In vitro and *In Vivo* Immunomodulatory Activity of *Physalis angulata* Concentrated Ethanolic Extract

Authors
Sérgio Ricardo Teixeira Daltro[1], Ivanilson Pimenta Santos[1], Paula Ladeia Barros[1], Diogo Rodrigo Magalhães Moreira[1], Therezinha Coelho Barbosa Tomassini[2], Ivone Maria Ribeiro[2], Ricardo Ribeiro dos Santos[1], Cássio Santana Meira[1], Milena Botelho Pereira Soares[1]

Affiliations
1 Instituto Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Bahia, Brazil
2 Laboratório de Química de Produtos Naturais-PN2-Extração, Isolamento e Purificação, Farmanguinhos, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, RJ, Brazil

Key words
lymphocyte function, *Physalis angulata*, Solanaceae, macrophage activation

received March 30, 2020
accepted after revision July 30, 2020
published online

Bibliography
Planta Med 2020
DOI 10.1055/a-1237-4268
ISSN 0032-0943

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

Correspondence
PhD. Milena Botelho Pereira Soares
Instituto Gonçalo Moniz, Fundação Oswaldo Cruz
Rua Waldemar Falcão 121 – Candeal, 40296-710
Salvador – Bahia, Brazil
Phone: + 55 71 31 76 22 60, Fax: + 55 71 31 76 22 72
milena@bahia.fiocruz.br

ABSTRACT

The need for new immunomodulatory drugs is due to the side effects associated with the prolonged use of the currently used immunomodulatory drugs. In this context, the present work aimed to investigate the immunomodulatory effect of an ethanolic concentrated extract from *Physalis angulata*. The cytotoxicity of samples was determined using peritoneal macrophages though the Alamar Blue assay. The immunomodulatory activity of the ethanolic extract from *P. angulata* on activated macrophages was determined by measurement of nitrite and cytokine production. The immunosuppressive effects of the ethanolic extract from *P. angulata* was evaluated on lymphocyte proliferation and cytokine production. The effects of the extract on cell cycle progression and cell death on lymphocytes were evaluated by flow cytometry. Lastly, the ethanolic extract from *P. angulata* was tested *in vivo* in toxicological tests and in models of peritonitis and delayed-type hypersensitivity response. The ethanolic extract from *P. angulata* decreased nitrite, interleukin-6, interleukin-12, and TNF-α production by activated macrophages without affecting the cell viability. In addition, the ethanolic extract from *P. angulata* inhibited lymphoproliferation and the secretion of interleukin-2, interleukin-6, and IFN-γ, and increased interleukin-4 secretion by activated splenocytes. Flow cytometry analysis in lymphocyte cultures showed that treatment with the ethanolic extract from *P. angulata* induces cell cycle arrest in the G1 phase followed by cell death by apoptosis. Moreover, mice treated with the extract from *P. angulata* at 100 or 200 mg/kg did not show signs of toxicity or alterations in serum components. Finally, the ethanolic extract from *P. angulata* significantly reduced neutrophil migration and reduced paw edema in bovine serum albumin-induced the delayed-type hypersensitivity response model. Our results demonstrate the potential of the ethanolic extract of *P. angulata* as an alternative for the treatment of immune-inflammatory diseases.

Introduction

Physalis angulata L. (Solanaceae) is an annual herb widely distributed in tropical and subtropical countries and used in folk medicine to treat several conditions such as asthma, hepatitis, and rheumatism [1, 2]. Previous phytochemical analysis of *P. angulata* extracts demonstrated the presence of a variety of substances such as flavonoids, alkaloids and especially steroid compounds, such as physalins and withanolides [3, 4].

ABBREVIATIONS

CC_{50}	50% cytotoxicity concentration
Con A	concanavalin A
DTH	delayed-type hypersensitivity
EEPA	ethanolic extract from *Physalis angulata*
HTLV-1	human lymphotropic virus type 1
IFN-γ	interferon gamma
IL	interleukin
LPS	lipopolysaccharide
NFκB	nuclear factor kappa B
PI	propidium iodide

▶ **Table 1** Cytotoxic activity of the EEPA on mammalian cells.

Samples	MØ CC_{50} ± SD (µg/mL)
EEPA	6.9 (± 0.7)
Dexamethasone[a]	> 10
Gentian violet[b]	0.2 (± 0.1)

Cytotoxicity was evaluated on peritoneal macrophages (MØ) exposed to samples for 72 h by the Alamar Blue method. Values represent the mean (± standard deviation) of three independents experiments. SD: standard deviation. [a] Reference immunosuppressive drug. [b]Reference cytotoxic drug

Physalins hold several biological properties, including cytotoxicity, antioxidant antimicrobial, and antiparasitic activities [5–9]. In particular, physalins are well-known anti-inflammatory and immunosuppressant agents [10–13].

More specifically, physalins inhibit the production of several inflammatory mediators, such as nitric oxide, TNF-α, IL-6, and IL-12 [10, 14, 15]. Most of these effects are attributed to the inhibition of NFκB activation, a transcription factor involved in the regulation of several proinflammatory genes [12, 15]. In addition, physalin F is also known to have a potent antiproliferative effect on mouse lymphocytes stimulated with Con A and on peripheral blood mononuclear cells from patients with HTLV-1-associated myelopathy [11, 16].

Despite the immunomodulatory potential or these compounds, the purification of physalins is an expensive, time-consuming, and low-yielding process [17]. Because of these limitations, the use of a standardized EEPA becomes an attractive and viable alternative. The standardized ethanolic extract has proven to be non-mutagenic and is effective against different *Leishmania* species [17]. In addition, in a model of acute Chagas disease, treatment with EEPA decreased blood parasitemia and increased survival of infected mice [18]. In this context, the present work aimed to investigate the immunomodulatory effect of EEPA, seeking a new therapeutic alternative useful in the treatment of numerous inflammatory conditions.

Results

First, we determined the nontoxic concentrations of EEPA in peritoneal macrophages obtained from BALB/c mice. As shown in ▶ **Table 1**, EEPA presented a CC_{50} value of 6.9 µg/mL and did not display cytotoxicity in concentrations equal to or below 4 µg/mL (▶ **Fig. 1a**). Under the same conditions, gentian violet presented a CC_{50} value of 0.2 µg/mL. Dexamethasone was not cytotoxic in the tested concentrations. Based on that, subsequent tests were done in concentrations not superior to 4 µg/mL.

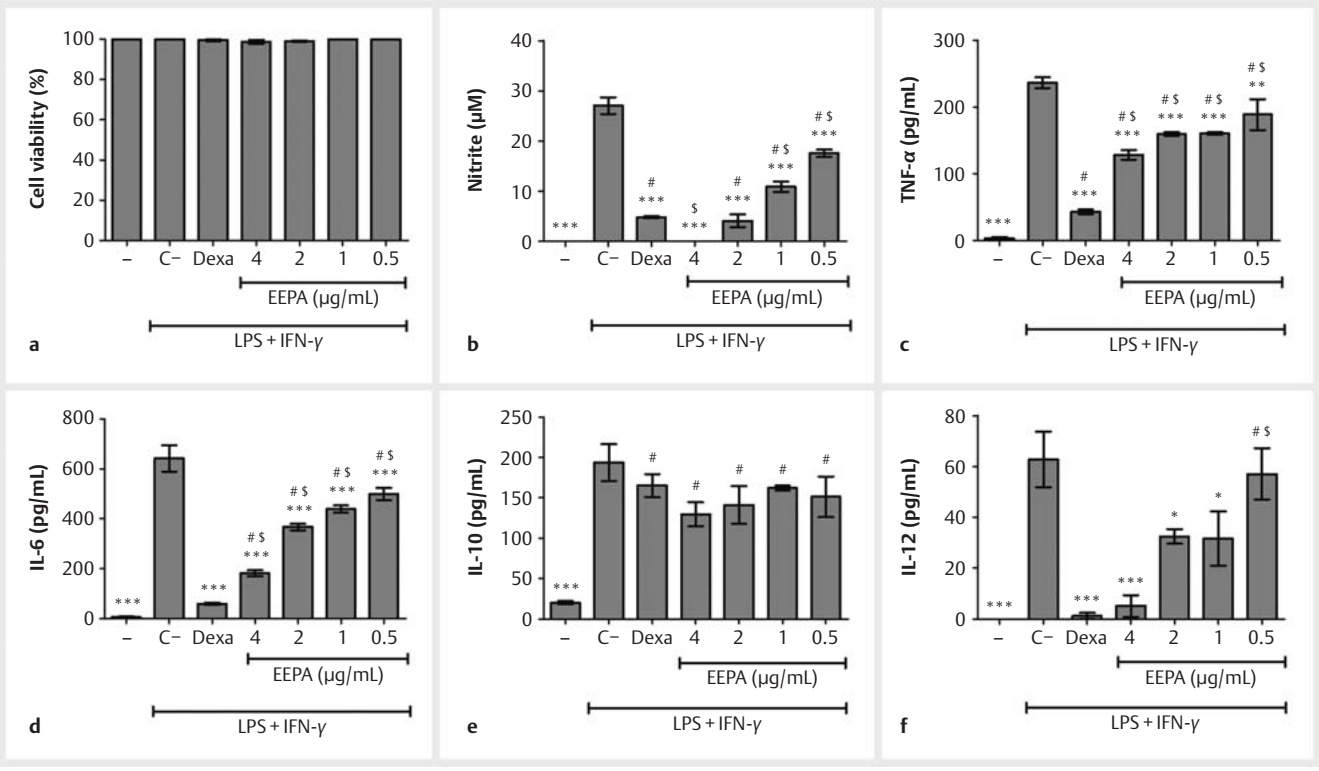
The immunomodulatory effect of EEPA was initially evaluated on cultures of peritoneal macrophages by analysis of nitric oxide production. As expected, macrophage activation with LPS plus IFN-γ increased the amount of nitrite production (▶ **Fig. 1b**). Treatment with EEPA inhibited, in a concentration-dependent manner, the production of nitrite ($p < 0.05$). Interesting, the inhibitory effect observed in cultures treated with EEPA at concentrations of 4 and 2 µg/mL was equipotent to the effect observed in cultures of activated macrophages treated with dexamethasone at 4 µg/mL (▶ **Fig. 1b**).

For a better characterization of the anti-inflammatory effect of EEPA on macrophage cultures, inflammatory cytokine levels were quantified by the ELISA method. Macrophage stimulation using LPS + IFN-γ induced a remarkable increase in the production of TNF-α, IL-6, IL-10, and IL-12 (▶ **Fig. 1c–f**). Treatment with EEPA significantly reduced ($p < 0.05$) the levels of TNF-α, IL-6, and IL-12. Under the same conditions, dexamethasone (4 µg/mL) promoted similar effects. Both EEPA and dexamethasone did not modulate IL-10 production (▶ **Fig. 1e**).

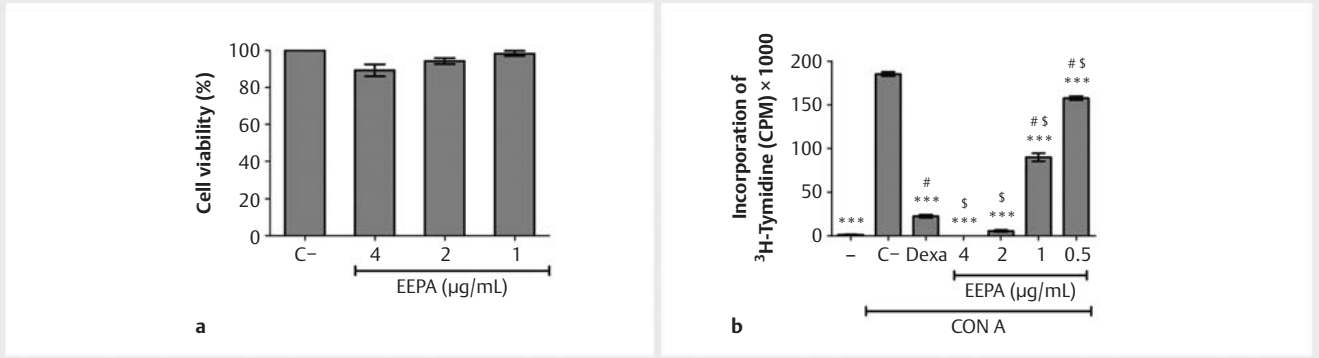
To investigate the immunosuppressive activity of EEPA, the antiproliferative effect of the extract was first evaluated in cultures of splenocytes stimulated with Con A. As shown in ▶ **Fig. 2a**, treatment with EEPA did not affect spleen cell viability. In cultures of splenocytes activated with Con A, treatment with the extract reduced lymphoproliferation in a concentration-dependent manner ($p < 0.05$). EEPA, at 4 and 2 µg/mL, had an equipotent effect to 4 µg/mL dexamethasone (▶ **Fig. 2b**). Additionally, EEPA reduced, in a concentration-dependent manner, the levels of IL-2, IL-6, and IFN-γ by activated splenocytes (▶ **Fig. 3a, c, e**). In contrast, EEPA induced a significant increase in IL-4 levels (▶ **Fig. 3b**) and did not modulate IL-10 levels (▶ **Fig. 3d**), while dexamethasone significantly reduced the levels of all cytokines evaluated (▶ **Fig. 3**).

Flow cytometry analysis was carried out in splenocyte cultures in order to determine the effect of EEPA on cell cycle progression and cell death. Treatment with EEPA induced, in a concentration-dependent manner, cell cycle arrest on the G1 phase, accompanied by an increase in the S phase (▶ **Fig. 4a, d**). An increase in the number of cells in the pre-G1 phase was found in the groups treated with EEPA (▶ **Fig. 4a, c**). Moreover, cell cycle arrest was accompanied by a concentration-dependent increase in Annexin V-positive cells, a hallmark of apoptosis (▶ **Fig. 4b, d**).

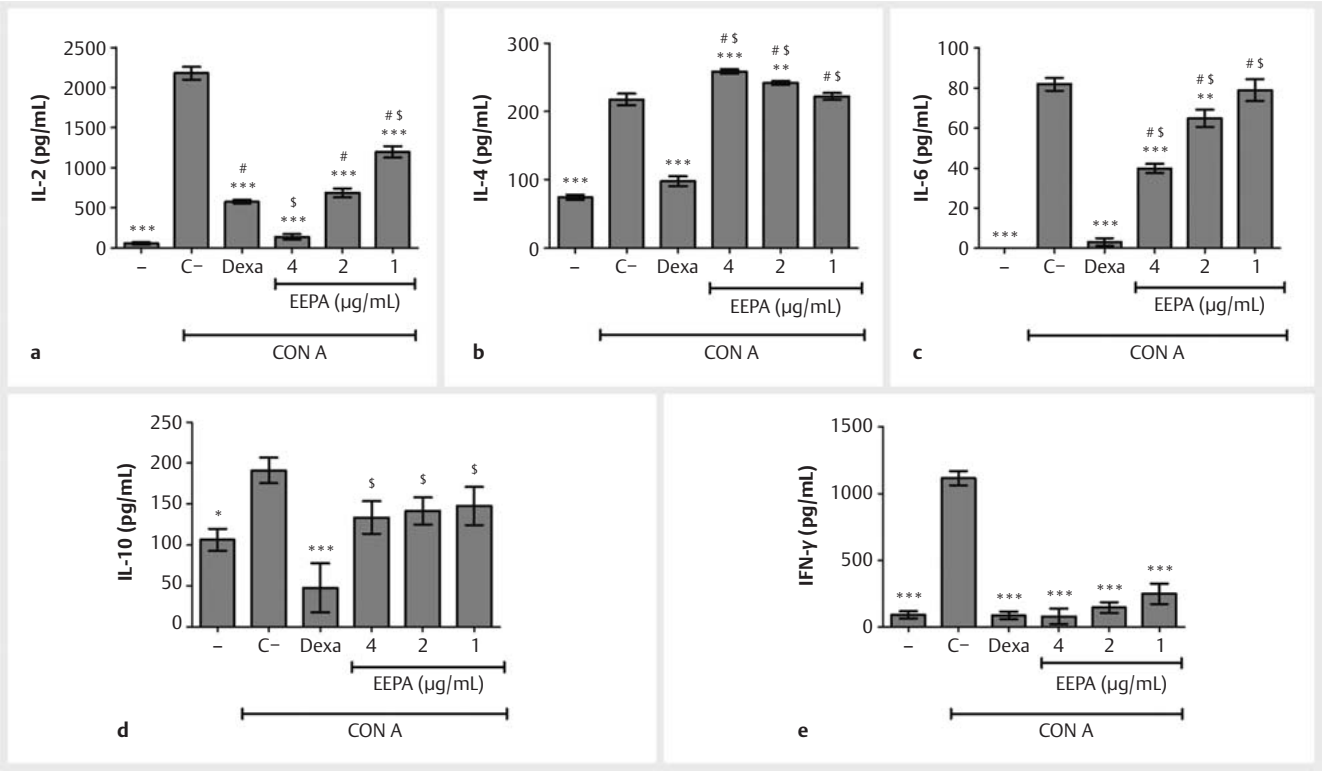
To evaluate the acute toxicity of EEPA, single oral treatment of EEPA to BALB/c mice (n = 6/group) at doses of 100 and 200 mg/kg was performed. Mice treated with EEPA did not show signs of toxicity, and no mortality was observed. We also measured serum biochemical components in uninfected mice treated with EEPA (▶ **Table 2**). In comparison to untreated mice, treatment with EEPA did not change serum components.



▶ **Fig. 1** Effects of EEPA on macrophages *in vitro*. Mouse peritoneal exudate macrophages stimulated or not with LPS + IFN-γ were cultured in the absence or presence of the ethanolic concentrated extract from *P. angulata* (EEPA; 4, 2, 1, or 0.5 μg/mL) or dexamethasone (Dexa; 4 μg/mL). Cell viability (**a**) was determined by the Alamar Blue method. Cell-free supernatants were collected after 24 h for nitrite (**b**), IL-6 (**d**), IL-10 (**e**), and IL-12 (**f**) quantification, or 4 h for TNF-α quantification (**c**). $^{-}$Group of untreated and unstimulated cells. $^{C-}$Group of untreated cells simulated with LPS + IFN-γ. Values represent the means ± SEM of nine determinations obtained in three independent experiments. *** P 0.001 compared to stimulated and untreated cells, ** $p < 0.01$ compared to stimulated and untreated cells, * $p < 0.05$ compared to stimulated and untreated cells, # $p < 0.05$ compared to unstimulated and untreated cells, and $ $p < 0.05$ compared to dexamethasone-treated cells.



▶ **Fig. 2** Inhibition of lymphocyte proliferation by EEPA. **a** Cell viability was determined by PI staining after 72 h of treatment with the ethanolic concentrated extract from *P. angulata* (EEPA; 4, 2, or 1 μg/mL). **b** Effect of EEPA (4, 2, or 1 μg/mL) or dexamethasone (Dexa; 4 μg/mL) on lymphoproliferation induced by concanavalin A. Proliferation rates were assessed by ^{3}H-thymidine incorporation. $^{-}$Group of untreated and unstimulated cells. $^{C-}$ Group of untreated cells but simulated with Con A. Values represent means ± SEM of nine determinations obtained in three independent experiments. *** $P < 0.001$ compared to stimulated and untreated cells, # $p < 0.05$ compared to unstimulated and untreated cells, and $ $p < 0.05$ compared to dexamethasone-treated cells.



▶ **Fig. 3** Assessment of cytokine production by splenocytes treated with EEPA. Concentrations of IL-2 (**a**), IL-4 (**b**), IL-6 (**c**), IL-10 (**d**), and IL-12 (**e**) were determined in cell-free supernatants from splenocyte cultures treated or not with the ethanolic concentrated extract from *P. angulata* (EEPA; 4, 2, or 1 µg/mL) or dexamethasone (Dexa; 4 µg/mL) in the presence of concanavalin A (Con A; 5 µg/mL) for 48 h. Cell-free supernatants were then collected for cytokine measurement by ELISA. $^{-}$Group of untreated and unstimulated cells. $^{C-}$ Group of untreated cells but simulated with Con A. Values represent the means ± SEM of nine determinations obtained in three independent experiments. *** $P < 0.001$ compared to stimulated and untreated cells, ** $p < 0.01$ compared to stimulated and untreated cells, * $p < 0.05$ compared to stimulated and untreated cells, # $p < 0.05$ compared to unstimulated and untreated cells, and $ $p < 0.05$ compared to dexamethasone-treated cells.

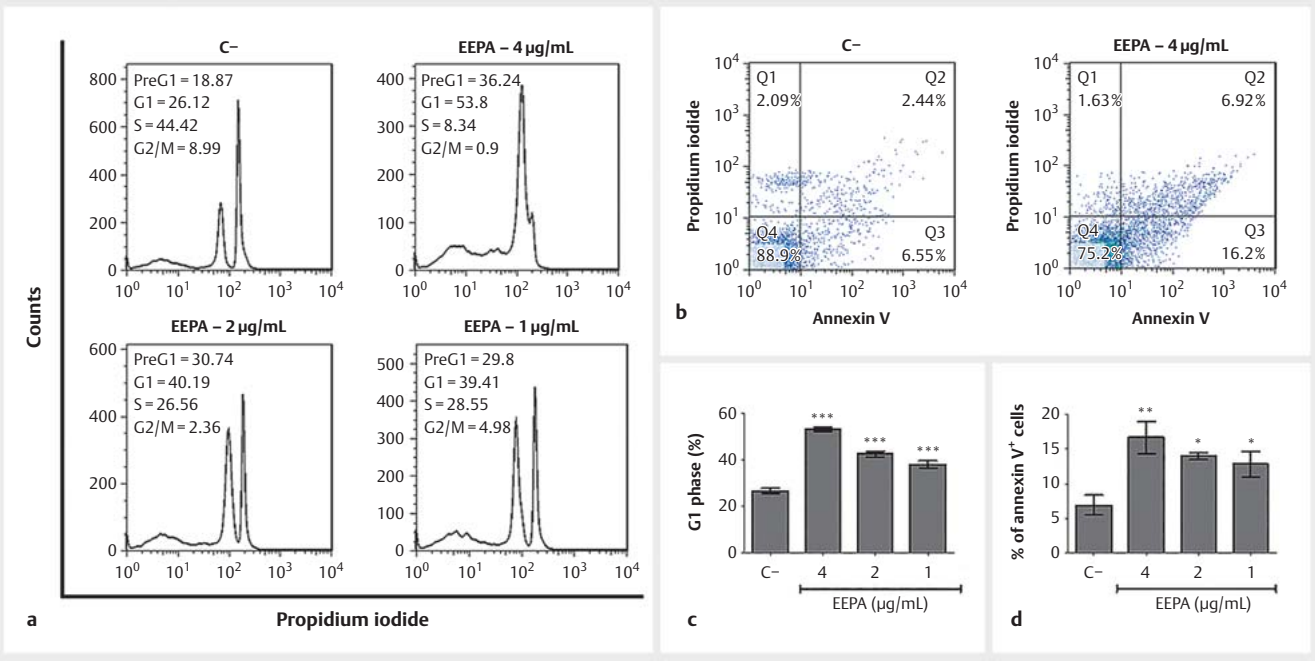
Next, the anti-inflammatory effect of EEPA was evaluated in a mouse model of acute peritonitis induced by carrageenan. In the vehicle-treated group, pretreatment with EEPA at doses of 100 and 50 mg/kg caused a reduction of 88.5 and 72.3% in the number of neutrophils, respectively, while dexamethasone at 25 mg/kg induced a 99.7% reduction (▶ **Fig. 5a**).

Finally, we investigated the effects of EEPA in a BSA-induced DTH reaction model in BALB/c mice. Following sensitization, mice were treated with EEPA or dexamethasone and the thickness of the paws was measured before and after challenge. As shown in ▶ **Fig. 5b**, treatment with 100 mg/kg of EEPA, but not 50 mg/kg, caused a significant reduction ($p < 0.05$) of paw edema. Under the same conditions, dexamethasone (25 mg/kg) also promoted a significant reduction in paw edema (▶ **Fig. 5b**).

Discussion

Natural products, such as physalins, which have a broad spectrum of action, are of interest for pharmacological development [8, 10, 19]. Due to the high cost and low yield of the production of pure physalins, the use of a standardized extract may be an alternative [17]. In this context, the present work investigated the immunomodulatory effect of a standardized ethanolic extract of *P. angulata* in experimental models *in vitro* and *in vivo*. We demonstrated here the immunomodulatory potential of EEPA, *in vitro* and *in vivo*, in down-modulating both macrophage and lymphocyte functions. Importantly, the preparation was nontoxic, indicating a safe profile.

Macrophages play numerous roles in immune responses, including the production of several inflammatory mediators that act in the induction and resolution of inflammation [20]. Among these are proinflammatory molecules, such as IL-6, IL-12, TNF-α, and nitric oxide, which play important roles in inflammatory processes [20]. IL-6 is a key cytokine in the production of acute inflammation proteins [21]. IL-12 acts mainly by inducing an increase in the production of IFN-γ by lymphocytes and inducing a Th1-type response [22]. TNF-α is considered a "master regulator" of proinflammatory responses, since it plays a critical role in the recruitment and activation of macrophages and lymphocytes, as well as in the production of other inflammatory molecules [23]. Nitric oxide, when produced in excess, promotes vasodilation, vascular permeability, and edema, classic signs of acute inflammation [24]. The production of these inflammatory mediators was suppressed by treatment with EEPA in a concentration-dependent manner. The results obtained in *in vitro* experimental models corroborate with the potent anti-inflammatory effect of

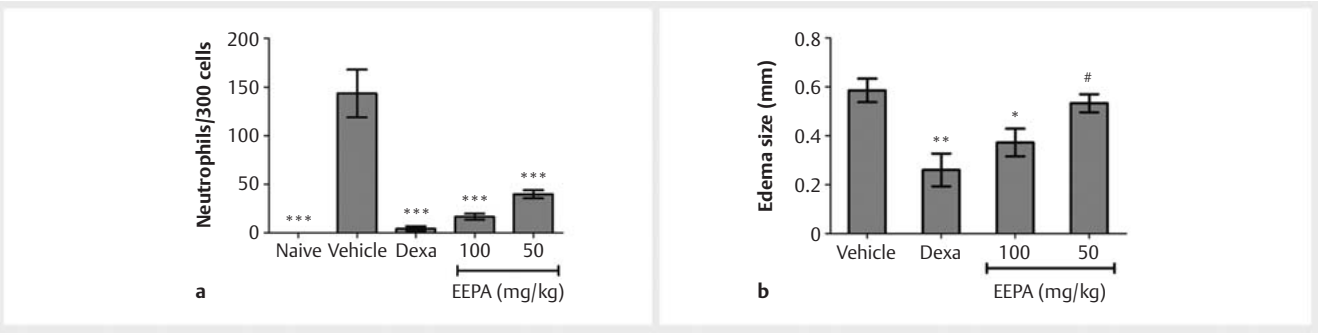


▶ **Fig. 4** Analysis of cell cycle progression and cell death after treatment with EEPA. Splenocytes activated with Con A were treated or not with the ethanolic concentrated extract from *P. angulata* (EEPA; 4, 2, or 1 µg/mL) for 24 and 48 h for cell death determination and cell cycle analysis, respectively. **a** The distribution and percentage of cells in pre-G1, G1, S, and G2/M phases of the cell cycle are indicated. **b** Representative dot plots of untreated and treated activated splenocytes with EEPA (4 µg/mL). **c** ercentage of cells in the G1 phase of the cell cycle. **d** Percentage of stained cells for Annexin V after 24 h of treatment with EEPA. Values represent the means ± SEM of nine determinations obtained in three independent experiments. *** $P < 0.001$ compared to stimulated and untreated cells, ** $p < 0.01$ compared to stimulated and untreated cells, and * $p < 0.05$ compared to stimulated and untreated cells.

▶ **Table 2** Assessment of biochemical parameters in sera from BALB/c mice after 24 h of treatment with the EEPA.

Parameters	Vehicle	EEPA	
		200 mg/kg	100 mg/kg
ALK PHOS (U/L)	190.8 (± 20.5)	216.4 (± 14.5)	194.8 (± 23.5)
AST (U/L)	199 (± 42.2)	172.5 (± 52.2)	230 (± 26.9)
ALT (U/L)	38.2 (± 27.9)	39.6 (± 13.7)	46.5 (± 16.1)
AML (U/L)	1359 (± 56.4)	1547 (± 95.1)	1507 (± 100.1)
BUN (mg/dL)	18.6 (± 6.3)	19.3 (± 4.5)	26.3 (± 2.7)
GLU (mg/dL)	223 (± 17.2)	244.5 (± 19.7)	241.3 (± 40.0)
PHOS (mg/dL)	15.7 (± 1.0)	14.9 (± 1.2)	14.4 (± 0.4)
Ca^{+2} (mg/dL)	9.3 (± 0.6)	9.2 (± 0.7)	9.3 (± 0.4)
CHO (mg/dL)	88.6 (± 5.7)	94 (± 15. 8)	87.8 (± 19)
URIC (mg/dL)	1.2 (± 0.5)	1.1 (± 0.1)	1.1 (± 0.1)
TPRO (mg/dL)	5.2 (± 0.2)	5.2 (± 0.4)	5.3 (± 0.2)
ALB (g/dL)	3.4 (± 0.2)	3.4 (± 0.3)	3.6 (± 0.3)
GLOB (g/dL)	1.6 (± 0.4)	1.7 (± 0.2)	1.7 (± 0.1)
ALB/GLOB	2.3 (± 0.7)	2 (± 0.2)	1.8 (± 0.6)

Animals were treated orally with a single dose of EEPA (200 or 100 mg/kg) or vehicle (10 % of DMSO in saline) 24 h before biochemical analysis. ALB: albumin, ALK PHOS: alkaline phosphatase, ALT: alanine aminotransferase, AML: amylase, AST: aspartate aminotransferase, BUN: urea, Ca^{+2}: calcium, CHO: cholesterol, GLU: glucose, PHOS: phosphorus, GLOB: globulin, TPRO: total proteins, URIC: uric acid



▶ **Fig. 5** Immunosuppressive effects of EEPA in mouse models of peritonitis and delayed-type hypersensitivity. **a** BALB/c mice were treated orally with the ethanolic concentrated extract from *P. angulata* (EEPA; 50 or 100 mg/kg), dexamethasone (25 mg/kg), or vehicle (saline solution containing 10% DMSO) by gavage 1 h before challenge with carrageenan (1%; v/v). Peritoneal exudate cells were collected 4 h after the injection of carrageenan. **b** Male BALB/c mice were treated orally with EEPA (50 or 100 mg/kg), dexamethasone (Dexa; 25 mg/kg), or vehicle (saline solution containing 10% DMSO) and challenged with 30 µL of a 2% suspension of heat-aggregated BSA in saline administrated in the footpad. At 3 h post-challenge, footpad thickness was measured with calipers and the extent of swelling was calculated by subtracting the thickness of the footpad after challenge from that before challenge. Dexa at 25 mg/kg was used as an anti-inflammatory reference via the oral route in both models. The naïve group consisted of untreated and unchallenged animals. Values represent the means ± SEM of six mice/group. *** $P < 0.001$ compared to vehicle group, ** $p < 0.01$ compared to vehicle group, * $p < 0.05$ compared to vehicle group, and # $p < 0.05$ compared to dexamethasone-treated mice.

EEPA observed in a carrageenan-induced murine model of peritonitis.

In agreement with these data, the aqueous extract formulated from roots of *P. angulata* also induced a potent anti-inflammatory effect in a rat model of carrageenan-induced peritonitis, where it significantly reduced the exudate volume, number of inflammatory cells, and production of nitric oxide and prostaglandin E_2 [25]. Some physalins, such as B and F, also have a potent inflammatory effect on activated macrophages, inhibiting the production of IL-6, IL-12, TNF-α, and nitric oxide [10]. The anti-inflammatory effect of physalins B and F was also demonstrated in a mouse model of endotoxic shock, where pretreatment with these molecules reduced serum levels of TNF-α and protected the animals against a lethal dose of LPS [10]. Altogether, these data reinforce the potential use of *P. angulata* for the treatment of inflammatory conditions, considering that extracts and pure substances obtained from this plant act on important inflammatory mediators produced by activated macrophages.

Although there are several reports about the anti-inflammatory activity of extracts or fractions obtained from *P. angulata*, little is known about the effects of these samples on lymphocyte function. In the present work, we showed that EEPA is a potent inhibitor of lymphocyte proliferation. In fact, the antiproliferative effect of physalins purified from *P. angulata* has been previously reported [11, 16, 26, 27]. In particular, physalin F has potent antiproliferative effects on different tumor cell lines, including acute T and B lymphoid leukemia cells lines, PBMC from patients with HTLV-1-associated myelopathy, and mouse spleen cells stimulated with Con A [1, 11, 16]. The antiproliferative activity of physalin F and other natural products from *P. angulata* may be due to cell cycle alterations followed by cell death by apoptosis [16, 27, 28]. Interestingly, in the present study, a similar pathway of cell death was observed in lymphocytes treated with EEPA. In addition, EEPA reduced the production of Th1-type cytokines (IL-2, IL-6, and IFN-γ) and increased a Th2 type cytokine (IL-4). Similar effects were obtained with physalin B and with withangulatin A in activated spleen cells [11, 29]. Altogether, these data suggest that the modulation of the Th1/Th2 balance in mouse T lymphocytes by natural products from *P. angulata* might be an important component of their immunosuppressive effects.

Finally, we observed a reduction of paw edema in a mouse model of BSA-induced DTH. Previous studies also detected the potential use of *P. angulata* for the treatment of inflammatory diseases mediated by T lymphocytes [11]. Physalins B, F, and G demonstrated potent immunosuppressive activity in a mouse model of acute allogeneic transplant rejection and type II collagen-induced arthritis [11, 13]. Moreover, treatment with EEPA did not modify critical hallmarks of toxicity, such as albumin (ALB) alanine aminotransferase (ALT), alkaline phosphatase (ALK PHOS), aspartate aminotransferase (AST), and urea (BUN) [30, 31].

As a part of an ongoing search for immunomodulatory agents from natural products, we validated EEPA as a potent and selective immunomodulatory agent, acting by immunosuppressing lymphocytes and macrophages, suggesting a strong potential of the ethanolic extract of *P. angulata* as an alternative for the treatment of immune-inflammatory diseases.

Materials and Methods

Preparation of the ethanolic extract of *Physalis angulata*

P. angulata stems were collected during the drier season (from June until November) in the city of Belém, Pará, Brazil. Plant identification was performed by Dr. Lucia Carvalho from the Botanical Garden of Rio de Janeiro. A voucher specimen (voucher number 15) was deposited in the Herbarium of the Department of Physiology from the University Federal of Pará. EEPA was prepared according to the methodology previously described [17]. A chemical profile of the extract, previously characterized, revealed four main

compounds: physalins B, D, F, and G. Using HPLC-UV, it was possible to find approximately 0.84% of physalin B, 0.90% of physalin D, 0.37% of physalin F, and 0.36% of physalin G in every 100 mg of EEPA [17].

Drugs

Dexamethasone (Sigma-Aldrich), a synthetic glucocorticoid, was used as a positive control in immunomodulatory assays. Gentian violet (Synth) was used as a positive control in the cytotoxicity assays. All compounds were dissolved in DMSO (PanReac) and diluted in DMEM (Life Technologies, GIBCO-BRL) for use in the *in vitro* assays. The final concentration of DMSO was less than 0.1% in all *in vitro* experiments and less than 10% in all *in vivo* experiments.

Animals

Male BALB/c mice (6 to 10 weeks old) were provided by the animal breeding facility of Gonçalo Moniz Institute and maintained in sterilized cages under a controlled environment, receiving water *ad libitum* and a balanced diet for rodents at the Gonçalo Moniz Institute (Oswaldo Cruz Foundation, Bahia, Brazil). All animal experiments and procedures were approved (April 7, 2016) by the institution's committee on the ethical handling of laboratory animals (approved number: L-IGM-018/15).

Cytotoxicity to mammalian cells

Peritoneal exudate macrophages were obtained by washing, with cold DMEM medium, the peritoneal cavity of BALB/c mice 4–5 days after injection of 3% thioglycolate (Sigma-Aldrich) in saline (1.5 mL per mice). Cells were plated into 96-well plates at a cell density of 1×10^5 cells/well in DMEM medium supplemented with 10% FBS (GIBCO) and 50 µg/mL of gentamicin (Life) and incubated for 24 h at 37 °C and 5% CO_2. After that time, each sample was added (10–0.04 µg/mL), in triplicate, and incubated for 72 h. Then, 20 µL/well of Alamar Blue (Invitrogen) were added to the plates for 10 h. Colorimetric readings were performed at 570 and 600 nm. Gentian violet was used as a positive control at the same concentrations. CC_{50} values were calculated using data from three independent experiments.

A second set of experiments was performed using peritoneal macrophages activated with LPS (500 ng/mL; Sigma-Aldrich) and IFN-γ (5 ng/mL; Sigma-Aldrich). Cells were seeded into 96-well plates at a cell density of 2×10^5 cells/well in DMEM supplemented with 10% FBS and 50 µg/mL of gentamicin and incubated for 24 h at 37 °C and 5% CO_2. EEPA (4, 2, 1, or 0.5 µg/mL) was then added in triplicate and incubated for 24 h, followed by the addition of 20 µL/well of Alamar Blue. After incubation of the plates for 10 h, the colorimetric readings were performed at 570 and 600 nm.

Finally, cell viability on splenocytes was also evaluated. For this purpose, splenocytes (5×10^6 cells/well) were seeded in 24-well plates, in triplicate, in the absence or presence of different concentrations of EEPA (4, 2, or 1 µg/mL). After 72 h of incubation, cells were centrifuged, and the pellet was washed twice with cold PBS and labeled with PI (2 µg/mL) in the dark at 37 °C for 30 min. The cell preparations were analyzed using a FACS Calibur flow cytometer (Becton Dickinson). A total of 10 000 events were acquired and data were analyzed using FlowJo software (Tree Star).

Macrophage cultures

Peritoneal exudate macrophages were plated in 96-well plates at 2×10^5 cells/well in DMEM medium supplemented with 10% of FBS and 50 µg/mL of gentamicin for 24 h at 37 °C and 5% CO_2. Cells were then stimulated with LPS (500 ng/mL) and IFN-γ (5 ng/mL) in the absence or presence of samples at different concentrations (4, 2, 1, and 0.5 µg/mL) and incubated at 37 °C. Cell-free supernatants were collected at 4 h (for TNF-α measurement) and 24 h (for IL-6, IL-10, IL-12, and nitrite quantifications) and kept at − 80 °C until use.

Splenocyte cultures

For lymphoproliferation assays, BALB/c splenocyte suspensions were prepared in DMEM medium supplemented with 10% of FBS and 50 µg/mL of gentamicin. Splenocytes (1×10^6 cells/well) were plated in 96-well plates, in triplicate, and stimulated or not with Con A (2 µg/mL; Sigma-Aldrich). To evaluate the lymphoproliferation, splenocytes were activated in the absence or presence of various concentrations of EEPA (4, 2, 1, or 0.5 µg/mL). After 48 h of incubation, 1 µCi of ^{3}H- thymidine was added to each well, incubated for 18 h and read, as described above. Cell proliferation was measured as the percent of ^{3}H-thymidine incorporation for treated cells in comparison to untreated cells. Dexamethasone was used as a positive control.

Splenocytes from BALB/c mice were also plated into 24-well plates at a cell density of 5×10^6 cells/well in DMEM medium supplemented with FBS containing or not 5 µg/mL of Con A in the absence or presence of different concentrations of EEPA (4, 2, and 1 µg/mL). After 24 h, cell-free supernatants were collected and kept at − 80 °C until use.

Assessment of cytokine and nitric oxide production

Cytokine concentrations in supernatants from peritoneal macrophages cultures collected at 4 h (for TNF-α) and 24 h (for IL-6, IL-10, and IL-12) and from splenocyte cultures (IL-2, IL-4, IL-6, IL-10, and IFN-γ) were determined by ELISA using DuoSet kits from R&D Systems, according to the manufacturer's instructions. Nitric oxide production was estimated in macrophage culture supernatants harvested at 24 h using the Griess method for nitrite quantification [32].

Flow cytometry analysis

Splenocytes from BALB/c mice were plated into 24-well plates at a cell density of 5×10^6 cells/well in DMEM medium supplemented with 10% FBS containing 5 µg/mL of Con A in the absence or presence of different concentrations of EEPA (4, 2, and 1 µg/mL) for 24 and 48 h for cell death determination and cell cycle analysis, respectively. For cell death determination, cells were centrifuged and then the pellet was washed twice with cold PBS and labeled with PI and Annexin V using the Annexin V-FITC apoptosis detection kit (Sigma-Aldrich) according to the manufacturer's instructions. For cell cycle analysis, cells were stained with a solution of PBS with PI (2 µg/mL), RNAase (100 µg/mL), and 0.1% of Triton X-100 in the dark at 37 °C for 30 min. The cell preparations were analyzed using a FACS Calibur flow cytometer. A total of 10 000 events were acquired and data were analyzed using FlowJo software.

Toxicity in mice

Male BALB/c mice (6–8 weeks old; n = 6/group) were orally treated with EEPA (single administration) at doses of 200 and 100 mg/kg. Animals were monitored for signs of general toxicity, including behavior and feeding, until 24 h after treatment. Heparinized blood samples were collected after 24 h of treatment, centrifuged for 3 min to separate out from cells, and then serum components were analyzed using the Analyst (Hemagen) platform system.

Induction of acute peritonitis in mice

Male BALB/c mice (8–10 weeks of age; n = 6/group) were randomized into four groups and treated orally with EEPA (100 or 50 mg/kg), dexamethasone (25 mg/kg), or vehicle (10% of DMSO in saline) 24 and 1 h before challenge. Next, animals were challenged with a 250 µL injection of carrageenan (1 mg/mL; intraperitoneal route). After 4 h, the animals were euthanized, and peritoneal exudates were harvested by peritoneal lavage using 2.5 mL of saline solution. Cells were centrifuged at × 400 *g* for 10 min at 4 °C. The pellet was resuspended in saline (1 mL). Total leukocytes in peritoneal fluid were determined in a Neubauer chamber after dilution in Trypan blue stain. Differential counting of neutrophils was carried out in rapid panotype-stained cytospin preparations. A differential count of 300 cells was made in a blinded fashion and according to standard morphologic criteria.

Delayed-type hypersensitivity assay

Male BALB/c mice (8–12 weeks of age; n = 6/group) were sensitized by injecting 50 µg of crystallized BSA (Sigma-Aldrich) emulsified in 20 µL of complete Freund's adjuvant (CFA; Sigma-Aldrich) subcutaneously into each side of the base of tail. Seven days later, animals were randomized into four groups and treated with EEPA (100 or 50 mg/kg), dexamethasone (25 mg/kg), or vehicle (10% of DMSO in saline) by the oral route 24 and 3 h before challenge. DTH was elicited by injection of 30 µL of a 2% suspension of heat-aggregated BSA in saline subcutaneously into the footpad, according to a previously reported method [33]. Before challenge and at 3 h post-challenge, footpad thickness was measured with a digital caliper and the extent of swelling was calculated by subtracting the thickness of the footpad after challenge from that before challenge.

Statistical analyses

To determine the CC_{50} of BALB/c mice macrophages, we used nonlinear regression. One-way analysis of variance and Newman-Keuls multiple comparison tests were employed by using Graph Pad Prism version 5.01 (Graph Pad Software). Differences were considered significant when the values of p were < 0.05. The data are representative of at least two or three experiments.

Contributors' Statement

The extract production was done by T. C. B. Tomassini and I. M. Ribeiro. The immunomodulatory experiments was designed by C. S. Meira, D. R. M. Moreira and M. B. P. Soares and conducted by S. R. T. Daltro, I. P. Santos and P. L. Barros. Data was analyzed by S. R. T. Daltro, P. L. Barros, C. S. Meira and R. Ribeiro-dos-Santos. The manuscript was written by S. R. T. Daltro, I. P. Santos, C. S. Meira, T. C. B. Tomassini and revised by D. R. M. Moreira, R. Ribeiro-dos-Santos and M. B. P. Soares.

Acknowledgements

This work was supported by grants from CNPq (grant number 562655/2010-7), PRONEX (grant number 0002/2014) and FAPESB (grant number 0042/2013).

Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Pio Corrêa M, Pena LA. Dicionário das Plantas Úteis do Brasil e das Exóticas Cultivadas, Primeira Edição. Rio de Janeiro: Ministério da Agricultura, Instituto Brasileiro de Desenvolvimento Florestal; 1984

[2] Lin YS, Chiang HC, Kan WS, Hone E, Shih SJ, Won MH. Immunomodulatory activity of various fractions derived from *Physalis angulata* L. extract. Am J Chin Med 1992; 20: 233–243

[3] Row LR, Sarma NS, Reddy KS, Matsuura T, Nakashima R. The structures of physalins F and J from *Physalis angulata* L. and *Physalis lancifolia*. Phytochemistry 1978; 17: 1647–1650

[4] Row LR, Reddy KS, Sarma NS, Matsuura T, Nakashima R. New physalins from *Physalis angulata* L. and *Physalis lancifolia* structures and reactions of physalins D, I, G and K. Phytochemistry 1980; 17: 1175–1181

[5] Guimarães ET, Lima MS, Santos LA, Ribeiro IV, Tomassini TBC, Ribeiro-dos-Santos R, Santos WLC, Soares MBP. Effects of seco-steroids purified from *Physalis angulata* L., Solanaceae, on the viability of *Leishmania* sp. Rev Bras Farmacogn 2010; 20: 945–949

[6] Helvaci S, Kokdil G, Kawai M, Duran N, Duran G, Guvenç A. Antimicrobial activity of the extracts and physalin D from *Physalis alkekengi* and evaluation of antioxidant potential of physalin D. Pharm Biol 2010; 28: 142–150

[7] Sá MS, Menezes MN, Krettli AU, Ribeiro IM, Tomassini TCB, Ribeiro-dos-Santos R, Azevedo WF jr., Soares MBP. Antimalarial activity of physalins B, D, F and G. J Nat Prod 2010; 74: 2269–2272

[8] Meira CS, Guimarães ET, Bastos TM, Moreira DRM, Tomassini TCB, Ribeiro IM, Ribeiro-dos-Santos R, Soares MBP. Physalins B and F, seco-steroids isolated from *Physalis angulata* L., strongly inhibit proliferation, ultrastructure and infectivity of *Trypanosoma cruzi.* Parasitology 2013; 140: 1811–1821

[9] Sun CP, Qiu CY, Zhao F, Kang N, Chen LX, Qiu F. Physalins V–IX, 16,24-cyclo-13,14-seco withanolides from *Physalis angulata* and their antiproliferative and anti-inflammatory activities. Sci Rep 2017; 22: 4057

[10] Soares MBP, Bellintani MC, Ribeiro IM, Tomassini TCB, Ribeiro-dos-Santos R. Inhibition of macrophage activation and lipopolysaccharide-induced death by seco-steroids purified from *Physalis angulata* L. Euro J Pharmacol 2003; 459: 107–112

[11] Soares MBP, Santos BD, Bellitani MC, Paiva FP, Ribeiro YM, Tomassini TCB, Ribeiro-dos-Santos R. Physalins B, F and G, seco-steroids purified from *Physalis angulata* L., inhibit lymphocyte function and allogeneic transplant rejection. Int Immunopharmacol 2006; 6: 408–414

[12] Jacobo-Herrera NJ, Bremner P, Marquez N, Gupta MP, Gibbons S, Muñoz E, Heinrich M. Physalins from *Witheringia solanacea* as modulators of the NF-kappaB cascade. J Nat Prod 2006; 69: 328–331

[13] Brustolim D, Vasconcelos JF, Freitas LA, Teixeira MM, Farias MT, Ribeiro IM, Tomassini TCB, Oliveira GG, Pontes-de-Carvalho LC, Ribeiro-dos-Santos R, Soares MBP. Activity of physalin F in a collagen-induced arthritis model. J Nat Prod 2010; 73: 1323–1326

[14] Ji L, Yuan Y, Luo L, Chen Z, Ma X, Cheng L. Physalins with anti-inflammatory activity are present in *Physalis alkekengi* var. *franchetii* and can function as Michael reaction acceptors. Steroids 2012; 77: 441–447

[15] Yang YJ, Yi L, Wang Q, Xie BB, Dong Y, Scha CW. Anti-inflammatory effects of physalin E from *Physalis angulata* on lipopolysaccharide-stimulated RAW 264.7 cells through inhibition of NK-κB pathway. Immunopharmacol Immunotoxicol 2017; 39: 74–79

[16] Pinto LA, Meira CS, Villarreal CF, Vannier-Santos MA, De Souza CV, Ribeiro IM, Tomassini TC, Galvão-Castro B, Soares MBP, Grassi MF. Physalin F, a seco-steroid from *Physalis angulata* L., has immunosuppressive activity in peripheral blood mononuclear cells from patients with HTLV1-associated myelopathy. Biomed Pharmacother 2016; 79: 129–134

[17] Nogueira RC, Rocha VPC, Nonato FR, Tomassini TCB, Ribeiro IM, Ribeiro-dos-Santos R, Soares MBP. Genotoxicity and antileishmanial activity evaluation of *Physalis angulata* concentrated ethanolic extract. Environ Toxicol Pharmacol 2013; 36: 1304–1311

[18] Meira CS, Guimarães ET, Santos JAF, Moreira DRM, Nogueira RC, Tomassini TCB, Ribeiro IM, Souza CVC, Ribeiro-dos-Santos R, Soares MBP. *In vitro* and *in vivo* antiparasitic activity of *Physalis angulata* L. concentrated ethanolic extract against *Trypanosoma cruzi.* Phytomedicine 2015; 22: 969–974

[19] Vieira AT, Pinho V, Lepsch LB, Scavone C, Ribeiro IM, Tomassini TCB, Ribeiro-dos-Santos R, Soares MBP, Teixeira MM, Souza DG. Mechanisms of the anti-inflammatory effects of the natural secosteroids physalins in a model of intestinal ischaemia and reperfusion injury. Br J Pharmacol 2005; 146: 244–251

[20] Dey A, Allen J, Hankey-Giblin PA. Ontogeny and polarization of macrophages in inflammation: blood monocytes versus tissue macrophages. Front Immunol 2015; 5: 683

[21] Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol 2014; 6: a016295

[22] Vecchio MD, Bajetta E, Canova S, Lotze MT, Wesa A, Parmiani G, Anichini A. Interleukin-12: biological properties and clinical application. Clin Cancer Res 2007; 13: 4677–4685

[23] Parameswaran N, Patial S. Tumor necrosis factor-α signaling in macrophages. Crit Rev Eukaryot Gene Expr 2011; 20: 87–103

[24] El-Haj L, Bestle MH. Nitric oxide and sepsis. Ugeskr Laeger 2017; 179: V011770086

[25] Bastos GN, Silveira AJ, Salgado CG, Picanço-Diniz DL, Nascimento JL. *Physalis angulata* extract exerts anti-inflammatory effects in rats by inhibiting different pathways. J Ethnopharmacol 2008; 118: 246–251

[26] Sun CP, Oppong MB, Zhao F, Chen XL, Qiu F. Unprecedented 22,26-seco physalins from *Physalis angulata* and their anti-inflammatory potential. Org Biomol Chem 2017; 15: 8700–8704

[27] Wu YW, Leu YL, Chang YL, Wu TS, Kuo PC, Liao YR, Teng CM, Pan SL. Physalin F induces cell apoptosis in human renal carcinoma cells by targeting NF-KappaB and generating reactive oxygen species. PLoS One 2012; 7: e40727

[28] Wang A, Wang S, Zhou F, Li P, Wang Y, Gan L, Lin L. Physalin B induces cell cycle arrest and triggers apoptosis in breast cancer cell through modulating p53-dependent apoptotic pathway. Biomed Pharmacother 2018; 101: 334–341

[29] Sun L, Liu J, Liu P, Yu Y, Ma L, Hu L. Immunosuppression effect of Withangulatin A from *Physalis angulata* via heme oxygenase 1-dependent pathways. Process Biochem 2011; 46: 482–488

[30] Sookoian S, Pirola CJ. Liver enzymes, metabolomics and genome-wide association studies: From systems biology to the personalized medicine. World J Gastroenterol 2015; 21: 711–725

[31] Carobene A, Braga F, Roraes T, Sandberg S, Bartlett WA. A systematic review of data on biological variation for alanine aminotransferase, aspartate aminotransferase and y-glutamyl transferase. Clin Chem Lab Med 2013; 51: 1997–2007

[32] Green LC, Wagne DA, Glogowski J, Skippe PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15 N] nitrate in biological fluids. Anal Biochem 1982; 126: 131–138

[33] Titus RG, Chiller JM. A simple and effective method to asses murine delayed type hypersensitivity to proteins. J Immunol Methods 1981; 45: 65–78