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Croton stipulaceus Kunth, a native Mexican medicinal plant with antioxidant and anti-inflammatory activities

[*Croton stipulaceus* Kunth, planta medicinal mexicana con actividad antioxidante y antiinflamatoria]

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Abstract: Leaves of *Croton stipulaceus* were extracted (EHex, $ECHCl_3$ and EEtOH extracts) to assess their antioxidant potential, anti-inflammatory activity in murine models and acute toxicity. EEtOH showed the highest effect in DPPH (37.80% inhibition), FRAP (1065.00 ± 55.30 µmol Fe^{2+}) and total polyphenols (231.24 ± 9.05 meq AG/gM). EHex was the most active, ~ 50% inhibition of TPA-induced ear edema; while EEtOH (dose of 2 mg/ear) showed the highest inhibition in the chronic model (97% inhibition), and inhibited MPO activity (48%). In carrageenan-induced edema, $ECHCl_3$ (dose 500 mg/kg) was the most active. None of the extracts showed acute toxicity (LD_{50}) at 2 g/kg (p.o.). This work is the first report that supports the traditional use of *C. stipulaceus* as an anti-inflammatory.

Keywords: *Croton stipulaceus* Kunth; Antioxidant; Anti-inflammatory; Medicinal plant; Myeloperoxidase.

Resumen: De las hojas de *Croton stipulaceus* se obtuvieron diferentes extractos (EHex, $ECHCl_3$ y EEtOH) evaluando el potencial antioxidante y la actividad antiinflamatoria en modelos murinos y la toxicidad aguda. El EEtOH mostró mayor efecto en DPPH (37.80% inhibición), FRAP (1065.00 ± 55.30 µmol Fe^{2+}) y polifenoles totales (231.24 ± 9.05 meq AG/gM). El EHex fue el más activo, cercano al 50% de inhibición del edema auricular inducido con TPA; mientras que el EEtOH (dosis de 2 mg/oreja) mostró la mayor inhibición en el modelo crónico (97% inhibición), e inhibió la actividad de la MPO (48%). En el edema inducido con carragenina, el $ECHCl_3$ (dosis 500 mg/kg) fue el más activo. Ninguno de los extractos mostró una toxicidad aguda (DL_{50}) mayor a 2 g/kg (p.o). Este trabajo es el primer reporte que sustenta el uso tradicional de *C. stipulaceus* como antiinflamatorio.

Palabras clave: *Croton stipulaceus* Kunth; Antioxidante; Antiinflamatorio; Planta medicinal; Mieloperoxidasa.

INTRODUCTION

The genus *Croton* (Euphorbiaceae) includes about 1300 plant species distributed mainly in tropical and subtropical regions around the world, have a varied foliar morphology and some species secrete a reddish exudate or latex (Ravanelli *et al*., 2016; Cucho-Medrano *et al*., 2021). In America, the distribution of the species is in southern Mexico (Veracruz, Puebla and Estado de México) and Central America to tropical and subtropical South American countries. The popular name of the species of this genus, including Mexico and the state of Veracruz are "sangre de drago", "sangregao", "palo sangre", "huampo", "palo de grado", "sangrado" (Webster, 1993; CONABIO, 2009). The leaves are commonly used for therapeutic purposes, such as healing, anti-inflammatory, antiseptic, hemostatic, antidiarrheal and gastrointestinal illnesses (Cucho-Medrano *et al*., 2021). In Mexico, the medicinal use of species of the genus *Croton* are numerous, *C. arboreous* Millsp., the aerial parts are used as inflammatory in respiratory ailments, the leaves of *C. californicus* Müll. Arg. are used for pain reliever for rheumatism and the *C. draco* Cham. & Schltdl. has different use, cough, flu, diarrhea and stomach ulcers, the topically aplication is used as wound healing, sores, herpes, anti-septic and others use (Salatino *et al*., 2007). It has been reported the effect of species of this genus as antimicrobial, antitumor, analgesic and healing (Cano-Asseleih, 1997; Martínez-Gordillo & Cruz-Durán, 2002; Tsacheva *et al*., 2004; Salatino *et al*., 2007). Additionally, various activities, including anti-inflammatory, antinociceptive, gastroprotective, healing, and cardiovascular of the essential oil from this genus were reported in various animal models (Salatino *et al*., 2007).

Chemically, from the *Croton* genus, diterpenoids are reported to be the major components with various biological activities such as cytotoxic, anti-inflammatory, antifungal, acetylcholinesterase inhibitory, and neurite outgrowth-promoting. Other reported chemical components include sesquiterpenoids, sesterterpenoid, triterpenoid, glycosides, alkaloids, benzoate derivatives, pyran-2-one derivatives, cyclopeptide, tropone derivatives and limonoids (Xu *et al*., 2018). Previous studies on species of the *Croton* genus have corroborated their traditional uses, for example, the latex of *Croton urucurana*, possess antifungal and antidiarrheal activity (Gurgel *et al*., 2001) and the extracts of the aerial parts and bark showed antiulcerogenic, analgesic and anti-inflammatory activity (Cordeiro *et al*., 2012; Cordeiro *et al*., 2016). Studies of the hydroalcoholic extract of the leaves of *C. echinocarpus* showed *in vitro* antiviral activity (Ravanelli *et al*., 2016) and the essential oil of the aerial part *C. heliotropiifolius* possesses antibacterial activity (Araújo *et al*., 2017). *Croton stipulaceus* Kunth is used by the population in Mexico as diuretic and anti-inflammatory; however, no study of these effects has been reported. For this reason, the present work, documents the first studies to support the popular use of this species as anti-inflammatory in experimental models of acute and chronic inflammation and its relationship with its antioxidant potential.

MATERIAL AND METHODS

Chemicals

The chemicals used in this study were analytical grade. Ferric chloride, acetone, sodium carbonate, sodium acetate, acetic acid, sodium picrate, acetic anhydride, methanol (MeOH), ethanol (EtOH), chloroform ($CHCl_3$), hexane (Hex), ethyl acetate (EtOAc), Grignard reagent, potassium hydroxide, potassium iodine, sulphuric acid, basic bismuth salts and cobalt chloride were purchased from Merck Co (Germany). 2,2diphenyl-1-pychrylhydrazyl (DPPH), Folin-Ciocalteu reagent, 2,4,6-tripyridyl-5-triazine (TPTZ), phorbol 12-myristate 13-acetate (TPA), indomethacin, carrageenan, ascorbic acid, gallic acid and TLC plates were purchased from Sigma-Aldrich Cod. 2193291 (St. Louis, MO, USA).

Collection material plant

The leaves of *C. stipulaceus* Kunth were collected in Landero y Coss municipality, Veracruz State, Mexico in July 2011. A voucher specimen (16785-UV) was deposited at the herbarium of the Centro de Investigaciones Biológicas, Universidad Veracruzana and was identified by Dr. Fernando Nicolas de Morejón.

Preparation of plant extracts

The leaves of the plant material (approximately, 500 g) were extracted by successive and exhaustive maceration with 3 L of solvents of ascending polarity using hexane (EHex, 9.3 g), chloroform ($ECHCl_3$, 7.8 g) and ethanol (EEtOH, 8.9 g), and kept in darkness at room temperature. The extracts were removed using a rotary evaporator (Heidolph LABOROTA 4000) and in a vacuum oven (ShelLab) at 25ºC, to a fully dried extract.

Phytochemical and chemical analyses

Phytochemical analyses of the plant extracts were carried out using standard qualitative methods (color test and/or Thin Layer Chromatography, TLC) to search for the presence of sterols, terpenoids, flavonoids, coumarins, lignans and alkaloids (Domínguez, 1973; Cseke, 2006).

Antioxidant activity

DPPH radical-scavenging activity

For this evaluation, Brand-Williams *et al*. (1995), method modified by Domínguez-Ortíz *et al*. (2009), was used. In an amber vial, 2.9 mL of prepared DPPH solution 9 x 10^{-5} M in MeOH were added, followed by the addition of 100 µL of plant extracts at different concentration (16.6 µg/mL) dissolved in MeOH. For the blank 100 µL of MeOH were added instead of the sample. After mixing, the samples were incubated for 30 min at 37ºC in a water bath. The extracts samples (A_E) and blank (A_B) were measured at 517 nm using a UV-Vis spectrophotometer (Varian Model Cary-100). The experiments were performed by triplicate and the activity was calculated using the formula:

$$\% \text{ inhibition} = [(A_B - A_E)/A_B] \times 100$$

FRAP "ferric reducing/antioxidant power"

The preparation of FRAP reagent was employed 100 mL of sodium acetate buffer solution (300 mM, pH 3.6), 10 mL of TPTZ (10 mM) in HCl 40 mM, 10 mL of $FeCl_3 \cdot 6H_2O$ (20 mM) and was incubated at 37ºC for 4 min. Then an amber vial, were transferred 2.7 mL of FRAP solution and added 150 µL of distilled water and 150 µL of a methanol solution of plant extract (1 mg/mL). The absorbance of the samples was measured at 593 nm for triplicate. The blank solution was prepared by adding 300 µL of distilled water to 2.7 mL of FRAP solution (Benzie & Strain, 1996). The results were expressed in µmol de Fe_2/L and based on a calibration curve from different concentrations of $FeSO_4$ (50-1000 µmol/L, R_2 = 0.99408)

$$\text{Absorbance} = 0.001\ [\mu\text{mol } Fe^{2+}/\text{L}] + 0.11128$$

Total phenolic content

The Folin-Ciocalteu reagent with some modifications for the determination the total phenolic concentration. 50 µL of each sample, 2.5 mL (1/10 dilution of Folin-Ciocalteu reagent) and 2 mL of Na_2CO_3 (7.5%, w/v) were mixed and incubated at 45°C for 15 min. The experiments were made in triplicates (Cai *et al*., 2004) and all samples were measured at 765 nm of the absorbance using a UV-Vis spectrophotometer (Varian, model Cary 100). Results were expressed as gallic acid equivalent GAE (mg/L) using a standard gallic acid graph (range 10 to 500 mg/L, R_2 = 0.99496).

$$\text{Absorbance} = 0.00109\ [\text{GAE mg/L}] + 0.07547$$

In vivo assays animals

Male CD1 mice (20 - 25 g) were used in acute and chronic inflammation model, myeloperoxidase assay and acute toxicity test. The storage and housing conditions of the animals were standard laboratory conditions (25ºC, 12-h dark/12-h light, 50% relative humidity) with food and water *ad libitum*. All procedures and handling of the animals were in accordance with the Mexican Official Regulation (NOM ZOO–062-200-1999) entitled "Technical Specifications for Production, Care, and Use of Laboratory Animals".

Anti-inflammatory activity

TPA induced acute ear edema in mice

The ear edema was induced applying topically 2.5 µg of TPA dissolved in 25 µL of acetone in both faces of the right ear; the left ear only received acetone (n = 6, for group). The groups with extracts or indomethacin (0.5 mg/ear, 1 mg/ear and 2 mg/ear dissolved in 50 µL of acetone) were applied topically 30 min after TPA. The animals were sacrificed 6 h after the induction edema with TPA by cervical dislocation. In both ears were cut and weighted 6 mm diameter in all groups. The edema inhibition was obtained with the weight difference between the right and the left ear respect the control group (Young & De Young, 1989; Moreno-Quirós *et al*., 2017).

TPA induced chronic ear edema in mice

Induction of ear chronic edema was performed using repeated doses of TPA and treatment according to García-Rodríguez *et al*. (2012). Ear edema was induced with the topical application of 2.5 µg of TPA dissolved in 25 µL of acetone for 5 alternate days in the left ear. The extracts (2 mg/ear) or indomethacin (0.5 mg/ear) were dissolved in acetone or ethanol and were applied 30 min after TPA in the same ear that edema induced. The right ear only was applied with acetone in all group. In the last application, the animals were processes same to acute ear edema with TPA by obtained de tissue ear of 6 mm of both ears. The inhibition percent was calculated with respect to control group.

Myeloperoxidase assay (MPO)

Tissue myeloperoxidase (MPO) activity was assessed 24 h after TPA application to the mouse ear according to Brandley *et al*. (1982). In this evaluation, the extract or indomethacin (1 mg/ear and 2 mg/ear dissolved in 50 µL of acetone) were applied to different times with respect to TPA, before 30 min, immediately after or after 30 min. The animals were sacrificed 24 h after of the induction edema with TPA by cervical dislocation. In both ears were cut and weighted 6 mm diameter in all groups. Biopsies (6 mm) of the ears were placed in 0.75 mL of 80 mM phosphate-buffered saline (PBS, pH 5.4) containing 0.5% HTAB and then homogenized (45 s at 0°C). The homogenate was decanted into a microfuge tube, and the vessel was added 0.75 mL aliquot of PBS/HTAB buffer, after was added to the tube and the 1.5 mL sample was centrifuged at 10,000 × g at 4°C for 20 min. Triplicate 30 µL samples of the resulting supernatant were added to 96-well microliters plates. For assay, 200 µL of a mixture containing 100 µL of 80 mM PBS pH 5.4, 85 µL of 0.22 M PBS pH 5.4, and 15 µL of 0.017% hydrogen peroxide were added to the wells. The reaction was started with addition of 20 µL of 18.4 mM tetramethylbenzidine HCl in dimethylformamide. Plates were incubated at 37 ◦C for 3 min and then the reaction was stopped by addition of 24 µL of 1.46 M sodium acetate, pH 3.0. Enzyme activity was determined colorimetrically using BIORAD iMark plate reader to measure absorbance at 655 nm (Bralley *et al*., 2008). Triplicate 30 µL samples of the resulting supernatant were added to 96-well microliters plates.

Carrageenan induced paw edema

The paw edema was induced with subcutaneous injection of the 50 µL of carrageenan solution of 1% in saline in the right hind paw. The edema was measured after the carrageenan applied at t = 1, 3, 5 and 7 h employed a digital micrometer, only the t = 0 was registered before the administration of carrageenan (Winter *et al*., 1962; Beloeil *et al*., 2005). The indomethacin (8 mg/Kg) or plant extracts (250 and 500 mg/Kg) were administration the oral route (p.o.) 60 min before of carrageenan, the control group only received the vehicle (tween 80-water 1:9) by the same route. The results were expressed as the difference between the edema at different times whit respect to the t = 0 (Tt) and the percentage of inhibition was calculated according to Olajide *et al*., 1999.

Acute toxicity (LD_{50})

Twelve hours before of the study the animals were fasted, the administration of the plant extracts (1000 and 2000 mg/kg) or control vehicle (tween 80-water 1:9) was intragastric route on a single dose, n=3. Daily for 14 days the animals were observed registering behavioral changes and deaths; after this period of days, mice were euthanized by clavicle dislocation. The liver, lungs, heart, spleen and kidney were excised and macroscopically examined (Lorke, 1983; OECD, 2016).

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). For statistical evaluation, comparison between experimental and control groups were performed by one-way analysis or variance (ANOVA) followed by Dunnett test. Values $p \leq 0.05$ were accepted as statistically significant.

RESULTS

Antioxidant activity

DPPH and FRAP tests were carried out to evaluate the antioxidant potential of extracts of *C. stipulaceus* (Table No. 1). The EEtOH extract showed both, the highest radical scavenging capacity of DPPH (37.80%) and the highest ferric reducing (FRAP) ability (1065.00 ± 55.30 µmolFe^{2+}/L). The same extract showed the highest concentration of phenolic content (231.24 ± 9.05 meqAG/gM). In both tests, the ascorbic acid was employed as reference.

A qualitative phytochemical analysis carried out on the organic extracts of *C. stipulaceus* leaves (EHex, E$CHCl_3$, EEtOH) showed the presence of sterols in all extracts, additionally, in the extracts E$CHCl_3$ and EEtOH, flavonoid compounds are also observed.

Anti-inflammatory effect

TPA induced acute ear inflammation

Table No. 2 shows the effect of the extracts of *C. stipulaceus* when applied topically on the surface of the mouse ear, noting that the extract EHex and E$CHCl_3$ in all the doses used (0.5 - 2 mg/ear), inhibited the edema significantly with respect to the control group. The EEtOH extract only showed significant inhibition at the higher dose (2 mg/ear) of 31%. The indomethacin showed to doses of 1 mg/ear the mayor inhibition of 72%.

Table No. 1
Antioxidant activity of *C. stipulaceus* extracts

Extract	DPPH Inhibition (%)	FRAP (µmol Fe^{+2} / L)	Total Phenolics (meqAG / gM)
EHex	10.40 ± 1.22	179.20 ± 28.20	16.32 ± 2.54
$ECHCl_3$	10.80 ± 0.47	465.50 ± 35.30	79.36 ± 10.12
EEtOH	37.80 ± 0.75	1065.00 ± 55.30	231.24 ± 9.05
Ascorbic acid	100	3276.50 ± 102.60	ND

Results are shown as mean ± standard deviation of the radical scavenging effect of DPPH, extracts were tested and ascorbic acid. Total phenolic content is expressed in miliequivalents of galic acid per gram of extract (meqAG/gM). Ferric reduction power is expressed in µmoles of ferric ions reduced per liter (µmol Fe^{+2}/L). ND=No determinated

Table No. 2
Anti-inflammatory effect of *C. stipulaceus* TPA induced ear acute edema in mice

Treatment	Doses (mg/ear)	TPA Edema (mg)	Inhibition edema (%)
Control		12.40 ± 0.22	0
EHex	0.5	5.20 ± 0.13*	58
	1.0	6.60 ± 0.06*	47
	2.0	6.00 ± 0.04*	52
$ECHCl_3$	0.5	7.40 ± 0.10*	40
	1.0	6.40 ± 0.05*	48
	2.0	7.50 ± 0.11*	39
EEtOH	0.5	10.20 ± 0.14	18
	1.0	11.00 ± 0.12	11
	2.0	8.60 ± 0.11*	31
Indomethacin	0.5	6.33 ± 0.09*	49
	1.0	3.48 ± 0.77*	72
	2.0	4.86 ± 0.19*	61

Results are shown as mean ± standard error of the mean. Extracts and indomethacin were administered topically 30 min after TPA. We used one-way ANOVA-Dunnett-test, * $p<0.05$, difference from the control group (n=6)

TPA induced chronic ear inflammation

The topical application of repeated doses of TPA irritant agent and extracts of *C. stipulaceus* (doses of 2 mg/ear), show that the anti-inflammatory effect of the three extracts is significant with respect to the control group (Table No. 3), the EEtOH extract being the one that inhibits edema (97%), the EHex extract showed lower inhibition of edema (79%), similar to that shown with indomethacin (74%).

Table No. 3
Anti-inflammatory effect of *C. stipulaceus* TPA induced ear chronic edema in mice

Treatment	Doses (mg / ear)	TPA Edema (mg)	Inhibition edema (%)
Control	-	7.66 ± 0.98	0
EHex	2.0	1.60 ± 0.40*	79
$ECHCl_3$	2.0	1.40 ± 0.96*	82
EEtOH	2.0	0.20 ± 0.66*	97
Indomethacin	0.5	2.00 ± 0.44*	74

Results are shown as mean ± standard error of the mean. Extracts and indomethacin were administered topically 30 min after TPA. We used one-way ANOVA-Dunnett-test, * $p<0.05$, difference from the control group (n=6)

Myeloperoxidase (MPO) activity on ear inflammation with TPA

Table No. 4 shows the anti-inflammatory effect and the activity of the myeloperoxidase enzyme when the extract EEtOH of *C. stipulaceus* or indomethacin is applied at different times with respect to the irritating agent TPA (30 min before, 30 min after and immediately afterwards). The anti-inflammatory effect was present in the application of both EEtOH and indomethacin in both doses (1 and 2 mg/ear) and in all times (- 30, + 30 and 0 min). However, the EEtOH does not decrease the activity of the MPO enzyme as the indomethacin, which presents a high inhibition in all the doses and times employed.

Table No. 4
Effect of the EEtOH of *C. stipulaceus* on the MPO activity

Treatment	Time to applied TPA (min)	Doses (mg/ear)	Inhibition edema (%)	Inhibition MPO (%)
Control		0	0	0
Indomethacin	- 30	1	89*	62*
		2	71*	77*
EEtOH		1	76*	5
		2	67*	0
Indomethacin	+ 30	1	66*	90*
		2	50*	95*
EEtOH		1	35*	7
		2	83*	48*
Indomethacin	0	1	86*	47*
		2	76*	86*
EEtOH		1	74*	4
		2	71*	26*

Results are shown as mean ± standard error of the mean. We used one-way ANOVA-Dunnett-test, * $p<0.05$, difference from the control group (n=6). Where: - 30 = applied 30 min before of TPA, + 30 = applied 30 min after to TPA, 0 = immediately of TPA

Carrageenan induce acute ear inflammation

The extracts $ECHCl_3$ and EEtOH (doses of 250 and 500 mg/Kg, p.o.) of *C. stipulaceus* on the plantar edema induced with carrageenan are shown in Table No. 5. Both extracts have anti-inflammatory effect, $ECHCl_3$ being the best at the highest dose (500 mg/Kg), inhibiting edema greater than 50% throughout the study, this effect is mayor that the indomethacin compound. On the other hand, the EEtOH showed an inhibition greater than 50% only at the lower dose (250 mg/Kg) and until after 3 and 5 h induced the edema.

Table No. 5
Anti-inflammatory effect of *C. stipulaceus* in carrageenan induced paw edema in mice

Treatment	Doses mg / Kg	Edema formation (mm) Time (h)			
		1	2	3	5
Control		0.57 ± 0.03	0.57 ± 0.02	0.66 ± 0.01	0.72 ± 0.07
$ECHCl_3$	250	0.33 ± 0.03* (42.10%)	0.40 ± 0.03* (29.82%)	0.28 ± 0.02* (57.57%)	0.38 ± 0.04* (47.22%)
	500	0.21 ± 0.02* (63.15%)	0.25 ± 0.01* (56.14%)	0.27 ± 0.02* (59.09%)	0.32 ± 0.04* (55.55%)
EEtOH	250	0.41 ± 0.03* (28.07%)	0.37 ± 0.02* (35.08%)	0.27 ± 0.02* (59.09%)	0.26 ± 0.03* (63.88%)
	500	0.50 ± 0.01 (12.28%)	0.42 ± 0.05* (26.31%)	0.37 ± 0.02* (43.93%)	0.47 ± 0.02* (34.72%)
Indomethacin	8	0.37 ± 0.03* (39.14%)	0.36 ± 0.03* (53.61%)	0.37 ± 0.02* (54.17%)	0.35 ± 0.04* (50.48%)

Results are shown as mean ± error standard of the mean. We used one-way ANOVA-Dunnet test, * $p<0.05$, difference from the control group. (n=6). Values in parenthesis are inhibition percent to edema formation

For the acute toxicity test, the extracts of *C. stipulaceus* did not cause lethality when administered in the animals or alterations in their behavior, nor were they observed macroscopically. The determination of LD_{50} was greater than 2 g/Kg (data no show). This result was used to determine the doses at which the extracts were tested.

DISCUSSION

Croton stipulaceus does not have previous reports supporting any of the therapeutic properties attributed by the population of the central area of Veracruz, Mexico. Hence, it is important to study and report the therapeutic properties of popular medicinal flora in order to document the therapeutic effects of those plants. This is the first study reporting the antioxidant capacity of the organic extracts of the leaves of *C. stipulaceus* and its anti-inflammatory effects applied topically and orally.

Free radicals are generated by the oxidation reaction and incomplete reduction of an oxygen molecule, which leads to structural and functional damage of biomolecules or cell organelles (Akar *et al.*, 2017). The reducing power the FRAP assay and the DPPH radical inhibition test are spectrophotometric determinations that serve as a significant indicator of the potential as antioxidant (Thaipong *et al.*, 2006; Chen *et al*., 2013). The results obtained show that EEtOH has the best antioxidant capacity of the extracts examined in the FRAP assay and the DPPH radical inhibition test, this effect may be attributed to the presence of polyphenolic compounds, such as flavonoids found in the $ECHCl_3$ and EEtOH extracts. Flavonoids have been extensively studied for their antioxidant effect, these compounds are widely known to prevent free radical damage through an ion chelating activity activation of antioxidant enzymes, inhibition of oxidases, etc. (Procházková *et al*., 2011). They can show antioxidant activity by donating electrons or H^+ atoms, as well as capturing DPPH free radicals. Additionally, they have shown anti-inflammatory effect by inhibiting some mediators (NO radicals, PGE2, TNF-α, IL-1β and IL-6) (Chen *et al*., 2019).

Reactive oxygen species (ROS) play a dual role within the body, in moderate concentrations are

involved in defense against infectious agents, function of signaling of cellular pathways and induction of the apoptotic response; overproduction results in a state of oxidative stress, a process that can cause significant damage to the macromolecules of biological systems due to their great instability and reactivity, such as lipids, proteins or nucleic acids (Valko *et al*., 2007). During acute inflammation, there is an increase in ROS during the activation of the immune system that persists only for a short period and is generally beneficial (Rawdin *et al*., 2013). On the other hand, in chronic inflammation there is a continuous induction of enzymes such as cyclooxygenase-2 (COX-2) or nitric oxide synthase (iNOS), an exacerbated expression of inflammatory cytokines (tumor necrosis factor (TNF-α), interleukins (H_2O_2), hydroxyl radical (HO^-) and lipid peroxidation which contributes to tissue damage (Nardi *et al*., 2007; Chen *et al*., 2019). For this chronic process, we expect that the presence of antioxidant compounds in extracts of *C. stipulaceus*, may offer resistance against oxidative stress; so, our goal is not only to determine the anti-inflammatory activity of this plant but also to evaluate its antioxidant properties.

TPA is a phorbol ester extracted from Croton oil (*Croton tiglium* L.), it is the most potent of all phorbol esters belonging to this species (Wambier *et al*., 2019). The irritant action of this agent consists of the stimulation of epidermal cells producing chemokines, stimulates phospholipase A2 and the enzymes COX and LOX, this process increases vascular permeability and cellular infiltration (Marakawa *et al*., 2006; Bralley *et al*., 2008). The single topical application of TPA, in mouse ear occurs between the first and second hour after application, an erythema and vasodilation; at 3 and 4 h, the thickness of the atrial tissue increases as a result of fluid extravasation, with the maximum oedema occurring at 6 and 8 h (Marakawa *et al*., 2006). It is known that the cumarins as scopoletine in acute models of inflammation can decrease the myeloperoxidase enzyme, PGE2 and IL-β; while stigmasterol and β-sitosterol decrease the migration of the neutrophils (Gómez *et al*., 1999). This model is widely used for the search of new agents with anti-inflammatory properties administered mainly topically, either chemical compounds or extracts of natural products, as medicinal plants. The organic extracts (EHex and $ECHCl_3$) of the leaves of *C. stipulaceus* applied topically, has the ability to inhibit this oedematous process successfully.

The topical application of repeated doses of TPA (5 doses in 10 days) in the ear of the mouse is a technique developed by Stanley *et al*. (1991), to evaluate the anti-inflammatory activity of compounds, drugs or extracts in chronic inflammation processes, this administration produces a prolonged inflammatory response in which increased atrial tissue weight, infiltration of polymorphonuclear inflammatory cells, epidermal hyperplasia and fibrosis. The anti-inflammatory capacity of the extracts to inhibit processes of chronic inflammation by multiple doses, became relevant in the three extracts of *C. stipulaceus*, presenting an even greater effect than indomethacin (Table No. 3). Experimental evidence has shown that exposure of skin to TPA induces the activation of intracellular pathways through the activation of protein kinase C (PKC), including PI3K/AKT/NF-κB signaling, STAT3 signalling, as well as the generation of inflammatory cytokines (Lai *et al*., 2007; Yue *et al*., 2016; Rakariyathama *et al*., 2019).

MPO is an enzyme released during the degranulation of neutrophils and monocytes that allows the microbicidal action of these. However, in contrast to that beneficial activity, MPO also contributes to the development of many disorders, such as cardiovascular, inflammatory and neurodegenerative diseases (Begum *et al.,* 2017). Measurement of MPO activity was performed using colorimetric methods that are very reliable, easy to perform and low cost, based on the use of tetramethylbenzidine (TMB), which reduces hydrogen peroxide by generating a turquoise blue compound whose intensity is proportional to the MPO activity. Inhibition of the activity of this enzyme has been used in numerous settings, as an indicator of influx of inflammatory cells in tissue (Bralley *et al*., 2008). The effect of EEtOH extract of *C. stipulaceus* on MPO activity indicates that the percentage of enzyme inhibition was no better than the inhibition shown by indomethacin, although EEtOH has an anti-inflammatory effect similar to that of indomethacin, it does not act in the same way on the activity of MPO, indicating that its anti-inflammatory effect is not related to leukocyte expression but perhaps more with the inhibition of cyclooxygenase (COX) and lipooxigense (LOX) that reduce the edematous response (Franco *et al*., 2007). In the present study, the EEtOH extract applied at different times with respect to the induction of edema with TPA (-30, +30 and immediately after), was carried out to know the anti-inflammatory potentials and their possible relationship with the inhibition of the MPO enzyme (preventive, remedial and inhibitor

effect). However, despite the significant decrease in atrial edema, this effect is not directly related to the inhibition of the MPO enzyme as it occurs with indomethacin, which is the reference drug of the study.

In the carrageenan model, the progress of the paw edema is divided into three phases. The initial phase, (0-1.5 h) is attributed to the release of histamine and serotonin. The second phase (1.5-2.5 h), mediated by bradykinin and finally, in the third phase (2.5-6 h) PG, leukotrienes (LT) and other arachidonic acid derivatives are overproduced (Moreno-Quirós *et al.*, 2017). In the present study, showed that the $ECHCl_3$ extract to doses of the 250 mg/Kg and 500 mg/Kg possess a significant inflammation effect that it is maintained all times (1-5 h). The extract EEtOH at a dose of 250 mg/Kg, inhibited the formation of plantar edema from the first hour of the analysis, increasing its effect as the study time elapsed. The anti-inflammatory effect observed in both extracts, could be associated with an inhibitory effect of PG biosynthesis and the inhibition of other mediators such as histamine and serotonin.

CONCLUSION

Croton stipulaceus is a plant commonly used as anti-inflammatory in the traditional medicine of the central area of Veracruz and other States of Mexico. However, as observed with other medicinal plants, there are no reports to support its therapeutic uses. Hence, our results offer the first relevant insight to understanding the anti-inflammatory use of *C. stipulaceus* and the mechanisms involved in this pharmacological activity. Finally, because of a rapid loss of ecological habitats, it is important to study and report the therapeutic properties of popular medicinal flora in order to document and support the therapeutic effects of those plants.

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