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Phytochemical Composition, Anti-Inflammatory and Cytotoxic Activities of Chloroform Extract of *Senna crotalarioides* Kunth

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Abstract

Senna crotalarioides is used in traditional medicine to treat inflammation. The aim of this work was to investigate the anti-inflammatory and cytotoxic activities and the possible mechanism of action of the chloroform extract washed with hexane of *S. crotalarioides* (CESC). The anti-inflammatory effect was tested on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema in mice. The levels of TNF-α, IL-1β, IL-6 and IL-10 were determined in macrophages J774A.1 stimulated by lipopolysaccharide (LPS). The cytotoxic activity was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay against six human cancer cell lines: HeLa (cervical cancer), SKLU-1 and A549 (lung cancer), LNCaP (prostate cancer), SW620 (colon cancer) and MCF7 (breast cancer). The composition of the CESC was determined by GC-MS analysis, and standardized by HPLC-ELSD with ursolic acid as the phytochemical marker. CESC inhibited ear edema 61.45%. In chronic ear edema, CESC diminished the inflammation by 53.77%. CESC decreased TNF-α, IL-1β and IL-6 concentrations, and increased the concentration of IL-10. The extract showed IC_{50} values on HeLa, SKLU-1, A549, LNCaP, SW620 and MCF7 by 48, 21, 8.16, 6.82, 1.81, 4.06 and 12.5 μg/mL, respectively. The main components were ursolic acid, 1-octacosanol, stigmasterol, β-sitosterol, 1-triacontanol, (Z, Z) hexadec-9-enoic acid octadec-9-enyl ester. CESC might be useful for developing a phytomedicine with anti-inflammatory and cytotoxic activities.

Keywords

Senna crotalarioides, Anti-Inflammatory, Ursolic Acid, Composition, Cytotoxic

1. Introduction

Inflammation is a protective response to an attack on the body. However, if inflammation is persistent, it can cause several pathological conditions, such as arthritis, cancer, among others. The inflammation is induced by chemical and other mediators, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) [1]. These mediators are pro-inflammatory cytokines. The biological functions of TNF-α, IL-1β and IL-6 are similar, and they are secreted as a response to inflammatory stimuli.

Current therapies for inflammation include non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids; however, both of these approaches have severe side effects [2] such as gastric ulcers and an increased risk of heart attack [3]. Because of these risks, new sources of anti-inflammatory compounds are of great interest.

Products from medicinal plants, such as pure compounds or standardized extracts, are good sources for new drugs.

Senna crotalarioides Kunth (Fabaceae) is a puberulent plant that grows in the Nuevo León, Coahuila, and San Luís Potosí States of Mexico, and this species is used in traditional medicine to treat some types of inflammation [4].

Previously, we reported the antioxidant potential and anti-inflammatory effect of the chloroform extract of this plant on TPA-induced ear edema and carrageenan-induced rat paw edema. However, there have been no studies of the bioactive compounds in this plant or their mechanisms of action.

In the present study, the composition and standardization of the CESC were determined, and we assessed both the in vivo and in vitro anti-inflammatory effects of this extract and the possible mechanism of its anti-inflammatory properties. Additionally, its acute toxicity and cytotoxicity on six cancer cell lines were determined.

2. Material and Methods

2.1. Plant Material

S. crotalarioides was collected in August 2014 in "Las Comadres", Guadalcazar Municipality of San Luis Potosí State, Mexico. Then, the taxonomist José García Pérez identified the plant and a voucher specimen (SPLM43012) was deposited in the Herbarium Isidro Favela of the Universidad Autónoma de San Luís Potosí. S. crotalarioides is not an endangered species, and for this reason, a collection permit is not required by SEGAM-SLP.

2.2. Chemicals

From Sigma-Aldrich was acquire indomethacin (IND), TPA, and ursolic acid, and the immunoenzymatic kits (IL-1β, IL-6, IL-10 and the TNF-α) were purchase from PrepoTech Company. All other reagents were of the highest commercial grade.

2.3. CESC Preparation

The leaves and branches were dried in the shade at room temperature, then, they were powdered. A mixture of 300 g powdered plant and 3.5 L of chloroform, was heated under reflux for 4 h. After, the solvent was eliminated under reduced pressure to give a dry residue, and the solid was washed with hot hexane.

2.4. Derivatization

A mixture of 1 mL of isooctane, 10 mg of CESC, and 100 µL a solution of bis (trimethylsilyl) trifluoroacetamide with 10% of trimethylsilyl chloride was heated for 10 min at 100˚C in a microwave oven (CEM Discover) at 150 watts.

2.5. CESC Analysis

The analysis of the extract was carried out on a gas chromatograph coupled to a mass spectrometer (Agilent Technology, model 6890 N); which was coupled to a selective detector of mass (model 5973). A capillary column DB-5HT of 15 m in length, 0.25 mm internal diameter and 0.10 µm film thickness was used. The injector temperature was 320˚C. The next temperature program was used: the initial temperature 100˚C was maintained for 3 min, then, the temperature was increased at a rate of 10˚C/min up to 320˚C, and this temperature was held for 5 min. The splitless injection was performed at a ratio of 1:100. The spectrum was performed at 70 eV. The identification of the compounds was carried out by comparing their spectra with the mass spectra of standard samples and the Wiley14.1/NIST11 library.

2.6. Standardized Extract

The standardization of CESC was performed by HPLC (Varian Model Pro Star 310) coupled to an evaporative light scattering detector (ELSD) (Alltech model ELSD 2000). Nitrogen was used as drying gas, a flow rate of 1.7 L/min at 67˚C evaporation temperature. A Grace Smart RP C18 column (5 µm, 4.6 × 250 mm) was used. The temperature of column oven was 40˚C. The flow rate was 0.7 mL/min. The linear gradient of the mobile phase began at 0.5% (v/v) of acetic acid in a solution of water-methanol-acetonitrile with a ratio of 1:0:99 (v/v/v) and finished 20 min later with a solution of 0.5% acetic acid in a mixture of water-methanol-acetonitrile with a ratio of 1:10:89 (v/v/v). The calibration curve was obtained with solutions of ursolic acid at six concentrations (0.06, 0.12, 0.18, 0.24, 0.30 and 0.60 mg/mL). The result for each concentration was the average of three injections of 20 µL, using a loop. The integrals of the chromatographic

peaks at a retention time of 5.38 min were used to generate the standard calibration curve (R^2 = 0.9912). CESC (6.0 mg) was dissolved in 10 mL methanol, and the sample (20 µL) were injected in triplicate and then extrapolated to the standard calibration curve.

2.7. Experimental Animals

Male CD1 mice (20 - 25 g) were obtained from the animal facility of the Universidad Autónoma Metropolitana-Xochimilco. The animals were kept in isolated cages, and they had access to food (Lab Diet 5001) and water ad libitum, and they were housed at 24˚C under light-dark cycles of 12:12 h. The Research Bioethics Committee of the UAM-X approved all experimental (number project 140). The tests in vivo were carried out according to the official Mexican Norm (NOM-062-ZOO-1999), for the care of animals. The mice were maintained to laboratory conditions for 1 week prior to the test, which were done after 9:00 am, and finally were sacrificed in a CO_2 chamber.

2.8. Anti-Inflammatory Evaluation

2.8.1. TPA-Induced Mouse Ear Edema

The TPA-induced mouse ear edema procedure was described previously by de Young [5]. A solution of TPA (2.5 µg) in acetone (25 µL) was applied topically to the inner and outer surfaces of the right ears of the mice (groups of 8 mice), and acetone (25 µL) was administered to both surfaces to the left ear. Thirty minutes after a solution of CESC (2.0 mg/ear) or IND in acetone was topically applied to the right ear. After six h. the mice were sacrificed, and 6 mm plugs of the central portion of both ears were obtained and weighed. The following formula was used to determine the percent inhibition of the edema.

$$\%\ \text{inhibition} = \frac{W - W_o}{W' - W_o'} \times 100$$

where W = TPA + treatment; W_o = vehicle-treated ear; W' = right ear with TPA, without treatment; W_o' = left ear with vehicle.

2.8.2. Determination of Anti-Inflammatory Activity on Induced Mouse Ear Edema by Multiple Application of TPA

Groups of 8 mice were topically applied to the right ear on both the inner and outer surfaces with a solution of 2.5 µg TPA in 25 µL acetone. After thirty min, IND (0.5 mg/ear) or CESC (2 mg/ear) were administered topically. TPA and the treatments were applied on day 1, 3, 5, 7 and 9 after the treatment. After 6 h, the animals were sacrificed, and 6 mm plugs of the central portion of both ears of each mouse were obtained and the circles were weighed. The above formula was used to determine the percent inhibition of the edema.

2.9. Acute Toxicity

The acute toxicity was determined using the methodology described in the OECD Guideline for testing chemicals [6]. Groups of 5 mice were used. One

group was administered polivinilpirrolidone (PVP) and the others with CESC at doses of 5000 and 2500 mg/Kg in PVP in a 1:4 ratio. After 72 h the animals were sacrificed, and biopsies were carried out to identify possible signs of toxicity.

2.10. Cell Culture

The cell lines were used Macrophages J774A.1 (obtained from ATCC® TIB-67™), HeLa (ATCC® CCL-2™), MCF7 (ATCC® HTB-22™), SKLU-1 (ATCC® HTB-57™), LNCaP (ATCC® CRL-1740), A549 (ATCC® CCL-185™), SW620 (ATCC® CCL-227™) and HaCat (ATCC® PCS-200-011™), obtained from Instituto Nacional del Cáncer of México. The cells were maintained in DMEM (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/ml streptomycin. For Macrophages were used RPMI supplemented with 10% of FBS, penicillin (100 units/mL), and streptomycin (100 µg/mL) at 37˚C under CO_2 (5%).

2.11. Cell Viability Assay

Macrophages J774A.1 and the cancer cell lines were washed with a PBS buffer, then, the cells were seeded in DMEM in 96-well microplates (8×10^4 cells/well), they were incubated for 24 h, after this time a solution of CESC in DMSO (1 - 200 µg/mL) was added. 48 h after the treatment, 10 µL of MTT of a solution of 5 mg/ml in PBS were added, and the cells were incubated for 4 h at 37˚C. After this time, the medium was removed, and the formazan crystals were dissolved in 100 µL of DMSO. The optical density (OD) was obtained using an ELISA plate reader from BioRad at 540 nm. Six wells were used for each concentration of CESC. The viability was determined using the following equation:

$$\%\ \text{viability} = \frac{\text{Abs treated cells}}{\text{Abs control cells}} \times 100$$

2.12. Determination of the Levels of Cytokines

The serum levels of TNF-α, IL-6, IL-10, and IL-1β were determined with a commercially ELISA kit following the manufacturer's instructions. The OD was measured using a microplate reader at 405 nm with a wavelength correction set to 650 nm.

In plates of 6 wells macrophages J774A.1 were seeded at a density of 1×10^6/well and treated with CESC (25 µg/mL) or IND at a concentration of 17 µg/mL (50 µM) and incubated for 2 h. After this time, 5 µg/mL of LPS was added and the cells were incubated for 24 h, the supernatants were collected and stored at −80˚C until they were analyzed. The levels of IL-6, IL-1β, IL-10 and TNF-α in the supernatants of the cultures of macrophages were determined using a commercial immunoenzymatic kit (PeproTech).

2.13. Statistical Analysis

The results are expressed as the means ± SE, and statistical analyses were per-

formed using Student's t-test and ANOVA followed by Tukey's test. Differences were deemed significant at $p < 0.05$.

3. Results

In CESC was identified 27 compounds by GC/MS (Table 1), they represented 94.03% of the extract. The retention times were between 5.27 and 18.62 min, and the main compounds were ursolic acid (16.39%), 1-octacosanol (13.19%), stigmasterol (9.57%), β-sitosterol (12.86%), 1-triacontanol (14.54%) and (Z,Z)-hexadec-9-enoic acid octadec-9-enyl ester (9.90%). Then, we considered the main component, ursolic acid as an appropriate phytochemical marker for developing a method for standardizing CESC.

In HPLC, retention time of ursolic acid was 5.61 min; and in Figure 1 is shown representative chromatogram of ursolic acid and CESC. The calibration curve was linear in the 0.06 to 0.6 mg/mL range (R2 = 0.9912). We found that CESC has 170.5 mg/g (17.05%) of ursolic acid.

CESC at doses of 2 mg/ear diminished TPA-induced ear edema in mice by 61.45 ± 3.95, which was similar to the inhibition obtained with IND (67.62 ± 4.88). In chronic TPA-induced ear edema, CESC diminished the edema by 53.77% at a dose of 2 mg/ear, and this was not significant difference with the edema inhibition obtained with IND (50.05% ± 3.52%).

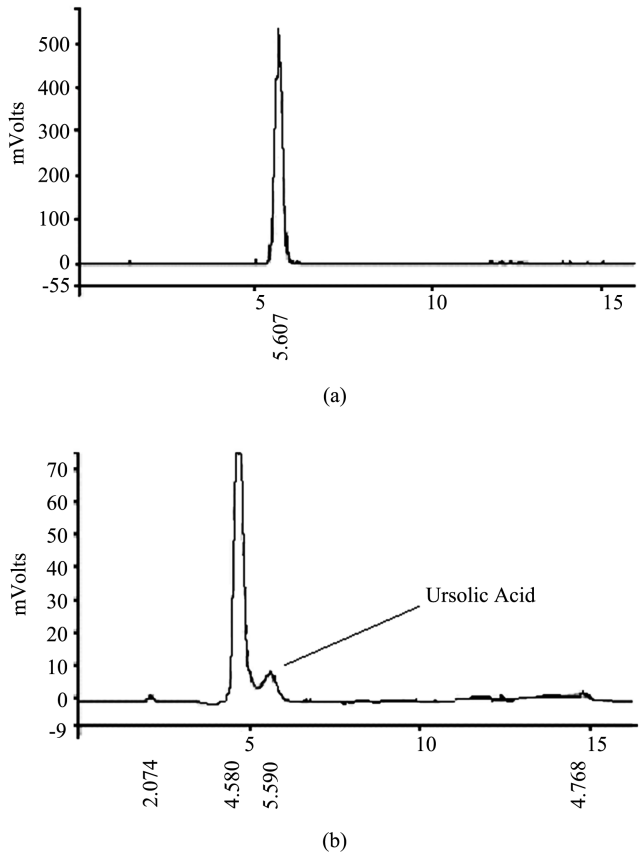

Figure 1. Chromatogram of commercial ursolic acid (a) and (b) CESC.

Table 1. Composition of CESC.

	Name of compound	Retention Time	%	KI
1	Neophytadiene	13.455	0.12%	1774
2	3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol	13.525	0.18%	2045
3	Palmitic Acid	15.621	4.95%	1987
4	Phytol	16.757	0.38%	2086
5	9, 12-Octadecadienoic acid (Z, Z)	17.087	0.06%	2202
6	*α*-Linolenic acid	17.165	0.14%	2210
7	Stearic acid	17.332	0.31%	2186
8	1-Heneicosanol	18.898	0.74%	2393
9	Arachidic acid	18.962	0.06%	2385
10	Cyclohexane, 1, 1-dimethyl-3-methylene-2-[3-(oxy)-2-propenyl)-, (Z)-	19.169	0.13%	1686
11	1-Monopalmitin	20.152	0.05%	2581
12	Behenic acid	20.451	0.05%	2584
13	Heptacosane	20.872	0.11%	2705
14	Lignoceric acid	21.872	0.30%	2782
15	Nonacosane	22.264	0.14%	2900
16	1-Hexacosanol	22.597	1.20%	2890
17	Octacosanal	23.352	6.61%	2993
18	1-Octacosanol	24.032	13.19%	3089
19	9-hexadecenoic acid. 9-octadecenyl ester, (Z, Z)	24.172	9.90%	3584
20	*α*-Tocopherol	24.340	0.62%	3226
21	17-Pentatriacontene	24.930	0.46%	3508
22	Stigmasterol	25.744	9.57%	2797
23	1-Triacontanol	25.966	14.54%	3287
24	*β*-Sitosterol	26.487	12.86%	2789
25	Lanostan-3*β*-ol, 11*β*, 18-epoxy-, acetate	27.063	0.79%	3003
27	Oleanolic acid	29.658	4.67%	3242
28	Ursolic acid	30.583	16.39%	3306

CESC at different concentrations (1, 5, 10, 25 and 50 μg/mL) did not affect cell viability of macrophages (**Figure 2**); however, at concentrations of 100 and 200 μg/mL of CESC the cell viability was 51.18% and 42% - 71% respectively. Therefore, the concentration of CESC used in further experiments was 25 μg/mL.

The activity of CESC and IND on the levels of cytokines TNF-*α*, IL-1*β*, IL-6 and IL-10 were measured in macrophages stimulated with LPS which increase the production of TNF-*α*, IL-1*β* and IL-6. In this study was found that CESC at

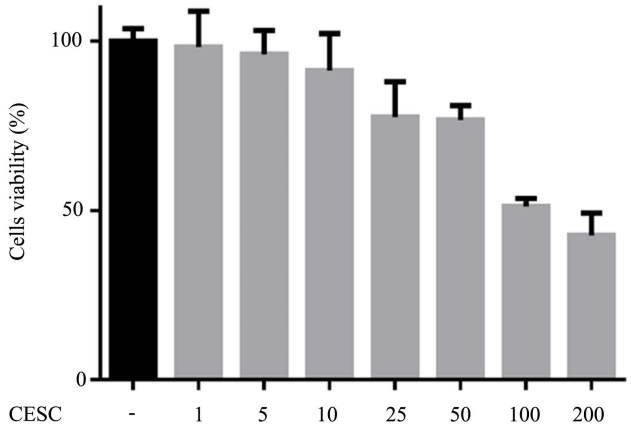

Figure 2. Effect on cell viability in macrophages treated with CESC at 1, 5, 10, 25, 50, 100 and 200 μg/mL. Determined with MTT assay. Results are expressed as the percentage of surviving cell relative to control cell. The results are the mean of three determinations ± SE.

concentration of 25 μg/mL (**Figure 3**) significantly reduced the concentrations of TNF-α (52.16%), IL-1β (55.21%) and IL-6 (54.86%), similar to the results obtained with IND. Moreover, CESC (25 μg/mL) increased IL-10 production (48.33%) in comparison with the control group, and the increase in production of this cytokine was similar to that obtained with IND.

The results obtained in this study indicated that CESC might be a useful agent for the treatment of inflammatory conditions.

The cytotoxic activity of CESC and ursolic acid, the major constituent of this extract, were evaluated against HaCat and six human cancer cell lines, HeLa, MCF7, SKUL-1, LNCaP, SW620 and A549, at concentrations of 1, 10, 25, 50, 100 and 200 μg/mL. CESC showed high cytotoxicities (**Table 2**) against SKUL-1, LNCaP, SW620 and A549 with IC50 values of 8.16, 1.81, 4.06 and 6.82 μg/mL respectively. The IC_{50} value of CESC toward HaCat was 80.3 μg/mL. These results showed that the cytotoxic activity of the extract on LNCaP was higher than that of cisplatin (CDDP). Ursolic acid exhibited the highest cytotoxicity against HeLa cells (IC_{50} 1.46 μg/mL), and the effect was higher than that of CDDP (IC_{50} value 3.2 μg/mL); the effects of this acid against the other cell lines was lower than those of CESC (**Table 2**).

4. Discussion

4.1. Standardized and Composition of the CESC

The CESC was standardized by HPLC using ursolic acid, because this compound is the main component of the extract, which makes it an excellent fingerprint to carry out the standardization of the extract by this analytical method.

The difference of percentages of ursolic acid in CESC obtained by the HPLC method with ELSD detector (17.05%) and the GC-MS (16.39%), might be due to that in the last method the extract was derivatized and the reaction could be not complete.

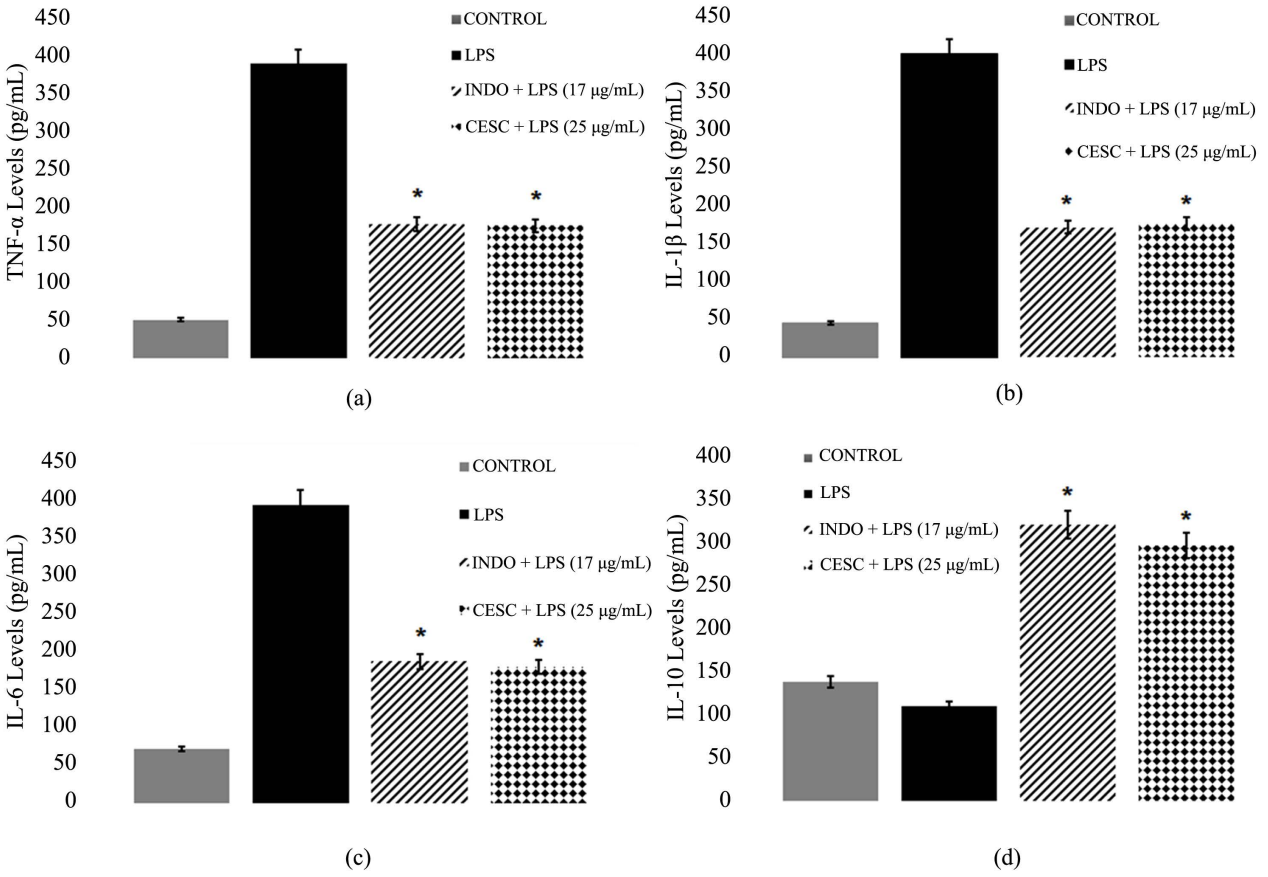

Figure 3. Effects of CESC on the (a) TNF-*α*, (b) IL-1*β*, (c) IL-6 and (d) IL-10 levels in macrophages LPS stimulated. The concentration was determined by ELISA. The results are the mean of three determinations ± SE. *p < 0.05 versus LPS group.

Table 2. Cytotoxic activity of CESC and Ursolic acid.

Cell line	IC_{50} μg/mL		
	CESC	Ursolic acid	CDDP
HaCat	80.3 ± 3.19	50.12 ± 1.96	3.9 ± 1.3
HeLa	48.21 ± 7.14	1.46 ± 0.49	3.2 ± 0.9
MCF7	12.5 ± 1.5	37.5 ± 2.43	1.11 ± 0.6
SKUL-1	8.16 ± 0.78	46.0 ± 3.69	4.2 ± 1.3
LNCaP	1.81 ± 0.34	>200	5.52 ± 2.3
SW620	4.06 ± 0.77	>200	3.81 ± 1.01
A549	6.82 ± 0.53	>200	3.17 ± 1.09

4.2. Anti-Inflammatory Activity *in Vivo*

After two h of topical application of TPA there are vasodilation, edema and platelet aggregation, and three h later the ear edema is increased by exudate action and after 6 h is observed the maximum expression of edema. The inflammation produced by topical administration of TPA is mediated the phospholitase A2 and cyclooxygenase stimulation [7]. Then, one way to control inflamma-

tion is the inhibition of the stimulation of phospholipase A2. CESC significantly diminished the edema, which suggests that it inhibits phospholipase A2 production, vasodilation, and platelet aggregation.

The multiple applications of TPA cause ear edema, epidermal hyperplasia [8], and infiltration of inflammatory cells, such as polymorphonuclear leukocytes. The results showed that CESC (2 mg/ear) significantly diminished TPA-induced ear edema, these facts suggested that the extract decreased the cellular infiltration and epidermal hyperplasia.

4.3. Anti-Inflammatory Activity *in Vitro*

Inflammation is a response to irritation or infection, and macrophages play an important role in the inflammation process [9]. These cells show a vigorous response to LPS, this compound increases the production of inflammatory modulators such as TNF-α, IL-6 and IL-1β.

TNF-α, also known as cachectin, is involved in immune response and can prevent infections and keep inflammation localized, but inappropriate or excessive production of this mediator can be harmful [10].

IL-6 is an inflammatory interleukin [11], and it is responsible for the induction and perpetuation of inflammation, and it can amplify the inflammatory cascade and cause injury.

IL-1β is also a mediator of inflammatory response and exacerbates damage during chronic and acute tissue injury [12].

IL-10 possesses anti-inflammatory activities and plays a crucial role in preventing inflammatory and autoimmune pathologies. IL-10 inhibits the production of IL-1β and TNF-α in LPS-activated macrophages. Therefore, compounds that regulate cytokines may have therapeutic effects. The inhibition of the mediators occurs by acting on NF-κB (nuclear factor kappa B), which regulates the release of inflammatory cytokines [13]. This fact suggests that CESC acts on transcription factors, but further studies are required to confirm this suggestion. Thus, the agents that inhibit the production of pro-inflammatory mediators and promote the synthesis of anti-inflammatory cytokines may be useful in treating inflammatory conditions.

The anti-inflammatory activity of ursolic acid has been attributed to its ability to suppress NF-ƙB activation [14]. Additionally, this compound suppresses the expression of COX-2 and iNOS [15] and inhibits the production of NO [16].

1-Octacosanol [17], triacontanol [18], stigmasterol [19], and β-sitosterol [20] also show anti-inflammatory activities, which suggests that the anti-inflammatory activity of CESC might be due to the presence of these five compounds.

4.4. Cytotoxic Effect

The cytotoxic activity of CESC and ursolic acid, the main component of this extract, were evaluated against HeLa, MCF7, SKUL-1, LNCaP, SW620 and A549, and HaCat (keratinocytes). CESC exhibited the highest activity on SKUL-1,

LNCaP, SW620 and A549, which was higher than that obtained with ursolic acid on these four cell lines.

According to the National Cancer Institute, pure compounds or extracts with IC values lower than 4 μg/mL and 30 μg/mL, respectively, are considered as cytotoxic [21], and CESC showed IC_{50} values lower than 30 μg/mL in 5 human cancer cell lines, and its IC50 value (80.3 μg/mL) in HaCat, a non-cancerous human cell line, was greater than those obtained for the cancer cell lines. The results suggest that the cytotoxic activity of CESC might be specific to cancer cell lines.

There are reports that indicate that ursolic acid shows cytotoxic activity against different cancer cell lines [22] [23]. This triterpene acts by inhibiting cell proliferation and inducing apoptosis by activating caspase-3. Cellular apoptosis is always accompanied by the disruption of the mitochondrial membrane, resulting in rapid collapse and the activation of downstream caspases, which induces cell apoptosis. In the case of MCF-7, this compound has a different mechanism action, including inhibition of growth and suppression of migration, and in this cell line, the signaling pathway is triggered by NFκB [24].

The cytotoxic effect of ursolic acid [25], 1-octacosanol [26], and *β*-sitosterol has been reported. Thus, the cytotoxic activity of CESC on SKUL-1, LNCaP, SW620, and A549 might be due to the mixture of these three compounds. More studies are requested to know the mechanism of action of CESC.

5. Conclusion

The results showed that CESC possesses anti-inflammatory and cytotoxic activities; therefore, standardized CESC might be useful for developing a phytomedicine.

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Author Contributions: Serrano R. prepared the extract (CESC) and evaluated the acute and chronic anti-inflammatory activity on TPA induced mouse ear edema, and cytotoxicity; Alarcón F. and Almanza J. determined the levels of inflammatory interleukines; Pérez C. and González M. determined the composition of CESC and standardized this extract; Pérez S. analysis of the results, and wrote the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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List of Abbreviation

DMEM: Dulbecco's Modified Eagle's Medium; IL-1β: Interleukin-1β; IL-6: Interleukin-6; IND: Indomethacin; LPS: Lipopolysaccharide Escherichia coli O111:B4; CESC: Chloroform extract of the aerial parts of *Senna crotalarioides*; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NO: Nitric Oxide; NSAIDs: Non-steroidal anti-inflammatory drugs; OD: Optical density; PVP: Polyvinylpyrrolidone; TNF-α: Tumor necrosis factor; TPA: 12-O-Tetradecanoylphorbol-13-acetate; CDDC: Cisplatin.