously reported [53] with some modifications. A solution of TPA (2.5 μ g) in ethanol (10 μ L) was applied topically to both faces (5 μ L each ear) of the right ear of the mice. After 10 min, solutions of the tested substances dissolved in ethanol-acetone 1:1 were applied (10 μ L in each face of the ear). The left ear received ethanol (10 μ L) first, and 20 μ L of the ethanol-acetone mixture subsequently. Four hours later mice were sacrificed in a CO_2 chamber. A 7 mm diameter plug was removed from each ear. The swelling was assessed as the difference in weight between the left and the right ear. Edema inhibition (EI %) was calculated by the equation: $\text{EI} \% = 100 - \left(\frac{B \times 100}{A} \right)$ where A is the edema induced by TPA alone and B is the edema induced by TPA plus sample. Indomethacin

dissolved in acetone:ethanol 1:1 was used as the reference compound. All animal procedures were approved by the CICUAL-IQ Institutional Ethics Committee (protocol CICUAL-IQ-004-17).

2.16. Myeloperoxidase (MPO) assay

Tissue MPO activity was measured in biopsies taken from ears 4 h after TPA administration using an adapted method of Bradley [54] and Suzuki [55]. Each mouse ear biopsy was placed in an eppendorf tube with 1.0 mL of 80 mM phosphate-buffered saline (PBS) pH 5.4 containing 0.5% hexadecyltrimethylammonium bromide (HTAB). Each sample was homogenized for 30 s at 4 °C with a small sample laboratory Tissue Tearor Homogenizer model 125 (OMNI International; Kennesaw, GA, USA). The homogenate was freeze-thawed at room temperature for 3 times, sonicated during 20 s and centrifuged at $13,400 \times g$ for 15 min at 4 °C. The resulting supernatant (10 μ L) were poured into 96-well plate and 180 μ L of 80 mM PBS (pH 5.4) without HTAB were added. Microtiter plate was heated at 37 °C then, 20 μ L of 0.017% hydrogen peroxide were added to each well. For the MPO assay, 20 μ L of 18.4 mM 3,3',5,5', tetramethylbenzidine in 50% aqueous dimethylformamide were added to start the reaction. Microtiter plates were incubated at 37 °C for 5 min. The reaction was stopped with 20 μ L of 2 M H_2SO_4 . MPO enzyme activity was assessed colorimetrically using a BioTek Microplate Reader (BioTek; Winooski, VT, USA) at absorbance wavelength of 450 nm. MPO activity (measured by quadruplicated in each sample) was expressed as percentage inhibition in respect with the control.

2.17. Statistical analysis

All data were represented as percentage mean $\pm$ standard deviation of mean (SD) using the GraphPad Prism® software version 6 (GraphPad Software; La Jolla, CA, USA). Statistical analysis was done by analysis of variance ANOVA one way followed by Dunnett test were used to compare several groups with a control additionally by analysis with Student's *t*-test and Kolmogorov-Smirnoff's test. Pearson's correlation coefficient was calculated for the edema and MPO. Values of $p \leq 0.05$ were considered to be significant.

3. Results

3.1. X-ray diffraction, NMR and HPLC analysis of Jacareubin

^1H and ^{13}C NMR data of Jacareubin isolated from *C. brasiliense* heartwood are in line with those previously reported from the related species *Garcinia xanthochymus*, and from the fungus *Amauroderma amoienis* [56,57]. Jacareubin is a tetracyclic polyphenol, it presents a core structure composed by C6–C3–C6 system with an extra ring derived from cyclization of a γ,γ -dimethylallyl substituent attached to C-2 and C-3 of ring A. This is the first time that Jacaraeubin crystal molecular structure is reported based on X-ray diffraction (Fig. 1A). The compound is planar and rigid with three hydroxyl groups, hydroxyl on C-1 is forming an intramolecular H-bond with O-4 of the carbonyl group. The H-atom on O6 is displayed in two positions, refined as 57/43% of site occupational factor, showing intramolecular H-bond with O2 and O5 atoms and intermolecular H-bond with O6. The crystal planar cell show that arrangement jacareubin molecules in a wedge arrangement, B ring of one molecule is overlapped with the B ring of another molecule through H bridge established by the hydroxyls on C5 and C6, another hydrogen bond is formed between the O2 of one molecule and the hydroxyl in C6 of another molecule (Fig. 1B). HPLC-MS showed a major peak (99.39%) corresponding to 16 min, which coincided with the molecular mass of Jacareubin m/z $\text{M}^+ - 1 = 325$ (Fig. 1C and D) indicating the purity of the used preparation.

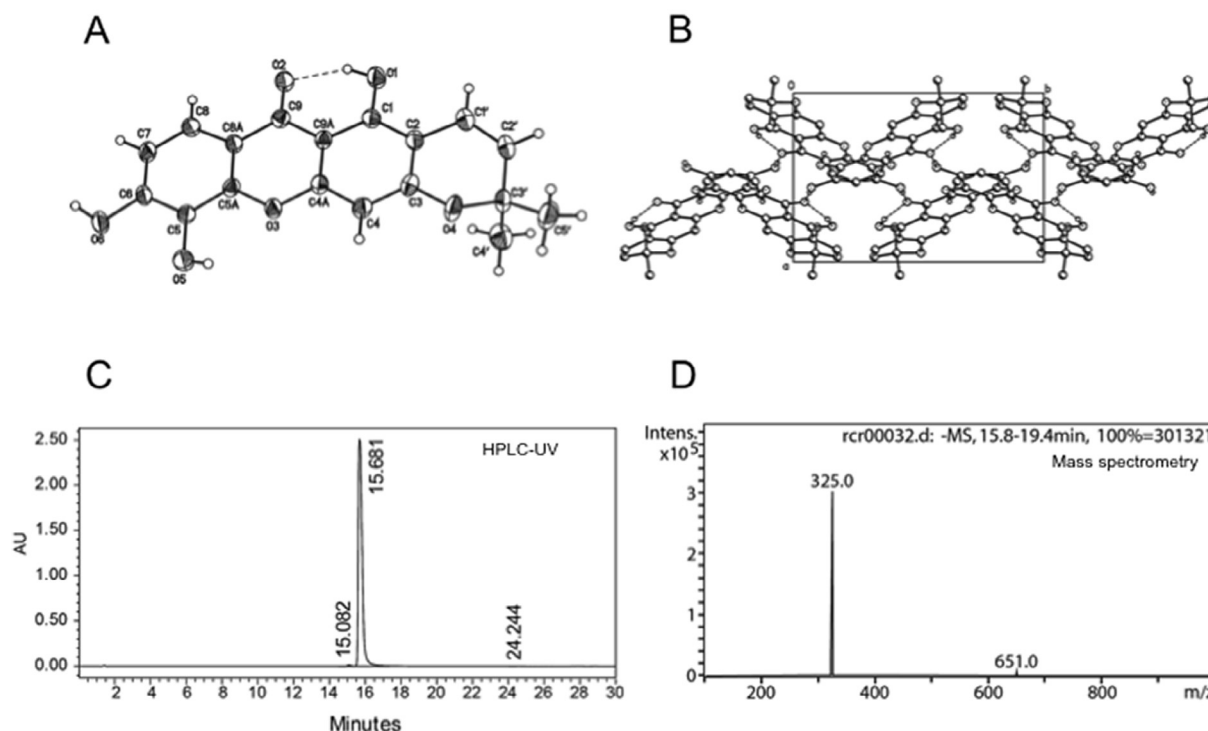


Fig. 1. X-ray structure and purity of Jacareubin utilized in this study. A) Tridimensional structure of Jacareubin obtained by X-ray diffraction. B) Crystallographic cell. Panels C) and D) Purity of Jacareubin used in this study determined by HPLC-UV and Mass Spectrometry.

3.2. Jacareubin inhibits mast cell degranulation activated by FcεRI triggering.

Utilizing the release of LDH to evaluate cell death, we found that jacareubin does not show cytotoxic activity on BMMCs at concentrations ranging from 3 nM to 30 μM. As can be observed on Fig. 1A, no significant release of LDH was observed after a 15 min incubation with the compound, compared with an important death detected with hydrogen peroxide treatment. Due to those results, this range of concentrations was therefore used for subsequent experiments.

To evaluate the actions of jacareubin on mast cell activation, BMMCs were sensitized overnight with 100 ng/mL of a monoclonal anti-DNP IgE. After this, cells were incubated in the presence of different concentrations of jacareubin at 37 °C. After 15 min, cells were stimulated with distinct concentrations of a specific antigen (DNP-HSA) and the activity of β-hexosaminidase in the supernatant was determined. As can be observed on Fig. 2B, jacareubin inhibited (IgE/Ag)-induced degranulation in a concentration-dependent fashion ($IC_{50} = 0.046 \mu M$). Also, effects of jacareubin were tested on degranulation induced by Thap, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), which activates secretion by increasing the intracellular calcium content in MCs [16] independently of FcεRI receptor crosslinking. Jacareubin was able to inhibit Thap-induced degranulation in a concentration-dependent manner (Fig. 2C). Finally, degranulation triggered with the PKC α/β activator (TPA) plus the calcium ionophore A23187 was also sensitive to the actions of jacareubin (Fig. 2D).

3.3. Jacareubin interferes with FcεRI-induced intracellular calcium rise in BMMCs

The effects of jacareubin on FcεRI receptor-induced calcium mobilization in BMMCs were evaluated. FURA2-AM-loaded cells were incubated with vehicle or different concentrations of jacareubin 15 min before to be stimulated with 27 ng/mL DNP-HSA and changes on FURA2-AM fluorescence were registered as described in the Material and

Methods section. As observed in Fig. 3A, B and C, addition of antigen caused a rapid increase in $[Ca^{2+}]_i$, which reached maximal values around 300 s after stimulation. Preincubation with jacareubin prevented calcium rise in a dose-dependent manner, reaching full inhibition at 30 μM (Fig. 3C). Quantification of the rate of calcium rise and maximal calcium levels reached with each treatment is shown on Table 1. Addition of jacareubin did not modify the immediate rate of calcium increase when used at 0.3 or 3 μM and this parameter was affected only with 30 μM. However, maximal increase on $[Ca^{2+}]_i$ was affected from 0.3 μM (Fig. 3A).

Intracellular calcium rise after FcεRI crosslinking in mast cells is controlled by the emptiness of intracellular stores and by the opening of membrane calcium channels. In order to dissect the point of action of jacareubin, we incubated BMMCs with vehicle or different concentrations of that compound in a calcium free buffer and stimulated them with IgE/Ag. As expected, after addition of calcium to the cuvette (to reach 1.8 mM extracellular Ca^{2+}), an important increase of intracellular calcium concentration was observed due to the calcium entry through the opened membrane channels (Fig. 4A). Pre-incubation with jacareubin blocked FcεRI-induced extracellular calcium entrance in a dose-response fashion. When 0.3 μM of Jacareubin was used, only a slight difference was observed with respect to untreated cells, but the addition of 3 μM blocked the maximal calcium concentration by 50% and 30 μM fully inhibited calcium influx (Fig. 4B and C). Closer analysis of the parameters of calcium entrance showed that Jacareubin decreased the rate and the maximal $[Ca^{2+}]_i$ (Table 2).

Intracellular calcium concentration after FcεRI increases due to the activation of two main pathways: the receptor-operated calcium entry (ROCE) and the store-operated calcium entry (SOCE) [58–60]. The first one occurs due to the activation of calcium channels with the participation of proteins and messengers that does not induce the emptiness of intracellular calcium stores [58,59]. On the other hand, SOCE occurs after the activation of the IP₃ receptor in the endoplasmic reticulum and is triggered when intracellular stores are mobilized and the cell requires to refills those storage compartments [14,15,61]. SOCE can be pharmacologically activated by Thap, a sesquiterpene lactone that inhibits

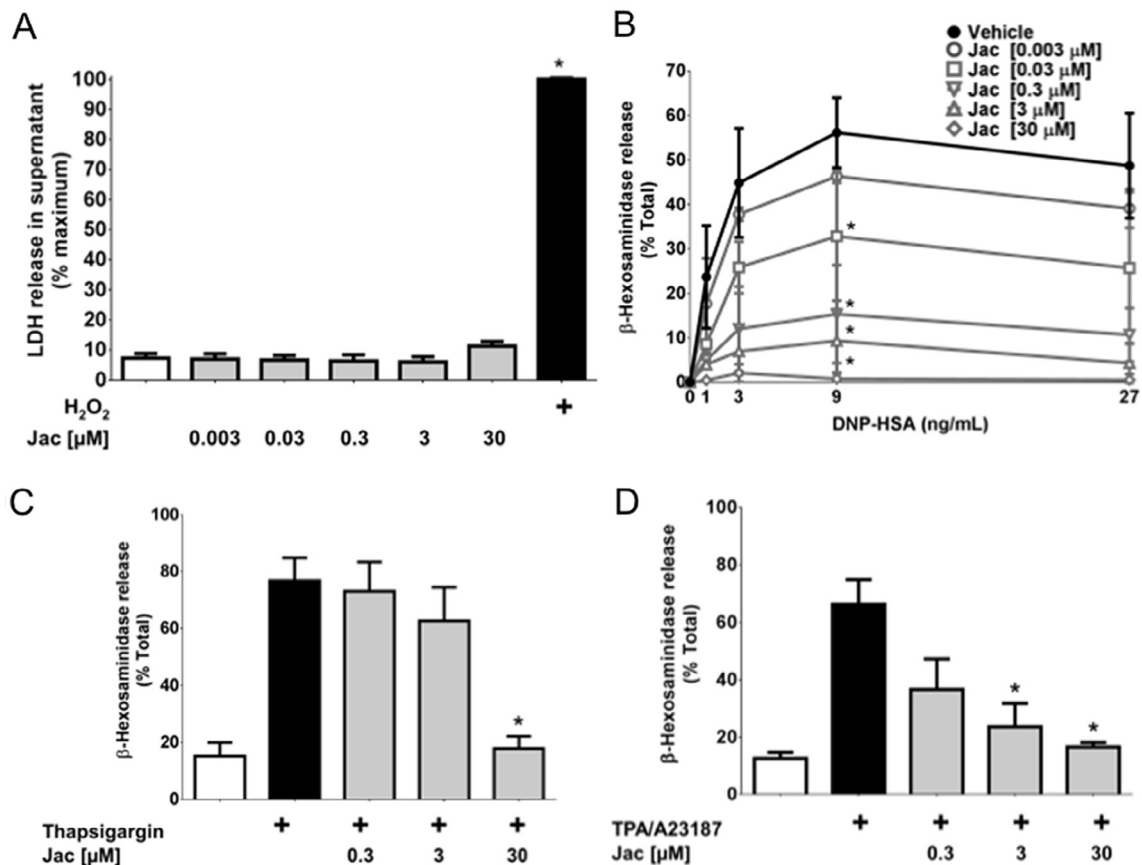


Fig. 2. Jacareubin inhibits degranulation stimulated by different agents in mast cells in a dose-dependent fashion. **A.** Toxicity of jacareubin in BMMCs measured by LDH release on cell supernatants, H_2O_2 treatment was included as a positive control. $n = 3$. **B.** Concentration-response curve of IgE/Ag-induced degranulation in BMMCs pre-treated with different concentrations of Jacareubin or with vehicle alone, $n = 3-6$. **C.** Effect of Jacareubin pre-treatment on Thap-induced degranulation in BMMCs, $n = 3$. **D.** Effect of jacareubin pre-treatment on TPA/A23187-induced degranulation, $n = 3$. One way ANOVA with Dunnett's multiple comparisons test ($^*p < 0.05$).

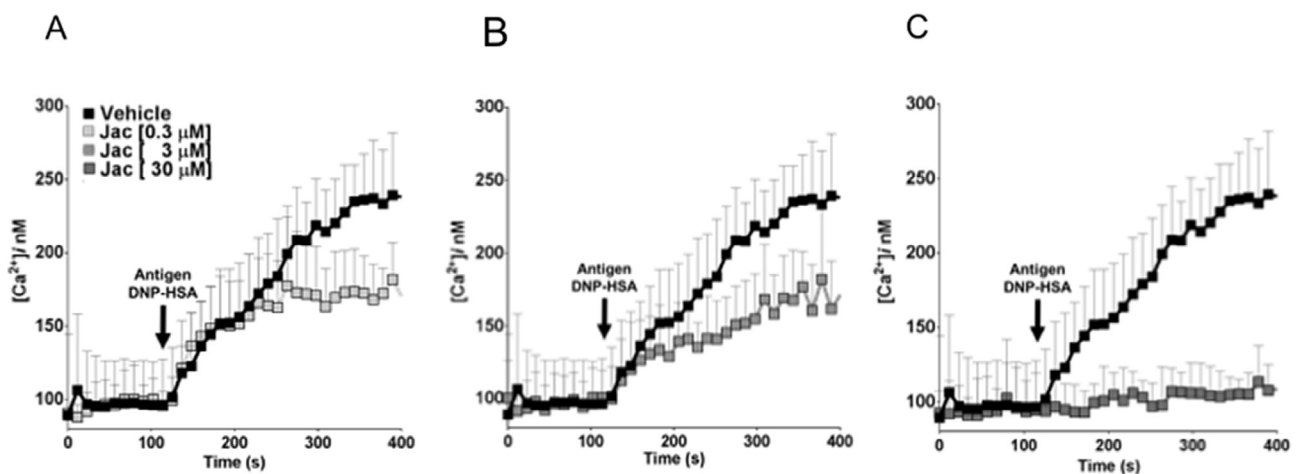


Fig. 3. Jacareubin inhibits Fc ϵ RI-induced calcium mobilization in mast cells in a concentration-dependent manner. **A) – C)** Representative traces of intracellular calcium rise induced by the specific antigen DNP-HSA added to BMMCs pre-treated with vehicle or different concentrations of jacareubin, $n = 3-6$. One way ANOVA with Dunnett's multiple comparisons test ($^*p < 0.05$).

the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA). In order to analyze if jacareubin could inhibit SOCE, cells were treated with vehicle or with different concentrations of jacareubin for 15 min in free Ca^{2+} buffer. Basal $[Ca^{2+}]_i$ was measured for 100 s before the addition of Thap (Fig. 5). After 100 s, Ca^{2+} was added to the cuvette to reach 1.8 mM and changes on $[Ca^{2+}]_i$ were followed for 200 s. As can be observed on Fig. 5, Thap induced, as expected, a discrete rise on $[Ca^{2+}]_i$

i corresponding to the emptiness of intracellular calcium stores and, after addition of extracellular Ca^{2+} , the entry of this ion through the STIM1/Orai-mediated mechanism of calcium replenishment of endoplasmic reticulum. Pre-treatment with jacareubin (0.3 μ M) diminished maximal Ca^{2+} increase (Fig. 5A), whereas at higher concentrations (3 and 30 μ M) importantly inhibited both the rate of Ca^{2+} rise and the maximal Ca^{2+} (Fig. 5B and C and Table 3).

Table 1
Effect of Jacareubin on FcεRI-induced calcium mobilization in BMMCs.

Treatment	Rate of increase $[Ca^{2+}]_i$	Max $[Ca^{2+}]_i$ (nM)
Vehicle	0.648 ± 0.222	239.3 ± 42.41
Jac [0.3 μM]	0.584 ± 0.334	181.8 ± 25.19
Jac [3 μM]	0.335 ± 0.065	182.1 ± 50.83
Jac [30 μM]	0.095 ± 0.122 [*]	113.4 ± 24.14 [*]

BMMCs were pre-treated with vehicle or distinct jacareubin concentrations and increase on intracellular calcium concentration after FcεRI receptor stimulation was registered. Data from those graphs (Fig. 3) were utilized for this table. One way ANOVA with Dunnett's multiple comparisons test (^{*}p < 0.05).

3.4. Jacareubin interferes with ROS production induced by IgE/Ag in mast cells

Since a common feature between ROCE and SOCE observed in distinct MC preparations is their modulation by the production of reactive oxygen-species (ROS) [59,62,63], we hypothesized that jacareubin could inhibit the production of ROS triggered by FcεRI receptor and this could cause its effect on intracellular calcium rise in MCs. To test this hypothesis, we first determined the antioxidant capacity of jacareubin and compared it with other molecules with reported antioxidant properties, exploring the possible relationship between antioxidant capacity and inhibition of MC activation. Fig. 6A shows that jacareubin reduces DPPH at lower concentrations than α-tocopherol and trolox, whereas, compared to quercetin, showed less antioxidant capacity. The antioxidant capacity of quercetin and trolox was related to their ability to block IgE/Ag-dependent degranulation, since lower concentrations of quercetin were needed to inhibit β-hexosaminidase release (Fig. 6B). However, when compared with jacareubin, the xanthone showed higher capacity of inhibition of IgE/Ag-induced degranulation and calcium influx than trolox and quercetin (Fig. 6B and C). When the mentioned compounds were tested on their ability to block the FcεRI-dependent production of reactive oxygen species, jacareubin inhibited DCF oxidation in a dose-response fashion (Fig. 6D).

In order to explore the mechanism by which jacareubin could inhibit ROS accumulation in BMMCs, cells were stimulated with IgE/Ag in the presence of buffer or different concentrations of the xanthone and the activity of the NADPH oxidase complex (NOX) was determined. As observed in Fig. 7A, jacareubin was not able to block the FcεRI-dependent stimulation of NOX at any of the concentrations tested. The NOX inhibitor DPI was used as a control and the expected effect of this compound was observed. However, in a cell-free assay, activity of xanthine oxidase was inhibited by jacareubin in a dose-response fashion

Table 2
Effect of Jacareubin on extracellular calcium flux stimulated by FcεRI receptor in BMMCs.

Treatment	Rate of increase $[Ca^{2+}]_i$	Max $[Ca^{2+}]_i$ (nM)
Vehicle	1.407 ± 0.221	264.7 ± 14.65
Jac [0.3 μM]	1.216 ± 0.181	266.3 ± 20.77
Jac [3 μM]	0.368 ± 0.106 [*]	161.4 ± 29.84 [*]
Jac [30 μM]	0.080 ± 0.047 [*]	104.5 ± 23.75 [*]

BMMCs were treated with vehicle or distinct jacareubin concentrations and increase on intracellular calcium concentration was registered after FcεRI stimulation. Data from those graphs (Fig. 4) were utilized for this table. One way ANOVA with Dunnett's multiple comparisons test (^{*}p < 0.05).

(Fig. 7B).

3.5. Jacareubin inhibits mast-cell dependent changes in vascular permeability and inflammatory reactions in vivo

In order to test the effects of Jacareubin on IgE/Ag-induced degranulation of mast cells *in vivo*, we utilized the murine passive anaphylaxis model, where the immediate changes on vascular permeability are entirely dependent on mast cell activation [28,52]. As can be observed on Fig. 8A, jacareubin inhibited FcεRI-dependent inflammation up to 80% at doses between 1 and 33.3 mg/kg i.p, when administered 2 h before DNP-HSA challenge. Utilized doses did not produce any effect on mice, since were below the concentrations where toxicity of this xanthone has been observed *in vivo* [64].

In order to evaluate the anti-inflammatory activity of jacareubin on conditions that have been associated with other immune cells (monocytes and neutrophils), we tested the effect of that compound on the TPA-induced inflammation model in mouse ear, determining the produced edema and the activity of myeloperoxidase. As can be observed on Fig. 8B, jacareubin inhibited TPA-induced edema in a dose-dependent fashion, from 7 to 68% and this blockage was similar to the observed with indomethacin. Regarding the MPO activity, jacareubin inhibited from 13 to 81% (Fig. 8C).

4. Discussion

In this study the structure of the xanthone Jacareubin was determined by X-ray diffraction for the first time, and its effects on mast cell degranulation were evaluated. Jacareubin showed high inhibitory activity on FcεRI-induced degranulation of mast cells by blocking the extracellular calcium influx activated either by IgE/Ag complexes,

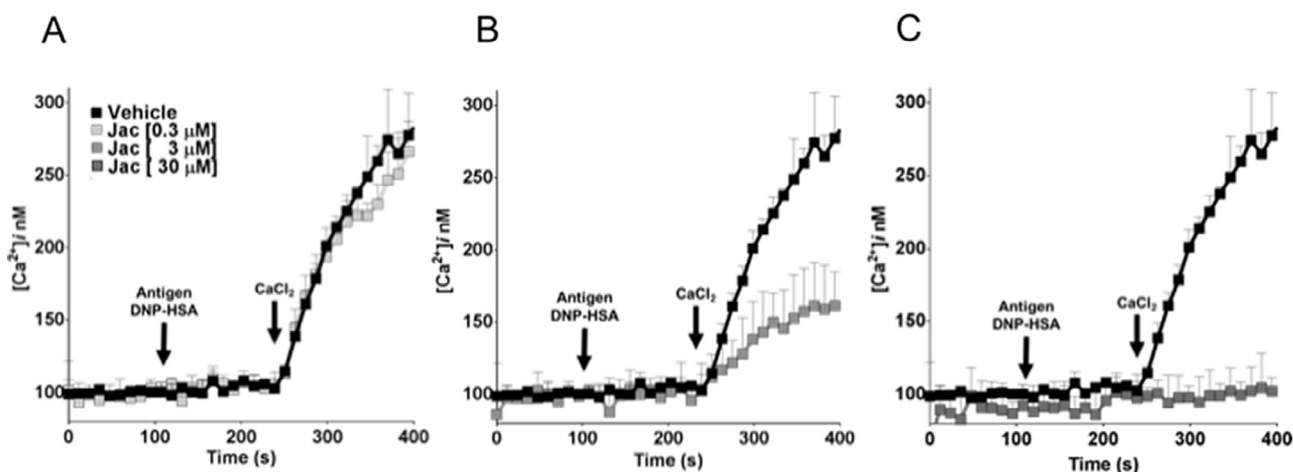


Fig. 4. Jacareubin inhibits extracellular calcium influx stimulated by FcεRI receptor in mast cells. A) – C) BMMCs were suspended in calcium-free buffer and the specific antigen DNP-HSA was added. After 100 s, calcium was added to the cuvette to reach 1.8 mM. A) – C). Representative traces of intracellular calcium rise in cells pre-treated with vehicle or different concentrations of jacareubin, n = 3. One way ANOVA with Dunnett's multiple comparisons test (^{*}p < 0.05).

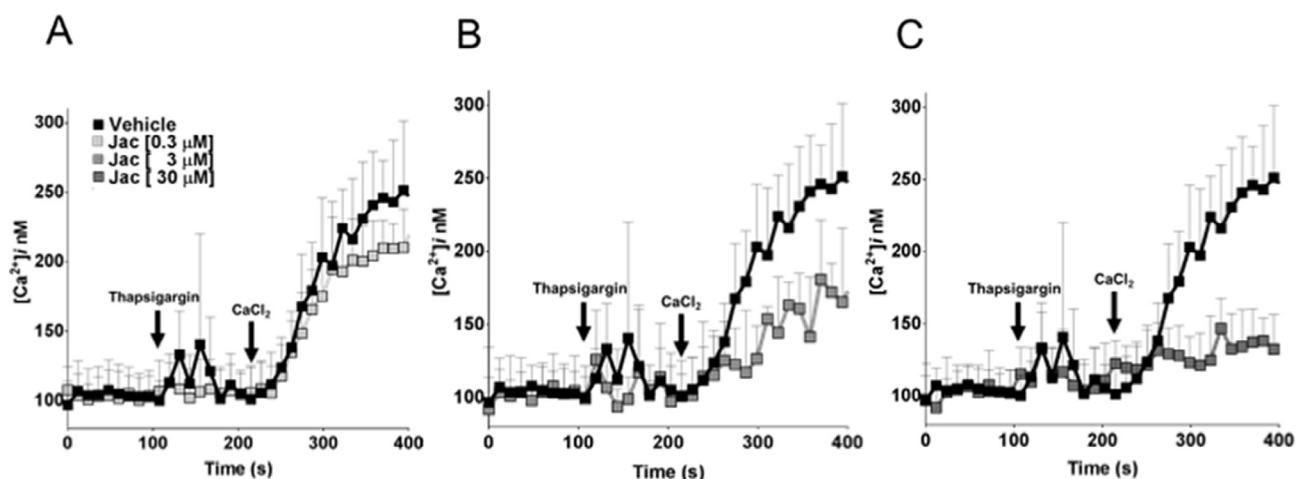


Fig. 5. Jacareubin inhibits Thap-induced calcium influx in mast cells in a dose-response fashion. A) – C) Representative traces of intracellular calcium rise induced by Thap (1 μ M) in BMMCs pre-treated with vehicle or different concentrations of jacareubin in Ca^{2+} -free medium, $n = 3$ –5. One way ANOVA with Dunnett's multiple comparisons test ($^{\circ}p < 0.05$).

Table 3

Effect of Jacareubin on extracellular calcium flux stimulated by Thapsigargin in BMMCs.

Treatment	Rate of increase $[\text{Ca}^{2+}]_i$	Max $[\text{Ca}^{2+}]_i$ (nM)
Vehicle	1.212 ± 0.491	251.3 ± 49.96
Jac [0.3 μ M]	0.931 ± 0.312	210.2 ± 27.60
Jac [3 μ M]	$0.423 \pm 0.164^{\circ}$	$180.7 \pm 40.68^{\circ}$
Jac [30 μ M]	$0.092 \pm 0.088^{\circ}$	$146.6 \pm 20.80^{\circ}$

BMMCs were treated with vehicle or distinct jacareubin concentrations and increase on intracellular calcium concentration was registered after Thapsigargin addition. Data from those graphs (Fig. 5) were utilized for this table. One way ANOVA with Dunnett's multiple comparisons test ($^{\circ}p < 0.05$).

Thapsigargin or TPA/A23187. Jacareubin also exhibited an important antioxidant activity and this could count to the effect on calcium mobilization, due the dependence of that phenomenon on ROS generation. Whereas jacareubin did not showed significant inhibitory activity on Fc ϵ RI-induced NOX activity, it inhibited the activity of xanthine oxidase in a cell-free assay. Finally, this compound exerts anti-inflammatory actions *in vivo*, blocking inflammatory reactions that depend on mast cells but also other immune cells activation.

The planar and rigid structure of jacareubin was clearly evidenced by X-ray diffraction, and may limit the possibility to block non-specific molecular targets. Xanthenes bearing a three ring skeleton have shown anti-inflammatory activity in mast cells, such as nujiangexanthone A, mangostins α , β , and γ , mangiferin and its aglycone norathyriol (Fig. 9) [34–37,65]. Our results show that Jacareubin, is the most potent xanthone, and the only one with an extra ring arising from cyclization of a dimethyl allyl substituent on C-2, conferring hydrophobic properties to the molecule. On the other side, hydroxyl on C-5 and C-6 may allow the interaction of jacareubin due to hydrogen bonds with carbonyls of membrane phospholipids, in a similar way to that reported for cholesterol [66]. Interestingly, less potent mangostins and nujiangexanthone bear uncyclized dimethyl allyl substituents, mangiferin is the less potent and the most hydrophilic due to four hydroxyls and a glucose residue. Emodin, an anthraquinone, also with a skeleton similar to xanthenes, due to a three ring system but with two carbonyls, is also able to inhibit β -hexosaminidase release stimulated by IgE/Ag in BMMCs [67].

Antioxidant activity of Jacareubin in the DPPH reduction test is similar to that previously reported [56,68]. Antioxidant activity has been proposed as the main mechanism responsible for inhibiting degranulation in mast cells associated for many natural compounds

[29,69]. Hydroxyl phenolic groups in xanthenes have been thought to importantly contribute to the antioxidant activity of those compounds, but this is not always the case. For instance, γ -mangostin and garcinone A and B, showed a greater antioxidant response than other xanthenes with higher number of hydroxyl substituents in the DPPH reduction test [34,56]. Jacareubin showed a better antioxidant activity than α -tocopherol and caffeic acid, despite the fact does not possess a high number of hydroxyl substituents.

Degranulation of mast cells after Fc ϵ RI crosslinking has been considered the main target of therapeutic approaches to control inflammation and allergy, since the release of preformed mediators occurs rapidly and the secreted compounds trigger immediate and intense reactions. The search for safety compounds able to inhibit mast cell degranulation has led to the identification of several molecules with different capacities of inhibition and distinct mechanisms of action. We found that jacareubin potently inhibits the secretion of the preformed mediator β -hexosaminidase in BMMCs. Our data show that Jacareubin is one of the most potent of the xanthenes so far tested on IgE/Ag-induced degranulation in mast cells. When utilized at 0.046 μ M and 30 μ M, this compound inhibited 50 and 97% β -hexosaminidase release respectively, while nujiangexanthone A showed maximal inhibitory activity at 50 μ M (causing 95% of inhibition of in BMMC) [38], but its IC_{50} was between 1 and 10 μ M, and α -mangostin exhibited inhibitory actions IC_{50} between 10 and 20 μ M, with maximal inhibition of histamine release on RBL-2H3 cells of 80% at 20 μ M [34]. Xanthenes are able to inhibit degranulation of mast cells in a wide range of concentrations, for example, mangiferin requires 118 μ M for inhibiting 50% of degranulation triggered by the 48/80 compound (a commonly used secretagogue in mast cells) [36]. It is noteworthy, that all concentrations of jacareubin here tested did not induce cytotoxicity in mast cells, this is in line with the observed with other immune cells, such as human blood mononuclear cells (PBMC) [64].

Besides xanthenes, other natural products, such as polyphenols, have been found to effectively inhibit the degranulation of mast cells by over 70% and in the same concentration ranges of the inhibitory activity of Jacareubin, those include flavonoids such as luteolin [25,27], tetramethoxyluteolin [28], and quercetin [25], however, given the polarity of the xanthenes, they exhibit better solubility than the mentioned flavonoids and this characteristic could favor to reach higher concentrations in tissues [70].

Although evidence showing that some xanthenes inhibit calcium mobilization in mast cells has been collected, the specific mechanism for this action has been elusive [34]. Description of the effects of xanthenes on the Fc ϵ RI receptor signaling system includes, for example, the

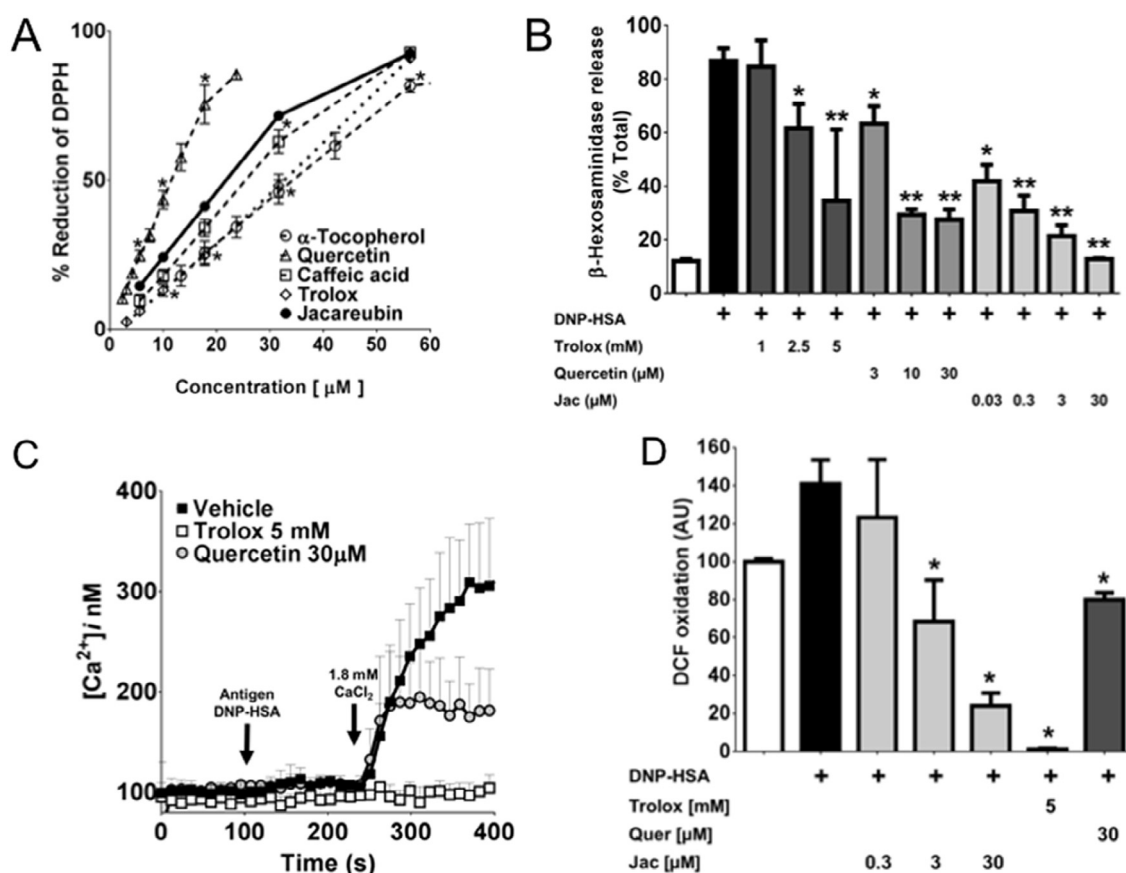


Fig. 6. Jacareubin exhibits important antioxidant activity: comparison with trolox and quercetin. A) Antioxidant activity of Jacareubin compared with other antioxidants, determined by DPPH reduction, $n = 3$. B) Inhibition of Fc ϵ RI-induced degranulation by trolox, quercetin and jacareubin (values obtained with jacareubin were taken from Fig. 2B), $n = 3$. C) Inhibition of IgE/Ag-induced calcium influx by trolox and quercetin, $n = 3$. D) Fc ϵ RI-induced DCF oxidation in BMMCs pre-treated with different concentrations of jacareubin (values obtained from cells pre-treated with trolox and quercetin were included as controls), $n = 3$. One way ANOVA with Dunnett's multiple comparisons test ($^*p < 0.05$).

blockage of the Tyr-phosphorylation of early signaling proteins, such as phospholipase A2, Src family kinases and MAPK [38]. Our results show that Jacareubin is able to efficiently block calcium mobilization in response to Fc ϵ RI triggering in BMMCs and seemed to be associated with the blockage of a plasma membrane calcium channels activated by

Fc ϵ RI and Thap.

In mast cells, Fc ϵ RI triggering leads to the activation of PLC which, in turn, produces DAG and IP_3 that activates the IP_3 calcium channel causing the emptiness of the intracellular calcium storage pools. This event leads to the entrance of calcium from the extracellular space by

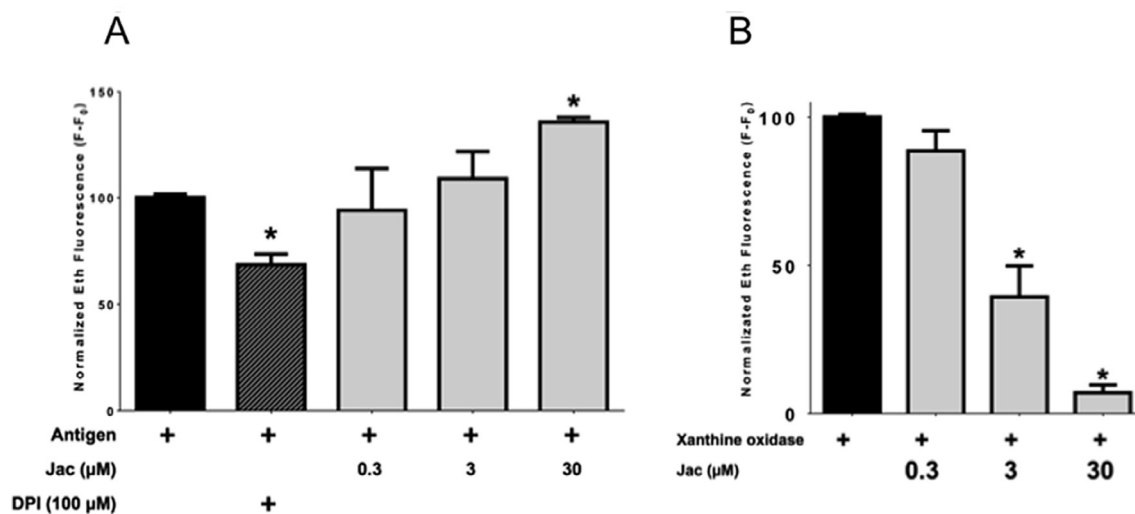


Fig. 7. Jacareubin does not inhibit Fc ϵ RI-dependent activation of NOX but is able to inhibit Xanthine Oxidase in a cell-free assay. A) IgE/Ag-induced activity of NOX in cells pre-treated with DPI or different concentrations of jacareubin, $n = 3$. B) Xanthine oxidase activity determined in the presence of distinct concentrations of jacareubin, $n = 3$. One way ANOVA with Dunnett's multiple comparisons test ($^*p < 0.05$).

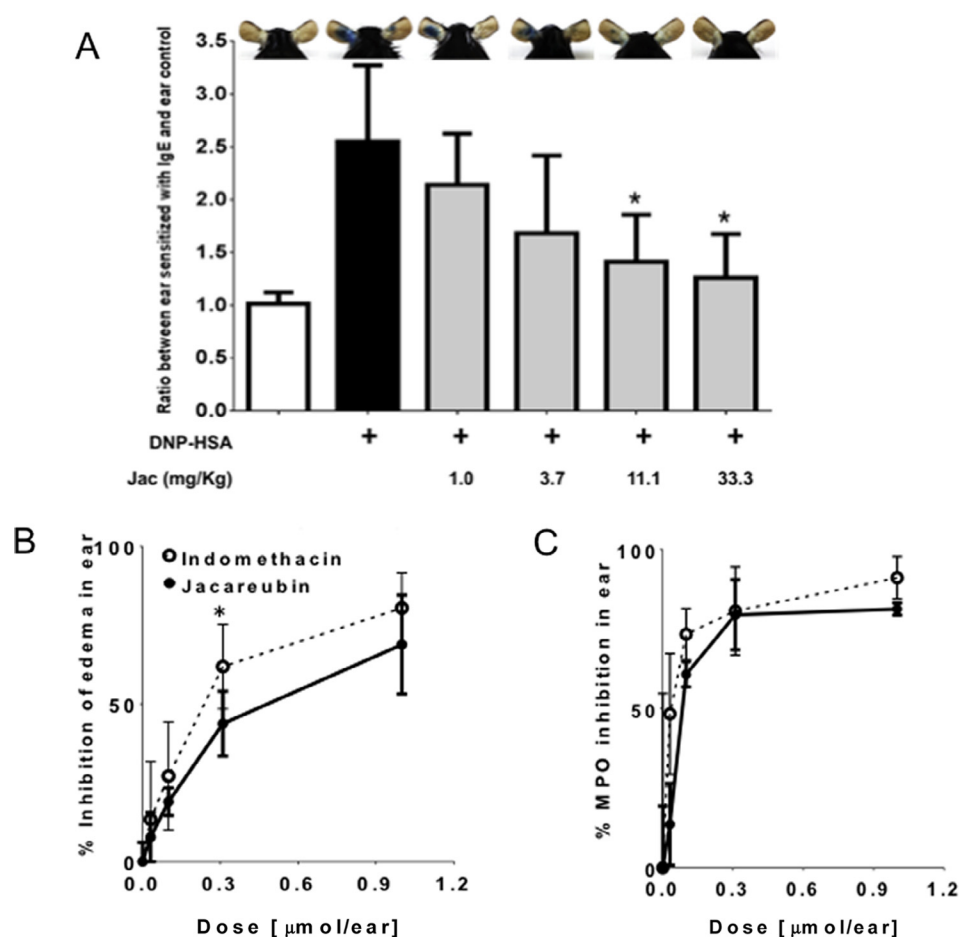


Fig. 8. Jacareubin reduces MC-dependent and MC-independent inflammation *in vivo*. A) Passive anaphylaxis observed in the ear of mice subjected to distinct Jacareubin doses, $n = 5$ –10. B) TPA-induced ear edema in mice pretreated with different doses of jacareubin topically administered in the left ear (results are shown as percentage of inhibition with respect to control (TPA only); indomethacin were use as reference. $n = 5$ –7. C) Tissue MPO activity was measured in biopsies taken from ears 4 h after TPA administration, results are expressed as percentage of inhibition with respect to control, indomethacin were use as reference. $n = 5$. One way ANOVA with Dunnett's multiple comparisons test and Pearson's correlation coefficient ($^*p < 0.05$).

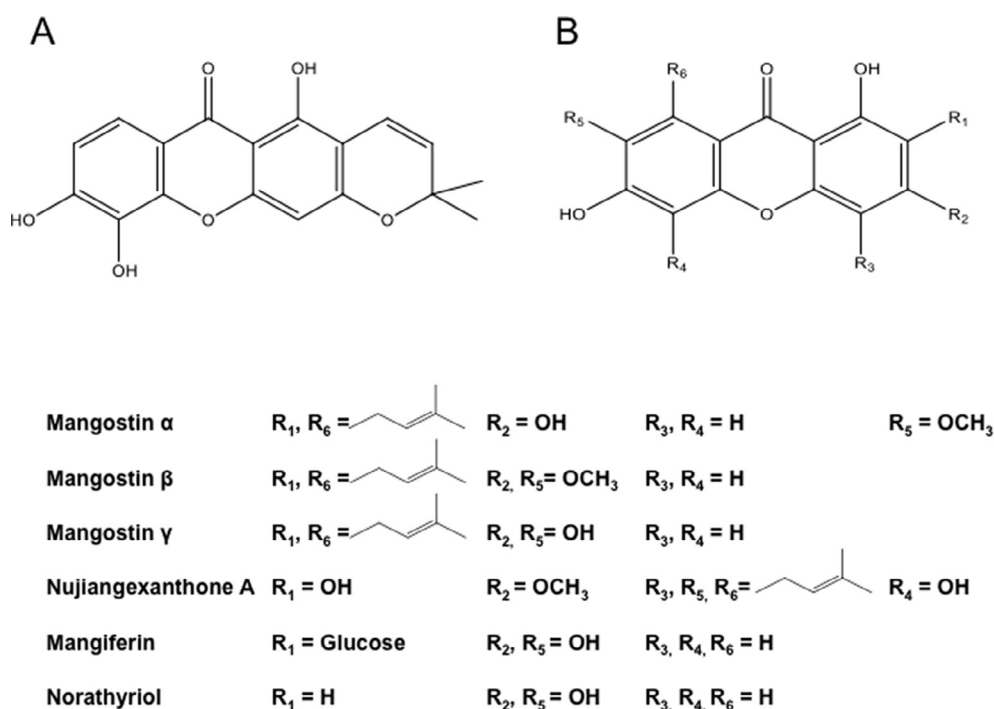


Fig. 9. Jacareubin present a unique structural signature for xanthones with anti-inflammatory activity. A) Jacareubin. B) Xanthones with anti-inflammatory activity in mast cells previously described (lower part, substituents on the specific structure of xanthones).

calcium channels that constitute the “store-operated calcium entry” (SOCE), which can be triggered by the inhibitor of the SERCA ATPase Thap. SOCE operates mainly through the Orai family of ion channels, being Orai1 the most relevant in immune cells [63,71]. After intracellular calcium pools emptiness, the Hand-EF region of the calcium sensor STIM located on the inner side of the endoplasmic reticulum loses the Ca^{2+} that keeps it stabilized, and provokes a conformational change that allows the oligomerization and extension of STIM and forms with Orai membrane channels the pore to facilitate the re-filling of intracellular Ca^{2+} pools in the endoplasmic reticulum [72,73].

Different studies have suggested that a very close relationship exists between the production of ROS and calcium entry in non-excitable cells [16]. For example, ROS production promotes glutathionylation of cysteines C49 and C56 in the inner region of the endoplasmic reticulum of STIM, which causes the affinity of Ca^{2+} to be reduced by the Hand-EF region of STIM, improving the extension of STIM and its oligomerization with Orai at the plasma membrane [17,72,73]. In mast cells, triggering of FcεRI leads to NADPH oxidase activation and the production of ROS that promote the activation of the LAT-PLCγ signaling axis [74]. Since Jacareubin inhibited the FcεRI-dependent production of ROS, it is possible to hypothesize that this compound could prevent the redox-dependent modifications of STIM needed for SOCE in mast cells.

Jacareubin-dependent blockage of ROS accumulation could also explain the inhibition of degranulation promoted by the combination of TPA plus A23187, since it has been shown that Thap and ionophore A23187 also produce the rapid production of ROS in mast cells [75]. Interestingly, although antioxidant activity of Jacareubin was higher than that showed by trolox and lower than the detected for quercetin, the xanthone was the most potent regarding to the inhibition of IgE/Ag-triggered degranulation. Antioxidant activity did not show a direct correlation with the capacity of the tested compounds to block calcium entry.

The mechanism by which jacareubin blocks IgE/Ag-induced degranulation and calcium mobilization in BMMC seems does not include the inhibition of NOX. However, xanthine oxidase was inhibited by this xanthone in a cell-free assay. Although the xanthone norathyriol is structurally related to jacareubin and show inhibitory activity on NOX and PLC in neutrophils [65], our data indicate that one possible mechanism of action of jacareubin, besides its capacity of chelating ROS, could be the inhibition of the xanthine oxidase. Antioxidant activity of jacareubin could contribute also to prevent IgE/Ag-triggered PLCγ activation, since it has been demonstrated that ROS production is essential to PLCγ phosphorylation in mast cells [74], see Fig. 10.

Jacareubin could exert inhibitory actions also by additional mechanisms. Evidence has been collected about the inhibition of the protein kinase Syk by other natural compounds such as piceatannol, morin, nujiangexantone A, mangostins α, β and γ, emodin [34,38,67,76,77], then, it is possible to speculate that jacareubin could also inhibit this important kinase involved in FcεRI signaling system.

The inhibitory actions of jacareubin on mast cell-dependent anaphylactic reactions are observed *in vivo*, since this compound suppressed extravasation of Evans blue stimulated by IgE/Ag (an effect mediated by histamine secreted by mast cells) and the edema induced by TPA in mice, a response dependent on prostaglandin production by other immune resident cells [78]. The blockage on the accumulation of ROS could importantly count for this effect, since ROS production is essential for FcεRI-induced mast cell degranulation [74] also for the production of prostaglandins in other cells [79]. This effect of jacareubin was similar or more potent to the observed with other natural compounds such as morin (30 mg/kg), teoritin (1 mg/kg) and theanine (1 mg/kg), that inhibited passive cutaneous anaphylaxis by 40, 42 and 44% respectively [77,80–82]. Xanthones similar to jacareubin such as mangostins do not have presented toxic effects in pre-clinical and clinical trials [83,84]. Jacareubin is presented here as a new drug with potential for the development of anti-inflammatory compounds derived

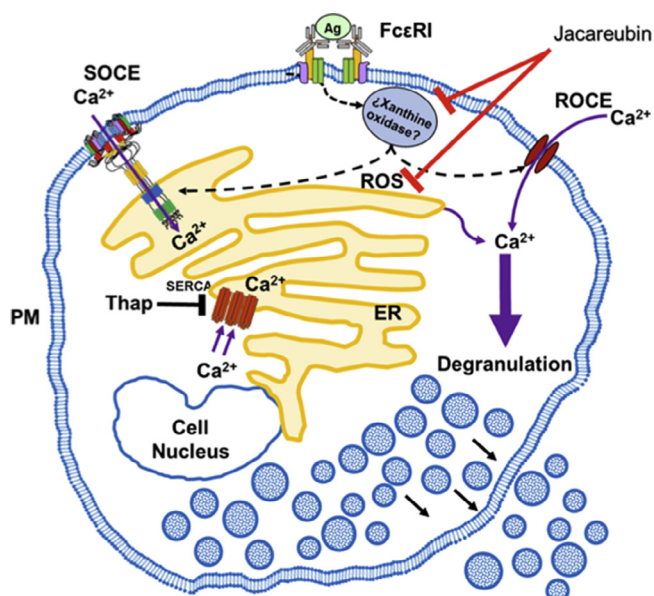


Fig. 10. Summary of the points of control of FcεRI signaling system that are modulated by Jacareubin in mast cells. Jacareubin inhibits degranulation and extracellular calcium influx triggered by IgE/Ag and Thap. This inhibition correlates with the blockage of the generation of ROS in this cell type.

from natural products to treat mast cell-related diseases.

5. Authorship

JICA performed the isolation, purification and chemical characterization of jacareubin. Additionally, performed degranulation assays, ROS production assays and $[\text{Ca}^{2+}]_i$ measurements, *in vivo* assays of passive cutaneous anaphylaxis in mice and wrote the first draft of the manuscript; SLGG contributed to the isolation and purification of jacareubin and also to the interpretation of the NMR spectra of the xanthone, together with the analysis and discussion of the data; AIS generated all full differentiated and functional BMMCs needed for the study and contribute to the discussion of the results; SHO carried out the structural identification experiments of the jacareubin by X-ray diffraction and contributed with the analysis and discussion of data; ANC performed a DPPH assay, TPA-induced edema model in mouse ear and myeloperoxidase assay; JPCh and ONMC performed NADPH and xanthine oxidase assays. RRCh and CGE performed the study conception and experimental design, they reviewed the main body of the paper and directed the activities of this work.

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6. Declarations of interest

None.

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