Sucrose Esters from *Physalis peruviana* Calyces with Anti-Inflammatory Activity

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Key words
- sucrose esters
- *Physalis peruviana*
- Solanaceae
- NO
- PGE2
- TNF-α
- antiinflammatory activity

received May 15, 2014
revised July 12, 2014
accepted Sept. 18, 2014

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DOI http://dx.doi.org/10.1055/s-0034-1383192
Published online October 22, 2014
Planta Med 2014; 80: 1605–1614 © Georg Thieme Verlag KG Stuttgart · New York · ISSN 0032-0943

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Abstract

Physalis peruviana is a native plant from the South American Andes and is widely used in traditional Colombian medicine of as an anti-inflammatory medicinal plant, specifically the leaves, calyces, and small stems in poultice form. Previous studies performed by our group on *P. peruviana* calyces showed potent anti-inflammatory activity in an enriched fraction obtained from an ether total extract. The objective of the present study was to obtain and elucidate the active compounds from this fraction and evaluate their anti-inflammatory activity *in vivo* and *in vitro*. The enriched fraction of *P. peruviana* was purified by several chromatographic methods to obtain an inseparable mixture of two new sucrose esters named peruviose A (**1**) and peruviose B (**2**). Structures of the new compounds were elucidated using spectroscopic methods and chemical transformations. The anti-inflammatory activity of the peruvioses mixture was evaluated using λ-carrageenan-induced paw edema in rats and lipopolysaccharide-activated peritoneal macrophages. Results showed that the peruvioses did not produce side effects on the liver and kidneys and significantly attenuated the inflammation induced by λ-carrageenan in a dosage-dependent manner, probably due to an inhibition of nitric oxide and prostaglandin E2, which was demonstrated *in vitro*. To our knowledge, this is the first report of the presence of sucrose esters in *P. peruviana* that showed a potent anti-inflammatory effect. These results suggest the potential of sucrose esters from the *Physalis* genus as a novel natural alternative to treat inflammatory diseases.

Abbreviations

COX-2:	cyclooxygenase 2
IL:	interleukin
iNOS:	inducible nitric oxide synthase
LPS:	lipopolysaccharide
m.a.s.l.:	meters above sea level
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO•:	nitric oxide
PGE2:	prostaglandin E2
RPMI:	Roswell Park Memorial Institute
SNP:	sodium nitroprusside
TNF-α:	tumor necrosis factor alpha
TPA:	12-O-tetradecanoyl-phorbol-13-acetate
1400 W:	N-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride

Supporting information available online at http://www.thieme-connect.de/products

Introduction

Inflammation is a frequent and immediate response to external and internal injurious stimuli including infections, chemicals, physical stress, and immune pathologies. It is a complex phenomenon that involves many cell types and cellular pathways [1]. Molecules produced during inflammation trigger pain receptors, induce local vasodilatation, and recruit phagocytic cells, especially neutrophils and macrophages, which then activate other immune system components [2]. Excessive or persistent inflammation leads to the overproduction of mediators that are a key factor for the development of severe pathologies such as rheumatoid arthritis [3], bacterial sepsis [4], asthma [5], atherosclerosis [6], inflammatory bowel disease [7], and cancer [8], which represents an important cause of morbidity worldwide. Thus, the reduction or elimination of the persistent in-

flammatory response and/or the overproduction of proinflammatory mediators is an important target to prevent or treat these diseases [9].

Plants represent an excellent source to obtain new drugs, as they constitute an immense reservoir of structurally diverse secondary metabolites that might potentially inhibit the inflammatory process by affecting different molecular targets [4]. In this sense, *Physalis peruviana* L. (Solanaceae), widely used in traditional medicine as a diuretic and hypoglycemic agent and to treat malaria, asthma, hepatitis, dermatitis, and rheumatoid arthritis [10], represents a promissory source of bioactive compounds. This has been experimentally validated, with extracts showing relevant antioxidant [11,12], antiproliferative [13–15], cytotoxic [16], anti-hepatotoxic [17], hipoglycaemic [18], immunomodulatory [19], and anti-inflammatory activities [20,21].

P. peruviana, also known as "uchuva" in Colombia or "gooseberry" in English speaking countries, is native to tropical South America and is characterized because the fruit grows enclosed in a papery husk or calyx, which is one of the best-known examples of persistent sepals due to its conspicuous post-floral growth and enlargement [22–24]. The *P. peruviana* calyx protects the fruit against insects, birds, diseases, and adverse climatic situations; this structure represents an essential source of carbohydrates during the first 20 days of growth and development of the fruit, and the conservation of it even after harvest, allowing for a shelf life of one month, whereas without the calyx, it is only lasts for 4 to 5 days or so [23,24]. Three varieties of *P. peruviana* are currently grown, originating from Colombia, Kenya, and South Africa. The ecotype Colombia, which has a great demand on the international markets, presents small and colorful fruits and different morphological characteristics in the calyx [25]. Although the fruit is highly appreciated for its commercial value, the calyx constitutes a waste generated in fruit production as well as an unexplored source of bioactive molecules.

The experimental studies of *P. peruviana* have been focused on the chemical and biological characterization of the whole plant, stems, leaves, and fruits, with only a few reports for calyces. Chemical studies on *P. peruviana*, mainly of the aerial parts, showed the presence of withanolides, steroids, alkaloids, and glycosides [15–17,23,26]. Phytochemical studies made by our research group on *P. peruviana* calyces indicated the presence of flavonoids, steroids and/or triterpenes, and lactones α-β unsaturated. Our previous studies also demonstrated that the major fraction obtained from the ether extract of *P. peruviana* calyces showed potent anti-inflammatory activity in a TPA-induced ear edema model, with inhibition to nearly 70% [20]. In this work, we elucidated the structure of two new sucrose esters from this major fraction and evaluated their anti-inflammatory activity *in vivo* in the λ-carrageenan-induced paw edema model and the *in vitro* effect on NO•, PGE2, and TNF-α production from LPS-stimulated murine macrophages. Sucrose esters have been isolated from *Physalis* species in fruits, flowers, and stems [27–29]. To our knowledge, this is the first report on the presence of sucrose esters in calyces of the *Physalis* genus.

Results and Discussion

Colum chromatographic purification of the methanol-water soluble fraction, obtained by liquid/liquid partition from the total ether extract of *P. peruviana* calyces, provided a major fraction which constituted 3.03% of the initial material and was subjected to preparative TLC to lead to the isolation of one spot that showed a single peak on reversed-phase HPLC. However, spectroscopic signals of this fraction revealed a mixture of two new sucrose esters, peruvioses A (**1**) and B (**2**), occurring in a 6 : 4 ratio, respectively. All attempts at the separation of the components with several combinations of solvent systems using preparative TLC and HPLC were ineffective. Therefore, we elucidated their structures by analyzing the spectroscopic data of the mixture.

Compounds **1** and **2** were isolated as a gummy solid, with a 99.05% purity based on HPLC that exhibited strong absorption bands for hydroxyl (ν = 3411 cm^{-1}) and saturated ester functions (ν = 1746 cm^{-1}) in the IR spectrum. Despite the fact that it was a mixture, NMR spectra displayed many duplicated peaks slightly displaced and MS showed similar but distinguishable fragmentation pathways, which allowed for the elucidation of both compounds separately.

^{1}H-NMR and ^{13}C-NMR signals and the connectivities observed in the HMBC and NOESY spectra for **1** revealed the presence of one pyranose and one furanose unit as the monomers of a disaccharide structure, as well as four acyl substituents: one decanoyl and three isobutanoyl chains (**Table 1** and **Fig. 1**). The relative configuration of the sugars was determined by comparing the ^{1}H-NMR coupling constants and ^{13}C-NMR chemical shifts with literature values [26–29]. The pyranose was identified as α-D-glucopyranose by the observed coupling constants ($J_{1,2}$ = 3.7 Hz, $J_{2,3}$ = 10.4 Hz, $J_{3,4}$ = $J_{4,5}$ = 9.4 Hz) that also established H-1 as equatorial, whereas the strong coupling observed between H-2 and H-3, H-3 and H-4, and H-4 and H-5 established these protons as axial. On the other hand, the furanose was identified as β-D-fructofuranose showing the following coupling constants: $J_{3'4'}$(H3′) = $J_{3'4'}$(H4′) = 8.2 Hz. The NOE contact between glucose H-1 to H-1a′ and H-1b′ of the fructose ring confirmed the glycosidic linkage with α- and β-orientations on the anomeric carbon for D-glucose and D-fructose, respectively. Moreover, the HMBC signal between H-1 and C-2′ unequivocally indicates the 1,2 linkage between D-glucose and D-fructose (**Fig. 1S**, Supporting Information).

The positions of all substituents in the disaccharide structure were determined from the analysis of the HMBC spectrum, which showed correlations between H-2 and the carboxylic carbon C-1″. This data evidences that the aliphatic decanoyl chain is placed in position 2 of the glucose unit. On the other hand, the HMBC spectrum showed correlations between H-3 of the glucopyranose and C-1‴ of an isobutanoyl group, which, in turn, was coupled with H-3‴. The same bidimensional spectrum showed connectivities of H-4 with C-1⁗ of another isobutanoyl group, which was coupled with H-3⁗. In the furanose moiety, a clear correlation between H-3′ and carboxylic C-1⁗⁗ was found, indicating that another isobutanoyl substituent was located in position 3 of this unit.

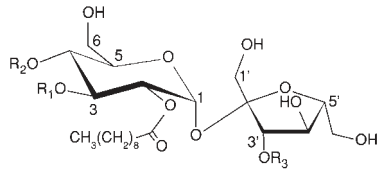
This analysis led us to elucidate the structure of the new compound as 2-*O*-decanoyl-3,3′,4-tri-*O*-isobutanoylsucrose, named peruviose A (**1**), $C_{34}H_{58}O_{15}$. The adduct ion peak at *m/z* 729 [M + Na]$^+$ obtained by FABMS, as well as fragments at *m/z* 155 (decanoyl) and *m/z* 71 (isobutanoyl), are in agreement with the proposed molecular formula and structure. Additionally, the ion peak at *m/z* 897 [M + Na]$^+$ (calcd. for $C_{42}H_{66}O_{19}Na$) of the corresponding acetylated derivate confirmed the presence of the four ester groups, while the fragment at *m/z* 233 supported the proposed substitution of the furanose moiety.

Compound **2** was identified on the basis of 1D and 2D NMR analyses and FABMS spectra in a similar manner. These analyses pointed out that both compounds shared an almost identical

Table 1 NMR spectroscopic data for peruviose A (**1**) in $CDCl_3$ (500 MHz for ^1H and 125 MHz for ^{13}C).

Number	^1H [δ (ppm), *m*, ^3J (Hz)]	^{13}C (δ, ppm)/DEPT	NOESY	HMBC
Glucose-1	5.59, *d, 3.7*	89.40/CH	H-2, H-1′a, H-1′b	C-5, C-2′
2	4.96–4.90*	70.06/CH	H-1′a	C-3, C-1′′
3	5.48, dd, 10.4, 9.4	69.02/CH	H-5	C-2, C-4, C-1′′′
4	4.96–4.90*	68.25/CH	H-6	C-3, C-5, C-6, C-1′′′′
5	4.19–4.12*	72.18/CH	H-6	
6	3.60, *m*	61.60/CH_2	H-4′	
Fructose-1′a	3.58, d, 12.0	64.69/CH_2	H-3′, H-5′	
1′b	3.45, d, 12.0		H-3′	
2′	–	103.81/C		
3′	5.20, *d, 8.2*	79.26/CH	H-5′	C-1′, C-4′, C-1′′′′′
4′	4.56, t, 8.2	71.19/CH	H-6′a, H-6′b	C-3′, C-5′, C-6′
5′	3.94, *m*	82.55/CH		C-4′
6′a	3.90, *m*	59.73/CH_2		C-4′
6′b	3.74, *m*			
1′′	–	172,88/C=O		
2′′	2.25, *m*	33.85/CH_2	H-3′′, H-4′′	C-3′′
3′′	1.54, *m*	24.55/CH_2	H-4′′	C-2′′
4′′-7′′	1.24, *s* wide	29.33–29.06/CH_2		
8′′	1.24, *s* wide	31.82/CH_2		
9′′	1.24, *s* wide	22.63/CH_2		
10′′	0.87, *t, 7.0*	14.06/CH_3		C-8′′, C-9′′
1′′′	–	175.74/C=O		
2′′′	2.45, *m, 7.0*	33.89/CH	H-3′′′a, H-3′′′b	
3′′′a	1.07, *d, 7.0*	18.79–18.70/CH_3		C-2′′′
3′′′b	1.08, *d, 7.0*			C-2′′′
1′′′′	–	175.98/C=O		
2′′′′	2.51, *m, 7.0*	33.93/CH	H-3′′′′a, H-3′′′′b	C-3′′′′
3′′′′a	1.13, *d, 7.0*	18.79–18.70/CH_3		C-2′′′′
3′′′′b	1.06, *d, 7.0*			C-2′′′′
1′′′′′	–	177.87/C=O		
2′′′′′	2.75, *m, 7.0*	34.02/CH	H-3′′′′′a, H-3′′′′′b	C-3′′′′′
3′′′′′a	1.32, *d, 7.0*	18.87/CH_3		C-2′′′′′
3′′′′′b	1.29, *d, 7.0*	18.87/CH_3		C-2′′′′′

* The multiplicity could not be determined



	1	2
R1	Isobutyryl	Isobutyryl
R2	Isobutyryl	Isobutyryl
R3	Isobutyryl	3-methylbutanoyl

Fig. 1 Structure of peruvioses A (**1**) and B (**2**) isolated from calyces of *P. peruviana*.

structure with an esterified sucrose core with four acyl groups (**Table 2** and **Fig. 1**). The only difference between these compounds was observed in the HMBC correlations of **2** with the presence of a connectivity signal between H-3′ and C-1′′′′′, corresponding to a 3-methylbutanoyl substituent. The quasimolecular ion peak *m/z* 743 $[M + Na]^+$ obtained by FABMS, as well as fragments at *m/z* 155 (decanoyl), *m/z* 71 (isobutanoyl), and *m/z* 85 (3-methylbutanoyl), supported the structural characteristics of the acyl groups. The peak at *m/z* 247 also corroborates the difference on the furanose moiety substitution and confirms the structure of the new sucrose ester derivative as 2-*O*-decanoyl-3,4-di-*O*-isobutyryl-3′-*O*-(3-methylbutanoyl)sucrose, named peruviose B, $C_{35}H_{60}O_{15}$. After acetylation of this compound, the ion peak at *m/z* 911 $[M + Na]^+$ (calcd. for $C_{43}H_{68}O_{19}Na$) also confirmed the presence of the four original ester groups on the sugar moiety.

Determination of the absolute configuration of the sugar moiety was performed by alkaline hydrolysis and comparison with a sucrose authentic standard. The hydrolysis of peruvioses A and B (**1** and **2**) with NH_4OH (2 M) yielded a product with an identical absolute configuration ($[\alpha]_D^{20\,°C}$ product = +62.2) in respect to the sucrose standard ($[\alpha]_D^{20\,°C}$ standard = +66).

Acylsucroses are considered the main protective constituents of the resin covering the inner parts of the calyces of several *Physalis* species, since other known sucrose esters exhibit aphicidal, molluscidal, and antifeedant activities [29]. Consequently, we performed an acute toxicity evaluation of the mixture of peruviose A and B, which did not produce mortality or visible signs of toxicity within 24 h when administered intraperitoneally (i.p.) at doses below 100 mg/kg. Doses higher than 200 mg/kg produced a toxic effect in a dose-dependent manner with slight hypoactivity, weakness, and labored breathing before animal death, accompanied by permanent piloerection (**Table 3**). The LD_{50} was estimated to be 223.59 (95% confidence interval 209.91–234.52) mg/kg.

The toxicity of peruvioses A and B was significantly high as expected, not only because of the known toxicity of sugar esters [30], but also because of the nearly complete access of the tested compounds to the general circulation. However, subchronic and

Table 2 NMR spectroscopic data for peruviose B (**2**) in $CDCl_3$ (500 MHz for ^1H and 125 MHz for ^{13}C).

Number	^1H [δ (ppm), *m*, ^3J (Hz)]	^{13}C (δ, ppm)/DEPT	NOESY	HMBC
Glucose-1	5.60, *d, 3.8*	89.42/CH	H-2, H-1′a, H-1′b	C-5, C-2′
2	4.96–4.90*	70.13/CH	H-1′a	C-3, C-1′′
3	5.46, dd, 10.3, 9.4	69.05/CH	H-5	C-2, C-4, C-1′′′
4	4.96–4.90*	68.30/CH	H-6	C-3, C-5, C-6, C-1′′′′
5	4.19–4.12*	72.01/CH	H-6	
6	3.60, *m*	61.55/CH_2	H-4′	
Fructose-1′a	3.58, *d, 12.0*	64.54/CH_2	H-3′, H-5′	
1′b	3.45, *d, 12.0*		H-3′	
2′	–	103.89/C		
3′	5.23, *d, 8.2*	79.12/CH	H-5′	C-1′, C-4′, C-1′′′′′
4′	4.56, *t, 8.2*	71.15/CH	H-6′a, H-6′b	C-3′, C-5′, C-6′
5′	3.96, *m*	82.55/CH		C-4′
6′a	3.92, *m*	59.79/CH_2		C-4′
6′b	3.71, *m*			
1′′	–	172,88/C=O		
2′′	2.25, *m*	33.85/CH_2	H-3′′, H-4′′	C-3′′
3′′	1.54, *m*	24.55/CH_2	H-4′′	C-2′′
4′′-7′′	1.24, *s* wide	29.33–29.06/CH_2		
8′′	1.24, *s* wide	31.82/CH_2		
9′′	1.24, *s* wide	22.63/CH_2		
10′′	0.87, *t, 7.0*	14.06/CH_3		C-8′′, C-9′′
1′′′	–	175.64/C=O		
2′′′	2.45, *m, 7.0*	33.88/CH	H-3′′′a, H-3′′′b	
3′′′a	1.08, *d, 7.0*	18.79–18.70/CH_3		C-2′′′
3′′′b	1.07, *d, 7.0*			C-2′′′
1′′′′	–	176.06/C=O		
2′′′′	2.52, *m, 7.0*	33.91/CH	H-3′′′′a, H-3′′′′b	C-3′′′′
3′′′′a	1.144, *d, 7.0*	18.79–18.70/CH_3		C-2′′′′
3′′′′b	1.119, *d, 7.0*			C-2′′′′
1′′′′′	–	174.06/C=O		
2′′′′′	2.39, ABX-system, *7.0, 14.8*	43.14/CH_2	H-4′′′′′a, H-4′′′′′b	C-4′′′′′a
3′′′′′	2.20, *m*	25.86/CH	H-4′′′′′a, H-4′′′′′b	C-2′′′′′
4′′′′′a	1.05, *d, 6.7*	22.42/CH_3		C-2′′′′′, C-3′′′′′, C4′′′′′b
4′′′′′b	1.04, *d, 6.7*	22.29/CH_3		C-2′′′′′, C-3′′′′′, C-4′′′′′a

* The multiplicity could not be determined

Table 3 Acute toxicity in mice after 24 h administration of a mixture of peruvioses A and B isolated from *P. peruviana* calyces.

Dose (mg/Kg)[a]	D/T[b]	Mortality latency[c]	Signs of toxicity observed
0	0/6	–	No toxic changes observed.
100	0/6	–	No toxic changes observed. Abdominal contractions.[d]
150	0/6	–	Abdominal contractions. Slight hypoactivity in the first 30 min.
175	0/6	–	
200	0/6	–	Abdominal contractions. Slight hypoactivity in the first 2 h.
215	3/6	> 5 h, < 24 h	Abdominal contractions. Piloerection. Slight hypoactivity in the first 2 h. Marked hypoactivity, peripheral cyanosis, and respiratory arrest before death.
230	4/6	> 5 h, < 24 h	Abdominal contractions. Piloerection. Marked hypoactivity. Peripheral cyanosis and respiratory arrest before death.
240	5/6	> 3 h, < 24 h	
250	5/6	> 5 h, < 24 h	
300	6/6	3–10 h	

[a] The mixture of peruvioses A and B was co-precipitated with PVP K-25, dissolved in saline, and administered as a single i. p. dose to groups of six mice, which were carefully examined for any signs of behavioral changes and mortality for 24 h; [b] D/T refers to the number of mice deaths/total number of mice; [c] Mortality latency refers to the time to death (in hours) after the injection; [d] Abdominal contractions were noted only after the administration of treatments (10–20 min)

chronic toxicological evaluations, employing several routes of administration, are needed before conducting further studies with these molecules.

During necropsy, a macroscopic examination did not show detectable changes in the shape, color, or size of the liver and kidneys due to the administration of peruvioses A and B. In addition, a histopathological evaluation did not reveal significant changes in tissue architecture, inflammatory cell infiltration, swelling, or necrosis in comparison to the control group (**Fig. 2**). Our results directly demonstrated that toxicity induced by peruvioses A and

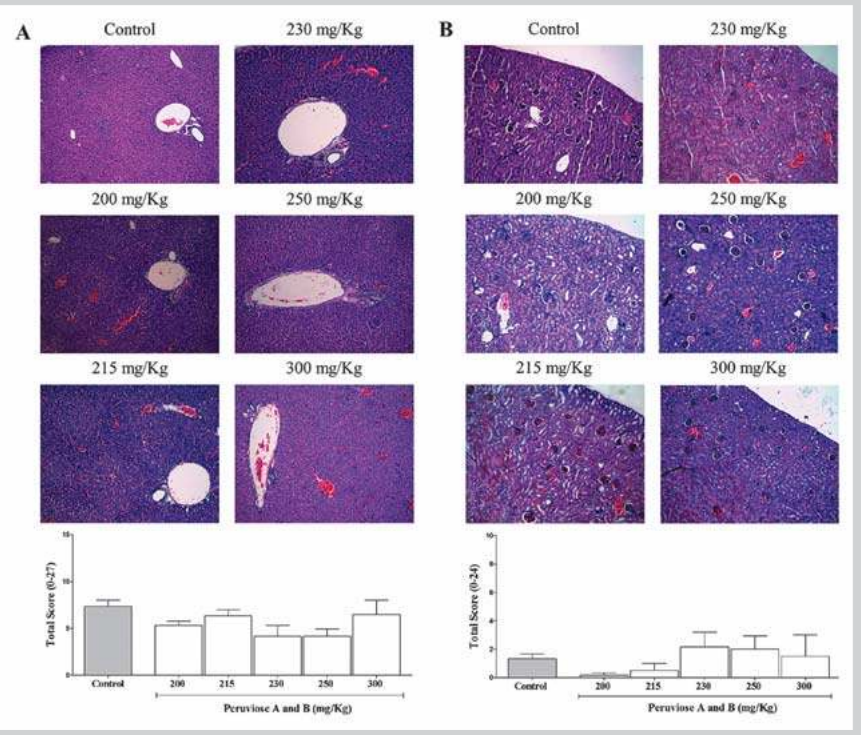


Fig. 2 Peruvioses A and B isolated from *P. peruviana* calyces did not produce an effect on the histological structure of the liver (**A**) and kidneys (**B**) of mice treated i. p. with doses of the mixture of sucrose esters ranging from 0 (control) to 300 mg/kg for 24 h. Micrographs are representative of a histological section of organs stained with hematoxylin and eosin from at least six different animals. Magnification 10×. Scores were assigned by a blinded pathologist according to the parameters established in **Table 1S**, Supporting Information. Each value represents the mean ± SEM. (Color figure available online only.)

B is not mediated through effects on liver or kidney function. Future studies should include examination of other vital organs like the brain, heart, lungs, and spleen.

Based on the experimental LD_{50}, we decided to select doses lower than 200 mg/kg to evaluate the anti-inflammatory effect of sucrose esters employing the λ-carrageenan paw edema test, a classical model of acute inflammation for the discovery of anti-edematous agents [31]. Subplantar injection of λ-carrageenan generated an increase in paw volume of the rats in the control group, which intensified progressively to reach a maximum peak at 5 h (134.15 % increase). As can be seen in **Fig. 3**, the peruviose A and B mixture (25, 50, and 100 mg/Kg, i. p.) significantly inhibited the edema induced by λ-carrageenan in a dose-dependent manner as early as 1 h after induction of inflammation. The highest effect of the mixture was produced at 3 h by the dose of 100 mg/kg (62.58 ± 3.35 % inhibition).

In vivo anti-inflammatory activity of four related sucrose esters isolated from *Physalis sordida* (CI_{50} = 0.24–0.35 µmol/ear) has been recently described [32]. We have also demonstrated the effect of the major glycosidic fraction from *P. peruviana* calyces, which includes peruvioses A and B among its constituents, using the TPA-induced ear edema model [20]. These experimental evidences suggest that the presence of sucrose esters can be highly related to the anti-inflammatory activity exerted by some plants of this genus [32]. However, the mechanism underlying this pharmacological activity has not been described.

Edema is an essential feature of acute inflammation caused by increased vascular permeability [33, 34]. The effect induced by λ-carrageenan is a biphasic response with multiple mediators acting in sequence. The first phase (0–1 h) is triggered through the rapid release of histamine, serotonin, and bradykinin. The more pronounced second phase (1–6 h) is elicited by the production of prostaglandins and NO• by inducible isoforms of COX-2 and iNOS, respectively [33, 35]. Since our results suggest that the anti-inflammatory effect produced by the mixture of peruvioses A and B is due to the inhibition of the synthesis or release of the proinflammatory mediators from the second phase, and considering a previous study which reported inhibition of LPS-induced NO• and PGE2 generation on RAW 264.7 macrophages by a supercritical fluid extract of *P. peruviana* leaves [21], we decided to further evaluate the effect of the mixture of sucrose esters from *P. peruviana* calyces in the production of some of the major mediators of acute inflammation, NO•, PGE2, and TNF-α, by LPS-stimulated mouse peritoneal macrophages.

Macrophages play a pivotal role in host defense against bacterial infection, being the principal cellular target for LPS, the major component in the outer membrane of gram-negative bacteria cell walls, which stimulates the secretion of NO• and PGE2 as well as proinflammatory cytokines including TNF-α, IL-1β, and IL-6 [36]. To verify whether peruvioses A and B are able to inhibit the production of NO• and PGE2, we first evaluated their effect on cell viability by employing the MTT assay. As shown in **Fig. 4 A**, the mixture of peruvioses inhibited the cell viability in a concentration-dependent manner, with an LC_{50} value of 25.41 (15.28–40.24) µg/mL, without exerting significant toxicity at 10 µg/mL. Therefore, concentrations employed in the subsequent experiments were equal or less than this concentration.

NO• is a gaseous signaling molecule that plays a crucial role in host defense mechanisms via its antimicrobial and cytoprotective activities. Stimulation of murine macrophages by LPS results in the increased expression of iNOS, which catalyzes the production of large amounts of NO•. We found that peritoneal macrophages produced a considerable amount of nitrite under basal conditions, 21.82 ± 1.25 µM. However, after stimulation with LPS, nitrite production was increased significantly to a concentration of 65.44 ± 3.42 µM. Nontoxic concentrations of the mixture of peruvioses A and B produced a significant reduction of nitrite production depending on the concentration, IC_{50} = 2.317 (1.368–4.055) µg/mL, showing a similar activity for that presented by 1400 W (**Fig. 4 B**), without exerting an important scavenging ef-

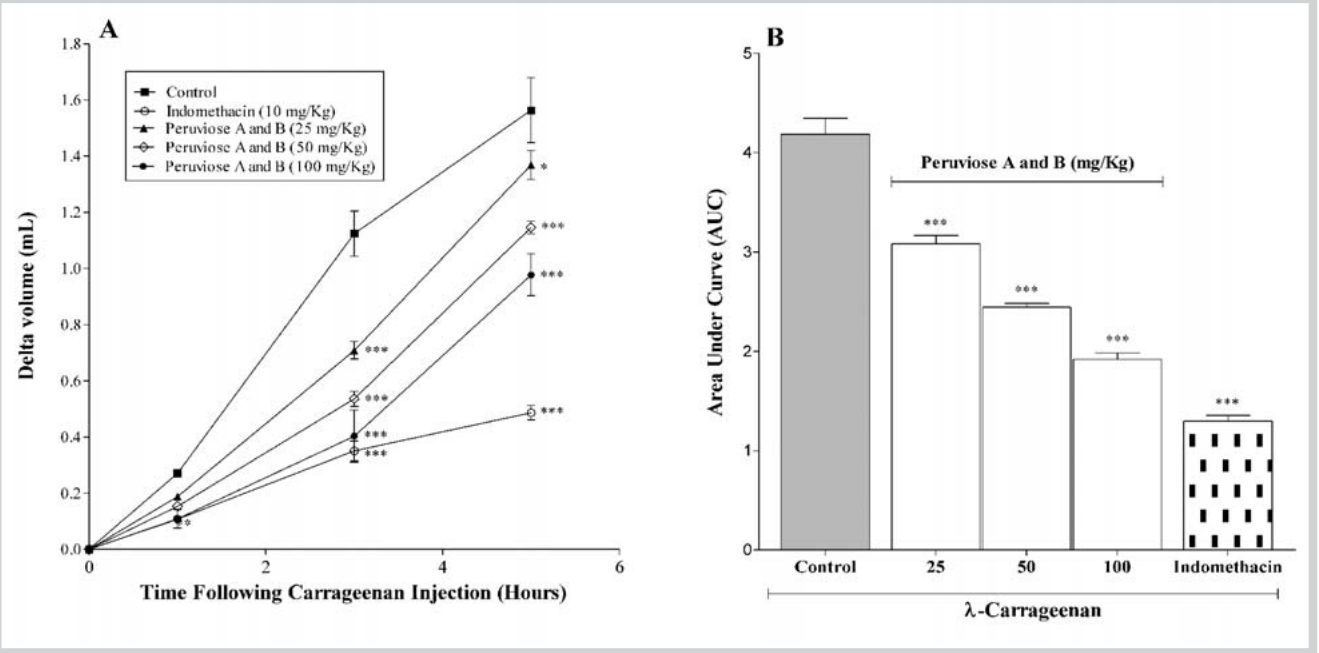


Fig. 3 Anti-inflammatory effect of the mixture of peruvioses A and B on λ-carrageenan-induced paw edema. Six rats per group were administered sucrose esters (25, 50, and 100 mg/kg, i. p.) or indomethacin (10 mg/kg, i. p.), 1 h before the λ-carrageenan injection. Paw volume was measured at 1, 3, and 5 h intervals, after phlogistic agent administration, and data expressed as (**A**) delta volume (mL), which denotes the degree of swelling after λ-carrageenan treatment or (**B**) area under curve (arbitrary units). Each value represents the mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ANOVA followed by the Dunnett test, statistically significant compared with the control group.

fect of NO• (**Table 4S**, Supporting Information), indicating that suppression of its release can be directly attributed to a blocked production by stimulated macrophages.

PGE2 is known to be a key mediator of immunopathology in chronic inflammatory diseases and cancer [37]. As can be seen in **Fig. 4C**, LPS produced a significant increase of PGE2 levels in cell culture, changing its concentration from 4.18 ± 0.38 ng/mL to 27.43 ± 0.61 ng/mL. The mixture of peruvioses A and B exhibited a potent inhibitory effect on PGE2 release in cell culture in a concentration-dependent manner, with IC_{50} = 0.072 (0.019–0.293) µg/mL. The effects of the mixture of sucrose esters are comparable to those of rofecoxib, which is a selective COX-2 inhibitor.

In vitro anti-inflammatory activity through the inhibition of PGE2 production of LPS-induced RAW 264.7 macrophages by three sucrose esters isolated from *Bidens parviflora* has also been described [38]. Taken together, these results suggest that the anti-inflammatory activity of sucrose esters might be related to the inhibition of this important prostanoid.

TNF-α is a potent proinflammatory cytokine released primarily from stimulated macrophages playing a critical role in the host response to infection and injury [39]. LPS-stimulated macrophages significantly increased TNF-α production in 2.93-fold (**Fig. 4D**). Unexpectedly, the mixture of peruvioses A and B did not affect the production of TNF-α, even at the highest tested concentration, which suggests that the anti-inflammatory activity of these sucrose esters is mediated through the selective downregulation of iNOS and COX-2, independently of common pathways or transcription factors with TNF-α.

Of the mediators that modulate PGE2 synthesis in macrophages, NO• seems to play a key role. A large body of evidence suggests that there is significant crosstalk between iNOS and COX-2 biosynthetic pathways, especially in biological systems like LPS-stimulated peritoneal macrophages [40]. However, the final effect of these interactions is often unclear, varying between different kinds of cells and tissues [41, 42]. Further studies are needed to clarify the effect of peruvioses A and B on iNOS and COX-2 in peritoneal macrophages.

In conclusion, we described the isolation of a mixture of two new anti-inflammatory sucrose esters from the calyces of *P. peruviana*. Overall, our results suggest that sucrose esters are important anti-inflammatory compounds of the *Physalis* genus, mainly through the downregulation of NO• and PGE2 production, without exerting significant acute toxicity.

Material and Methods

Experimental instrumentation and chemicals

Melting points were determined by differential scanning calorimetry-DSC7 (Perkin-Elmer) and are uncorrected. IR spectrum was recorded on a FTIR (Perkin Elmer 1600 series). NMR spectra were obtained on a Bruker AMX-500 spectrometer, with TMS as the internal standard. FAB-MS was obtained on a Kratos MS80-RFA mass spectrometer. A Hitachi-LaChrom Elite® apparatus equipped with PDA was used for analytical HPLC separations. Reversed-phase chromatography was performed with a 100 × 4.6 mm Chromolith® C-18 column (Merck). TLC was performed on silica gel 60 F254 plates (250 µm thickness; Merck). Silica gel 60 (0.063–0.200 mm) and NH_4OH were also obtained from Merck. All solvents were of analytical grade and purchased from Merck. A plethysmometer (model 7140 Ugo Basile) was used to determine the paw volume in rats. To work with cell cultures, thioglycollate broth, RPMI-1640 medium, FBS, PBS tablets, antibiotics, N-(1-naphthyl)-ethylenediamine, sulfanilamide, λ-carrageenan, LPS, 1400 W (purity > 98%), $NaNO_2$, SNP, indomethacin

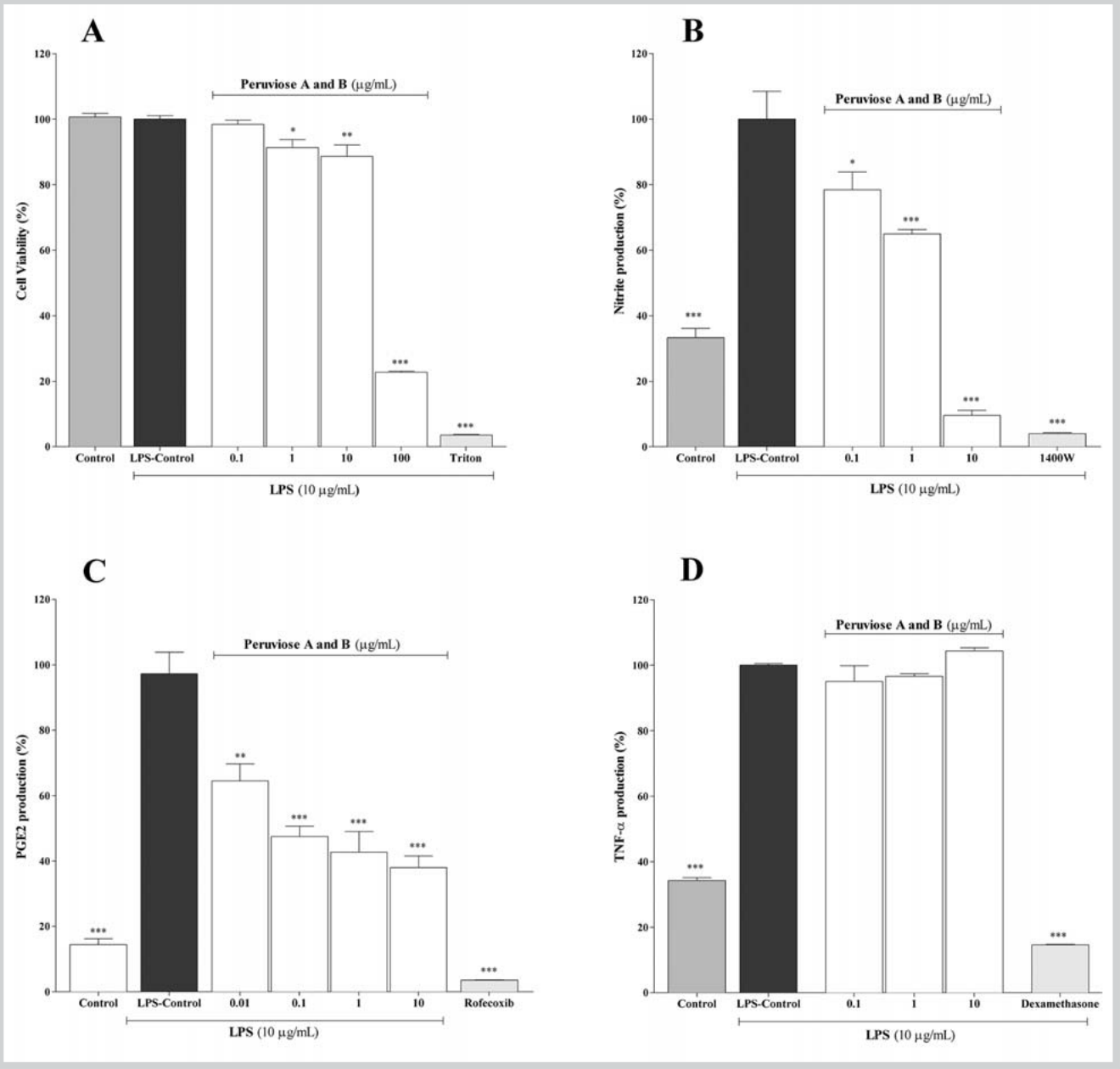


Fig. 4 Effect of the mixture of peruvioses A and B on lipopolysaccharide-induced mouse peritoneal macrophages viability and proinflammatory mediator production. Macrophages were treated with various concentrations of the mixture of sucrose esters (0.01–100 µg/mL) for 30 min, and activated with LPS (10 µg/mL) for 24 h. After incubation, cell viability was determined by the MTT assay (**A**) or culture supernatants were collected. Nitrite production was assessed using Griess reaction (**B**), and ELISA was employed to quantify PGE2 (**C**) and TNF-α release (**D**). Triton X-100® (20%), 1400 W (2.50 µg/mL), rofecoxib (6.29 µg/mL), and dexamethasone (7.85 µg/mL) were employed as positive controls, respectively. Results are expressed as the mean ± SME of at least two independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ANOVA followed by the Dunnett test, compared with the LPS-treated group.

(purity 98–101%), dexamethasone (purity ≥ 97%), curcumin (purity ≥ 80%), and DMSO were obtained from Sigma-Aldrich. MTT, rofecoxib (purity > 99%), and caffeic acid (purity ≥ 95%) were purchased from Calbiochem®.

Plant material

Calyces of *P. peruviana* L. were collected in La Mesa, Colombia (4°37′ 49.22″ N; 74°27′ 45.60″ W; elevation 1198 m. a. s. l.) in November 2003. Taxonomic identification was performed by Clara I. Orozco at Herbario Nacional Colombiano (Instituto de Ciencias Naturales, Universidad Nacional de Colombia), Bogotá, Colombia, where a voucher specimen (COL-512200) has been deposited.

Extraction and isolation

Dried calyces (2 kg) were powdered and extracted with petroleum ether by percolation at room temperature until exhaustion of the material. The concentrated extract (271.6 g) was partitioned with ether and methanol-water (9 : 1) to give a polar fraction (223.3 g). This fraction (22 g) was subjected to column chromatography (CC, 14 × 30 cm columns) on silica gel (600 g, 0.063–0.200 mm) and eluted with petroleum ether, dichloromethane, ethyl acetate, and methanol mixtures, gradually increasing the polarity. Fractions were combined based on TLC examination using a proper mobile phase and visualized by heating after spraying with Godin reagent [43] to obtain 38 main fractions with a

94.77% efficiency. The major fraction (8.23 g), named Pp-D_{28}-LF [20], was subjected to analytical HPLC analysis using a mixture of methanol and 0.07 M monobasic potassium phosphate buffer (6.5 : 3.5, pH 4.0) as the mobile phase, a 1 mL/min flow rate, and an operating temperature of 25 °C to show one component that constituted nearly 82% of the mixture. This major fraction (2 g) was purified by CC (gradient elution CH_2Cl_2 to EtOAc) followed by two successive preparative TLCs (eluent CH_2Cl_2-EtOAc 4 : 6) to yield 293 mg of an inseparable mixture of two new sucrose esters, peruviose A (**1**) and peruviose B (**2**). Their structures were elucidated trough IR, FAB-MS, and extensive 2D NMR methods including ^{1}H-NMR, ^{13}C-NMR, Dep90, Dep135, HMBC, and NOESY.

Peruvioses A and B (**1** and **2**): Light yellow gummy solid mixture of **1** and **2**; Rf 0.489 on silica gel 60 F-254 (CH_2Cl_2/EtOAc, 4 : 6); purity (HPLC) 99.05% [t_R 2.35 min; CH_3OH/KH_2PO_3 (0.07 M), 6.5 : 3.5, pH 4.0]; m. p. 20–22 °C (uncorrected); UV (EtOH) λ_{max} 220 nm; $[\alpha]_D^{20\,°C}$ = + 52.08, IR (KBr) ν_{max} 3411, 2927, 2858, 1746, 1191, 1155, 1063, 1017 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR, HMBC, and NOESY (125 MHz, $CDCl_3$) spectra, see **Table 1** and **Fig. 2S** and **3S**, Supporting Information. FAB-MS: (**1**) *m/z* 729 $[M + Na]^+$ (calcd. for $C_{34}H_{58}O_{15}Na$), *m/z* 233 $[C_{10}H_{17}O_6]$, *m/z* 155 $[C_{10}H_{19}O]$, and *m/z* 71 $[C_4H_7O]$; (**2**) *m/z* 743 $[M + Na]^+$, (calcd. for $C_{35}H_{60}O_{15}Na$), *m/z* 247 $[C_{11}H_{19}O_6]$, *m/z* 155 $[C_{10}H_{19}O]$, *m/z* 85 $[C_5H_9O]$, and *m/z* 71 $[C_4H_7O]$, **Fig. 4S**, Supporting Information.

Chemical modifications

To verify the presence of glycosylated esters and the absolute configuration of the sugar moiety, the mixture of peruvioses A and B (10 mg) was hydrolyzed with 2 mL of NH_4OH 2 M for 4 h at 50 °C. The reaction mixture was adjusted to pH 3 by the addition of formic acid 2 M and subjected to successive liquid-liquid extraction with ethyl acetate (3 × 3 mL). The aqueous solution was used to determine the absolute configuration of glycosides. Additionally, 90 mg of the compound mixture were acetylated by the usual procedure with acetic anhydride and pyridine (5 : 1 per gram of the compound) to yield 90.9 mg of the acetylated material (efficiency 81.9%), after the usual workup. Acetylated peruvioses A and B (**3** and **4**) were submitted to ^{1}H-NMR, ^{13}C-NMR, Dep90, Dep135, HMBC, NOESY, and FABMS to confirm the elucidation of their structure.

Acetylated peruvioses A and B (**3** and **4**): Light yellow gummy solid; NMR (500 MHz, $CDCl_3$) data are described in **Table 2S** and **3S**, Supporting Information. FAB-MS: *m/z* 897 $[M + Na]^+$ (calcd. for $C_{42}H_{66}O_{19}Na$) and *m/z* 911 $[M + Na]^+$ (calcd. for $C_{43}H_{68}O_{19}Na$).

Experimental animals

Female Wistar rats (140–170 g) and ICR mice (20–25 g) were provided by Instituto Nacional de Salud, Colombia. Animals were allowed to acclimatize for ten days before use and fed with standard rodent food and water *ad libitum*. They were housed in filtered-capped polycarbonate cages and kept in a controlled environment at 22 ± 3 °C and relative humidity between 65 to 75%, under a cycle of 12 h light/darkness. Animals were sacrificed by cervical dislocation at the corresponding time of each experiment. All experiments were designed and conducted in accordance with the guidelines of the Ethics Committee of the University of Cartagena (minutes of October 23, 2010) and the European Union regulations (CEC council 86/809).

Acute toxicity

The acute toxicity test was carried out to evaluate any possible toxic effect exerted by the mixture of peruvioses A and B from *P. peruviana* calyces. Mice were randomly divided into ten groups of six animals per group and treated intraperitoneally with graded doses of sucrose esters ranging from 0 (control) to 300 mg/kg. Mice were observed for 24 h post-treatment for mortality, behavioral changes, and signs of toxicity. The LD_{50} value was determined by the Miller and Tainter method [44]. At the end of experiment, all animals were sacrificed, and the kidneys and liver were carefully excised for histological examination, fixed in 4% buffered formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin using standard techniques. All slides were coded and evaluated in a blinded manner by a pathologist observer according to the parameters described in **Table 1S**, Supporting Information.

λ-Carrageenan paw edema

The anti-inflammatory activity was evaluated according to the method described by Winter et al. [45]. Edema was induced by subplantar injection of λ-carrageenan (0.1 mL of a 2% solution) in the right hind paw of each animal. Rats were randomly divided into three groups of six animals each, and saline (control), indomethacin 10 mg/kg (positive control), or the mixture of peruvioses A and B (100, 50, and 25 mg/kg) was prophylactically administered (i. p.) 1 h before the administration of λ-carrageenan. Paw volume was determined by means of a volume displacement method using a plethysmometer immediately prior to the injection of λ-carrageenan and 1, 3, and 5 h thereafter. Edema was expressed as the increase in paw volume (mL) after λ-carrageenan injection relative to the preinjection value for each animal.

Isolation and culture of peritoneal macrophages

Peritoneal macrophages were isolated from ICR mice three days after i. p. injection of 10% sterile thioglycollate broth (1 mL). Peritoneal exudate cells were obtained by lavage with 20 mL of cold sterile PBS, pH 7.4, supplemented with 2% antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL). The cells were placed in RPMI-1640 medium with 2% antibiotics and 10% FBS, seeded in 24-well plates (1×10^6 cells/mL), and incubated at 37 °C in a 5% CO_2 atmosphere. The isolation and purification of the macrophages were carried out by adherence to culture plates. After a period of 2 h, non-adherent cells were washed off with PBS. The adherent cells were treated for 30 min with various concentrations of the peruviose A and B mixture (0.01–10 µg/mL), 1400 W (2.50 µg/mL), dexamethasone (7.85 µg/mL), and rofecoxib (6.29 µg/mL), stimulated with LPS (10 µg/mL), and incubated for 24 h. Culture supernatants were collected and assayed for NO•, PGE2, and TNF-α levels as described below. Control cells were cultured under the same conditions but were not activated.

MTT assay

The mitochondrial-dependent reduction of MTT to formazan was used to assess the cytotoxic effect of the test compounds [46, 47]. Cells (1×10^6 cells/mL) were cultured at 37 °C with various concentrations of peruvioses A and B (0.1–100 µg/mL). Triton X-100 (20%) was used as a positive control. After 24 h, the medium was removed and cells were incubated with MTT solution (3 mg/mL). Four hours later, the medium was carefully aspirated and formazan crystals were dissolved in DMSO (100 µL). The OD_{550} was measured using a microplate reader (Multiscan EX Thermo®).

NO• production

NO• release was determined spectrophotometrically by the accumulation of NO_2^-, a stable metabolite of the reaction of NO• with oxygen, using the Griess reaction [48]. Briefly, 100 µl of cell culture supernatant were mixed with 100 µL of Griess reagent [1 : 1 mixture of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4], and incubated at room temperature for 5 min. The OD_{550} of the samples was measured using a microplate reader (Multiscan EX Thermo®) and compared with a standard curve prepared with $NaNO_2$ (1–200 µM).
Additionally, a direct NO• scavenging effect of peruvioses A and B was determined as described in Supporting Information.

Tumor necrosis factor-α and prostaglandin E2 release

Levels of TNF-α and PGE2 in culture supernatants were determined using commercially available competitive ELISA kits (R&D Systems) according to the manufacturer's instructions. The final results are expressed as ng/mL.

Statistical analysis

Data are presented as mean ± SEM. Concentrations that inhibited cell survival (LC_{50}) and inflammatory mediators (IC_{50}) by 50% were calculated employing nonlinear regression. Statistical comparisons between groups were established using analysis of variance (ANOVA), followed by Dunnett test post hoc analysis. P values less than 0.05 were considered statistically significant.

Supporting information

Methods to determine the NO•-scavenging effect of peruvioses A (**1**) and B (**2**), as well as the scoring criteria of liver and kidney sections for histological analysis are described in Supporting Information. Results of the radical scavenging effect, in addition to FABMS and NMR spectra for **1** and **2**, and NMR spectra for the acetylated compounds (**3** and **4**) are also included.

Acknowledgements

The authors thank Colciencias and the University of Cartagena for financial support of this research (grant 110751929179–2010). Professor Lia Barrios is also acknowledged for her collaboration with the histopathology analysis. Yanet Ocampo is deeply grateful to Colciencias and the University of Cartagena for her PhD fellowship through the National Program for Doctoral Formation (grant 597–2012) and the scholarship for excellent students (resolution 1446–2011), respectively.

Conflict of Interest

The authors declare no conflicts of interest.

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