SUPPLEMENTARY MATERIAL

Physalis angulata concentrated ethanolic extract suppresses nociception and inflammation by modulating cytokines and prostanoids pathways

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Abstract

Physalins are *seco*-steroids with a variety of pharmacological activities already described. In this study the pharmacological properties of a standardized concentrated ethanolic extract from *Physalis angulata* (CEEPA), rich in physalins B, D, F and G, were studied in models of pain and inflammation in mice. Inflammatory mediators were measured by radioimmunoassay and Real-Time PCR in mice paws after the CFA stimuli. Systemic administration of CEEPA produced antinociceptive effect on the writhing test and formalin test. In the writhing test, physalins B, D, F and G showed that the antinociceptive effect of CEEPA is more potent than that of these purified compounds. In addition, CEEPA reduced the levels of TNF-α, IL-1β, COX-2 and iNOS mRNA in the CFA-induced paw inflammation. Likewise, CEEPA decreased the TNF-α, IL-1β and PGE_2 paw levels. In conclusion, CEEPA induces antinociceptive and anti-inflammatory effects, with improved pharmacological potency relative to pure physalins, associated to modulation of cytokine and cyclooxygenase pathways.

Keywords

anti-inflammatory, antinociception, immunomodulation, physalins, herbal drug extracts.

Experimental

Plant material and production of CEEPA

P. angulata specimens were collected during the drier season (from June until November) in Belém, Brazil, and received botanic identification by Dr. Lúcia Carvalho. A voucher specimen was deposited in the Herbarium of the University Federal do Rio de Janeiro (Voucher number RFA23907/08). Stems of *P. angulata* (1 kg) were dried and crushed, followed by extraction with ethanol at 50 - 60^{o} C during 6 h. The ethanolic extract was concentrated under reduced pressure, yielding 100 g (10 %) of crude extract. The concentrated ethanolic extract was maintained in a desiccator under vacuum until weight stabilized. HPLC-UV analysis was determined in an Agilent HP1100 series system consisting of an auto-sampler, high-pressure mixing pup and UV detector (Agilent Technologies, Santa Clara, CA, USA). HPLC-UV conditions were: run in phase reverse column Hibar® LiChrospher® 100, RP-18 - 25cm X 4 mm - 5 μm (Merck; Darmstadt, Germany), oven temperature 30°C, and UV absorbance was measured at 225 nm by injecting 20 μl of CEEPA (10 mg/ml) in MeOH. The solvent system was: A, MeCN and B, H_2O containing 0.05% trifluoroacetic acid; gradient: 3–3% of A in 0–6 min, 3–10% of A in 3–6 min, 10–10% of A in 6–9 min, 10–18% in 9–12 min, 18–18% in 12–15 min, 18 to 21% in 15–18 min, 21–21% in 18–21 min, 21–35% in 21–45 min, 35–35% in 45–55 min, 35–80% in 55–70 min, 80–80% in 70–55 min, at flow rate 1 ml/min. The presence of physalins B, D, F and G in the CEEPA was confirmed by comparing with standard isolated physalin D, previously characterized (Nogueira et al. 2013). These procedures were executed in accordance with European Medicines Agency guidelines for herbal products.

Animals and treatments

Experiments were performed on male Swiss Webster mice obtained from the Animal Facilities at the Instituto Gonçalo Moniz (FIOCRUZ, Salvador, Brazil). Animals (22−28 g) were housed in temperature-controlled room (22−25 °C), under a 12/12 h light−dark cycle, with access to water and food *ad libitum* until experimental initiation. All behavioral tests were performed between 8:00 A.M. and 5:00 P.M., and animals were used only once. Animal care and handling procedures were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH, 8023). The protocol was approved by the Institutional Animal Care and Use Committee - Ethics Committee for Animal Experimentation of FIOCRUZ (CEUA/FIOCRUZ; Permit Number: L-IGM-015/2013). Every effort was made to minimize the number of animals used and any discomfort. Behavioral tests were performed without knowing to which experimental group

each mouse belonged. Mice were treated with physalins, CEEPA, vehicle (5% DMSO in saline; control group), or reference drugs by intraperitoneal (ip) route, 40 min prior to stimulus exposure. The reference drugs indomethacin (purity > 99 %) and dexamethasone (purity > 98 %) were purchased from Sigma (St. Louis, USA).

Writhing test

Acetic acid (0.8% v/v, 10 mL/kg) was injected into the peritoneal cavities of the animals and counted the total number of writhes between 0 and 30 min after a stimulus injection. Antinociceptive activity was expressed as writhing scores over 30 min. Indomethacin (10 mg/kg) was used as the reference drug in this test (Espírito-Santo et al. 2017).

Formalin test

An injection (20 μL) of diluted formalin (1:100 dilution of stock formalin solution, 37% formaldehyde in 0.9% saline) was administered subcutaneously (sc) to the dorsum of the hind paw. The response was the amount of time the animals spent licking the injected paw from 0 to 10 min (early phase) and 10 to 30 min (late phase). Indomethacin (10 mg/kg) and morphine (5 mg/kg) were used as reference drugs in this test (Espírito-Santo et al. 2017).

Rota-rod test

To evaluate possible deficit motor of the CEEPA, mice were submitted to the rota-rod test, as previously described (Gama et al. 2013). The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 120 s. Animals were treated intraperitoneally with diazepam (10 mg/kg; reference drug), CEEPA (150 mg/kg), or vehicle (5% DMSO in saline; control group) and, 40 min afterward, were placed on a rotating rod. The resistance to falling was measured for up to 120 s. The results are expressed as the average time (s) the animals remained on the rota-rod in each group.

Open field

To assess the possible effects of CEEPA on locomotor activity, mice were evaluated in an open field test, as described by Santos et al. (Santos et al. 2012). Mice were treated intraperitoneally with diazepam (10 mg/kg; reference drug), CEEPA (150 mg/kg), or vehicle (5% DMSO in saline; control group) and, 40 min afterward, were placed individually in a wooden box (40 × 60 × 50 cm) with the floor divided into 12 squares. The number of squares crossed with the four paws was

measured for a period of 3 min. Results were expressed as the number of squares crossed by the mice.

Inflammatory model

Mice were lightly anesthetized with halothane and received 20 µL of complete Freund's adjuvant (CFA 1 mg/mL of heat killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannidemonoleate; Sigma Chemical Company, St. Louis, MO, USA) in the plantar region of the right hind paw, according to a previously reported method (Lima et al. 2013). Dexamethasone (2 mg/kg) was used as the reference drug in this test. Paw edema and inflammatory mediator levels were measured by plesthismometer, qRT-PCR and immunoassay, as described below.

Plesthismometer test

The volume of each mouse paw was measured (mm^3) with a plesthismometer (Ugo Basile, Comerio, Italy) before (Vo) and after (VT) the CFA injection, as described previously (Lima et al. 2013). The amount of paw swelling was determined for each mouse and data were represented as paw volume variation (Δ, mm^3).

Real-time PCR

The transcription of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α) genes was evaluated by real-time quantitative polymerase chain reaction (qRT-PCR) in mice euthanized 6 h after the CFA injection (Chen et al. 2010). RNA was extracted from plantar skin of the hind paws with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the concentration determined by photometric measurement. A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize cDNA from 1 µg of RNA, according to the manufacturer's recommendations. Synthesis of cDNA and RNA expression analysis was performed by Real-Time PCR using TaqMan Gene Expression Assay for TNF-α (Mm00443258_m1), IL-1β (Mm0043228_m1), IL-6 (Mm00446190_m1), IL-10 (Mm00439616_m1), COX-2 (Mm01307320_m1) and iNOS (Mm01309898_m1). A no-template control (NTC) and no-reverse transcription controls (No–RT) were also included. All reactions were run in duplicate on an ABI7500 Sequence Detection System (Applied Biosystems) under standard thermal cycling conditions. The mean Ct (cycle threshold) values from duplicate measurements were used to calculate expression of the target gene, with normalization to an

internal control - Gapdh (Mm99999915_g1), using the ΔΔCt formula. Experiments with coefficients of variation greater than 5% were excluded.

Measurement of cytokines in paw skin

The paw levels of TNF-α and IL-1β were determined by ELISA, as previously described (Lima et al. 2013). Treatments were performed 40 min before the CFA injection. Skin tissues were removed from the paws 6 h after CFA, in mice terminally anesthetized with halothane from each experimental group. Tissue proteins were extracted from 100 mg tissue/mL phosphate buffered saline (PBS) to which 0.4 M NaCl, 0.05% Tween 20 and protease inhibitors (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KI aprotinin A/100 ml) were added (Sigma Chemical Company, St. Louis, MO, USA). The samples were centrifuged for 10 min at 3000 g and the supernatant was frozen at -70 ºC for later quantification. TNF-α and IL-1β levels were estimated using a commercially available immunoassay ELISA kit for mice (R&D System, Minneapolis, MN, USA), according to the manufacturer's instructions. The results are expressed as picograms of cytokine per milligram of protein.

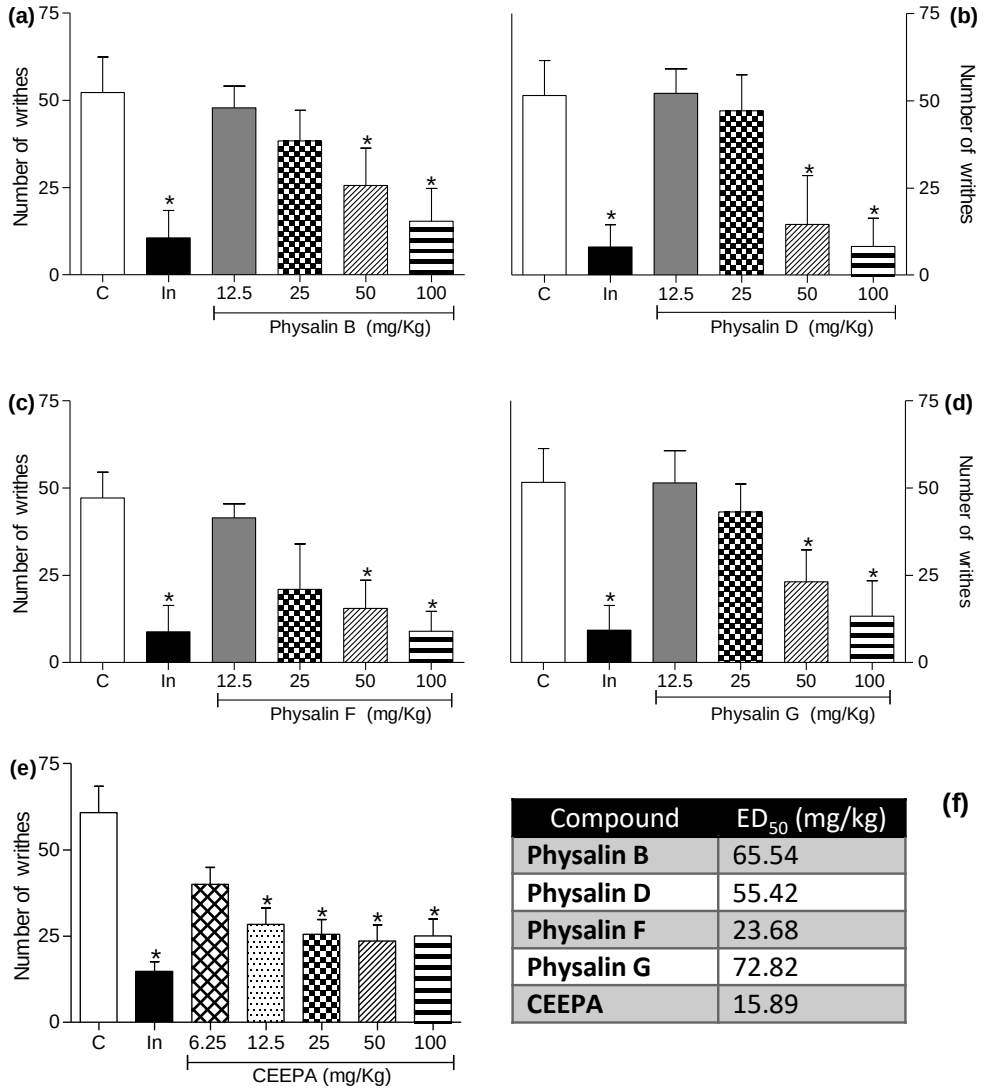
Measurement of PGE_2 in paw skin

Treatments were performed 40 min before the CFA injection. The plantar tissues were collected 3 h after CFA. The paws were injected with indomethacin (50 μg/paw) 10 min before tissue retrieval to block PGE_2 production during tissue processing. The PGE_2 levels were determined by radioimmunoassay, as previously described (Cunha et al. 2010). Briefly, the plantar tissue samples were homogenized in a mixture of 3.0 ml of extraction solvent (isopropanol/ethyl acetate/0.1 N HCl, 3:3:1) and 3.0 ml of distilled water contained 20 μg/ml of indomethacin. Homogenates were centrifuged at 1,500 g for 10 min at 4°C. The organic phase was aspirated and evaporated to dryness in a centrifugal evaporator. The pellet was reconstituted in 500 μl of 0.1 M phosphate buffer (pH 7.4) containing 0.8% sodium azide and 0.1% gelatin. The concentration of PGE_2 in the samples was then determined by radioimmunoassay using the PGE_2 enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA). The results are expressed as picograms of PGE_2 per milligram of protein.

Statistical analyses

Data are presented as means ± standard error of the mean (SEM) of measurements made on 6 animals in each group. Comparisons between three or more treatments were made using one-way ANOVA with Tukey's post-hoc test or, repeated measures of two-way ANOVA with Bonferroni's

post-hoc test, as appropriate. Data were analyzed using Prism 5 Computer Software (GraphPad, San Diego, CA, USA). Statistical differences were considered significant at $p < 0.05$. In the present study, to determine the ED_{50} values, the compounds were administered at doses ranging between 6.25 and 100 mg/kg. ED_{50} values and confidence limits were calculated by computer-assisted log-probit analysis according to Litchfield and Wilcoxon (Litchfield and Wilcoxon 1949).



(f)

Compound	ED_{50} (mg/kg)
Physalin B	65.54
Physalin D	55.42
Physalin F	23.68
Physalin G	72.82
CEEPA	15.89

Figure S1. Effects of systemic administration of concentrated ethanolic extract from *Physalis angulata* (CEEPA) and physalins B, D, F and G on acetic acid-induced writhing in mice. Mice were treated with physalin B (12.5 – 100 mg/kg, panel A), physalin D (12.5 – 100 mg/kg, panel B), physalin F (12.5 – 100 mg/kg, panel C), physalin G (12.5 – 100 mg/kg, panel D), CEEPA (6.25 – 100 mg/kg, panel E), vehicle (C; control group; 5% DMSO), or indomethacin (In, 10 mg/kg; reference drug) by intraperitoneal route 40 min before the administration of acetic acid. Panel F shows the ED_{50} values to physalins and CEEPA on the writhing test. Data are expressed as mean

writhing scored ± SEM; n=6 mice per group. *Significantly different from control group ($p < 0.05$), as determined by ANOVA followed by Tukey's test.

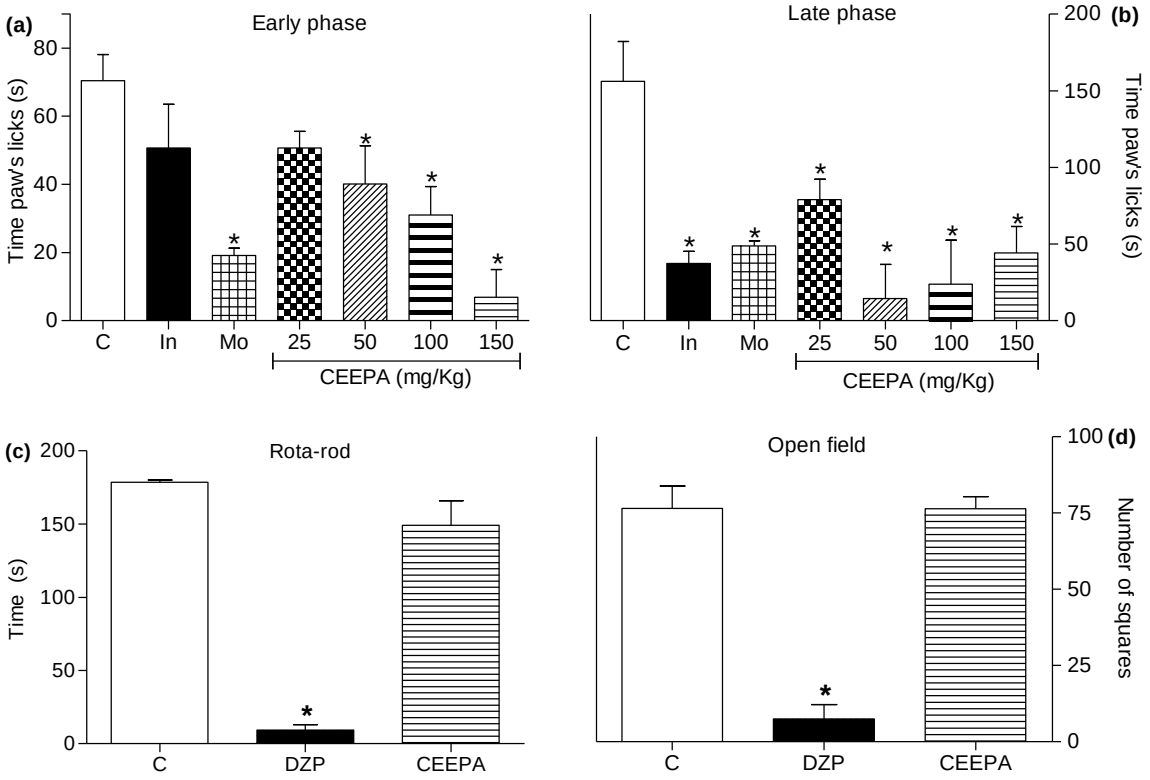


Figure S2. Effects of the systemic administration of concentrated ethanolic extract from *Physalis angulata* (CEEPA) on the formalin test and motor function of mice. Panels (A) and (B) represent effects of CEEPA on the early and late phases of formalin-induced nociception in mice, respectively. Mice were treated with CEEPA (25 – 150 mg/kg), vehicle (C, 5% DMSO in saline; control group), indomethacin (In; 10 mg/kg; reference drug) or morphine (Mo; 5 mg/kg; reference drug) by intraperitoneal route 40 min before the intraplantar injection of formalin. Bar graphs representing (C) the run time on the rota-rod and (D) the number of square crossings in the open field test, 40 min after the intraperitoneal injection of CEEPA (150 mg/kg), vehicle (C, 5% DMSO in saline; control group) or diazepam (DZP; 10 mg/kg, reference drug). Data are reported as

means ± SEM; n=7-10 mice per group. *Significantly different from the control group ($p<0.05$). One-way ANOVA followed by the Tukey's test.

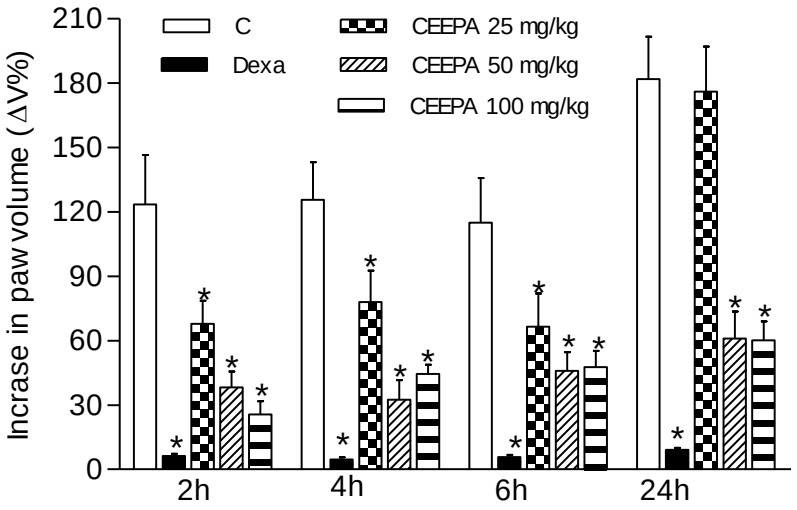


Figure S3. Effects of the concentrated ethanolic extract from *Physalis angulata* (CEEPA) treatment on complete Freund's adjuvant (CFA)-induced paw edema. Mice were injected with CEEPA (25 – 100 mg/kg), vehicle (C, 5% DMSO in saline; control group), or dexamethasone (Dexa, 2 mg/kg; reference drug) by intraperitoneal route 40 min before CFA (injected at time zero). Edema value is represented as percentage of increase in paw volume. Data are expressed as means ± SEM; n = 6 mice per group. *Significantly different from the control group ($p < 0.05$). Two-way ANOVA followed by Bonferroni's test.

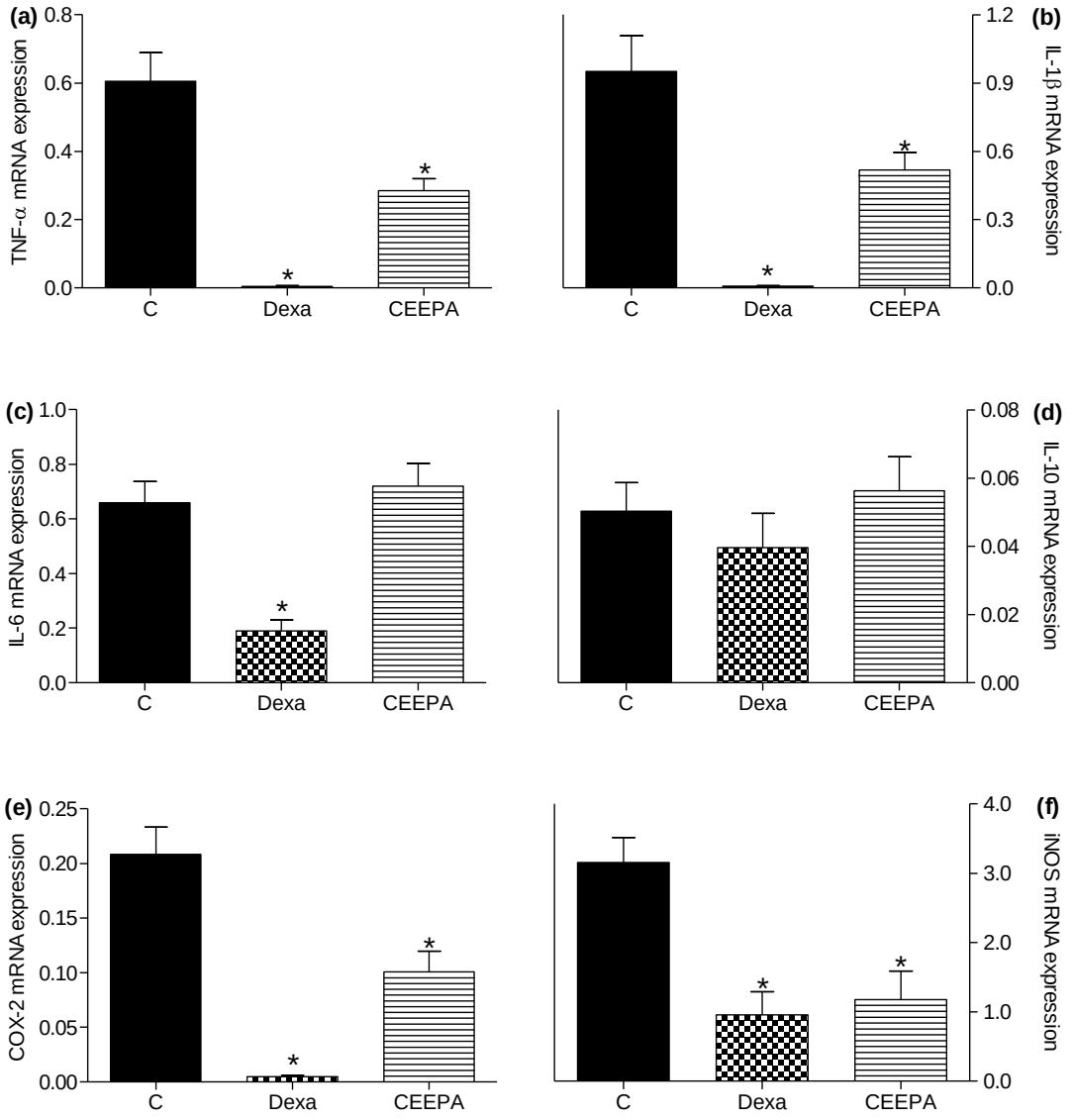


Figure S4. Effects of systemic treatment with concentrated ethanolic extract from *Physalis angulata* (CEEPA) on local expression of inflammatory mediators during inflammation. Mice were injected with CEEPA (50 mg/kg), vehicle (C, 5% DMSO; control group) or dexamethasone (Dexa, 2 mg/kg; reference drug) by intraperitoneal route 40 min before the intraplantar injection of complete Freund's adjuvant (CFA). The paw levels of mRNA were measured by RT-qPCR 6 h after the CFA stimulus. Panels show the paw levels of TNF-α mRNA (A), IL-1β mRNA (B), IL-6 mRNA (C), IL-10 mRNA (D), COX-2 mRNA (E) and iNOS mRNA (F). Data are reported as means ± SEM; n = 6 mice per group. *Significantly different from the control group ($p < 0.05$). One-way ANOVA followed by Tukey's test.

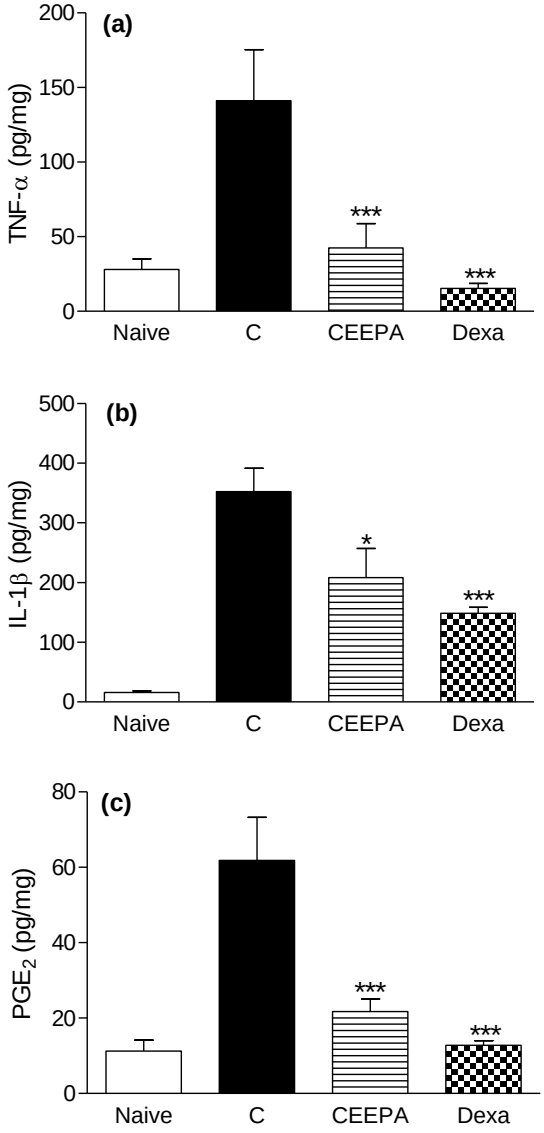


Figure S5. Effects of systemic treatment with concentrated ethanolic extract from *Physalis angulata* (CEEPA) on TNF-α, IL-1β and PGE_2 paw levels during inflammation. Mice were injected with CEEPA (50 mg/kg), vehicle (C; 5% DMSO; control group) or dexamethasone (Dexa; 2 mg/kg; reference drug) by intraperitoneal route 40 min before the intraplantar injection of complete Freund's adjuvant (CFA). The naive group received intraplantar injection of saline (10 µl/paw) instead of CFA. The paw levels of TNF-α (A), IL-1β (B) (6 h after the CFA injection) and PGE_2 (C) (3 h after CFA injection) was measured by ELISA and enzyme immunoassay, respectively. Data are reported as means ± SEM; n = 6 mice per group. *Significantly different from the control group ($p < 0.05$). One-way ANOVA followed by Tukey's test.

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