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Vasorelaxant activity of *Euphorbia furcillata* Kunth mainly by activation of NO/cGMP pathway and calcium channel blockade

[Actividad vasorrelajante de *Euphorbia furcillata* Kunth principalmente por activación de la vía NO/GMPc y bloqueo de canales de calcio]

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Abstract: The aim of current study was to determinate ex vivo and chromatographic fingerprint by HPLC of four extracts of *Euphorbia furcillata* K. Ethyl acetate extract of *Euphorbia furcillata* (EaEEf) was the most effective and potent extract (Emax=98.69±1.24%) and its effect was partially endothelium-dependent. Functional vasorelaxant mechanism of action of EaEEf was determinate, EaEEf showed efficient relaxation of KCl [80 mM]-induced contraction and norepinephrine and $CaCl_2$ contraction curves showed diminution of maximal contraction in the presence of EAEEf and EaEEf-relaxation curve was shifted to the right in the presence of L-NAME (nitric oxide synthase inhibitor) and ODQ (guanylate cyclase inhibitor). Chromatographic fingerprints analysis suggests presence of diterpenoid such as abietane, tigliane, and ingenane skeletons. Our experiments suggest the EaEEf vasorelaxant activity could be attributed to diterpenoid molecules whose mechanism involves nitric oxide production and calcium channel blockade.

Keywords: *Euphorbia furcillata*; vasorelaxant effect; NO/cGMP pathway; calcium channel blockade; chromatographic fingerprint

Resumen: Se determinó el efecto vasorrelajante ex vivo y los perfiles cromatográficos mediante HPLC de cuatro extractos de *Euphorbia furcillata* K.. El extracto de acetato de etilo de *E. furcillata* (EaEEf) fue el más eficaz y potente en la contracción inducida por norepinefrina (Emax=98.69±1.24%) y el efecto fue parcialmente dependiente del endotelio vascular. Se determinó el mecanismo de acción vasorrelajante para EaEEf, este mostró ser eficaz sobre la contracción inducida por KCl [80 mM] y la curva de contracción en respuesta a norepinefrina y $CaCl_2$ en presencia de EaEEf mostró disminución en la contracción máxima, mientras que la curva de relajación de EaEEf en presencia de L-NAME (inhibidor de óxido nítrico sintasa) y ODQ (inhibidor de guanilato ciclasa) se desplazó hacia la derecha. El análisis cromatográfico de EaEEf sugiere la presencia de moléculas diterpenoides como abietano, tigliano y esqueletos de ingenano. Nuestros resultados sugieren que el efecto vasorrelajante de EaEEf podría atribuirse a moléculas diterpenoides, cuyo mecanismo de acción involucra la producción de óxido nítrico y bloqueo de canales de calcio.

Palabras clave*: Euphorbia furcillata*; efecto vasorelajante; vía NO/cGMP; bloqueo del canal de calcio; huella digital cromatográfica

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INTRODUCTION

Plants have been utilized as medicines for thousands of years (Samuelsson, 2004) and remain today as an important source of treatment for nearly 80% of the world population (Jiménez, 2005). Their use has led to the isolation and characterization of active compounds that represent novel candidates for the development of phytomedicines, which can be used to treat chronic, degenerative, and infectious diseases with high prevalence in the world population (Mouhssen, 2013). Thus, more recently, drug discovery techniques have been applied to the standardization of herbal medicines (Butler, 2004).

Cardiovascular diseases are the cause of one third of the total amount of deaths worldwide. In Mexico, the prevalence of this disease has remained stable, affecting 30–35% of the population since 2006 according to the National Health and Nutrition Survey (Barquera *et al*., 2010). Due to the wide diversity and cultural inheritance of the country, Mexican traditional medicine has developed therapeutic strategies through the use of medicinal plant preparations. This knowledge is commonly utilized to provide alternative treatments to the existing drugs employed for hypertension or to contribute to the improvement of the lifestyle of patients with hypertension (Heinrinch, 2003).

There are many plant species used in Mexican traditional medicine and the majority of these belong to the Anacardiaceae, Apocynaceae, Asteraceae, Burseraceae, Cactaceae, Euphorbiaceae, Fabaceae, Malpighiaceae, and Rubiaceae families (Monroy-Ortiz & Castillo-España, 2007). *Euphorbia furcillata* Kunth, commonly known as "hierba del coyote" (coyote grass), belongs to the most important genus of the Euphorbiaceae family, *Euphorbia*. *Euphorbia furcillata* Kunth is utilized in traditional medicine for the treatment of diabetes, rheumatic and non-rheumatic pain, infertility, inflammation, and hypertension (Martínez *et al*., 2002). There are few systematic studies that support the therapeutic use of this medicinal plant; however, some studies report that other species from the *Euphorbia* genus present many pharmacological effects such as protection against nociceptive pain and inflammation (Ding *et al*., 2016) in an *in vitro* arthritis model (Palit *et al*., 2016), apoptosis mediated cytotoxicity in intestinal epithelial cells of rats (Gao *et al*., 2015) and Human Epidermoid carcinoma strain cells (Betancur-Galvis *et al*., 2002), as well as apoptosis and autophagy mediated cytotoxicity in HeLa human cervical carcinoma cells, and MDA-MB-231 and MCF-7 breast tumor cells (Lu *et al*., 2008; Gao *et al*., 2016). In addition, antibacterial (Elumalai *et al*., 2010; Mohsenipour & Hassanshahian, 2016), antidiabetic (Sunil *et al*., 2010; Pooja *et al*., 2011; Rahmatullah *et al*., 2012; Mansuri & Patel, 2013), antioxidant (Kumar *et al*., 2010), vasorelaxant (Wang *et al*., 2013), anti-parasitic (Amin *et al*., 2016), anti-fungal (Rawal *et al*., 2014; Xu *et al*., 2015) anti-viral (Zheng *et al*., 1998; Betancur-Galvis *et al*., 2002; Ávila *et al*., 2010; Zhao *et al*., 2014), anti-inflammatory (Xu *et al*., 2012; Liu *et al*., 2014), antidiarrheic (Gálvez *et al*., 1993), and multidrug resistance-modulating effects are reported (Jiao *et al*., 2015; Rédei *et al*., 2015).

Previous phytochemical studies reveal that terpenoids, coumarins, lignans, alkaloids, flavones, flavonoids, and glycosides have been isolated from the genus *Euphorbia* (Shi *et al*., 2008; Vasas & Hohmann, 2014; Rédei *et al*., 2015; Sheliya *et al*., 2015; Li *et al*., 2015; Nguyen *et al*., 2016).

The current study aimed to investigate the vasorelaxant effect and mode of action of *Euphorbia furcillata* Kunth in an *ex vivo* test. Additionally, the chromatographic fingerprints of *E. furcillata* extracts were determined.

MATERIAL AND METHODS

Chemicals and drugs

(+/-)-norepinephrine bitartrate hydrate (NE), carbamoylcholine chloride (carbachol), indomethacin, L-NG-nitroarginine methyl ester (L-NAME), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), potassium chloride (KCl), calcium chloride ($CaCl_2$), and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents and solvents were analytical grade and obtained from local sources. Stock extracts were prepared with distilled water on the same day of experimentation. The methanol (Fermont, México) was used in the chromatographic analysis was of chromatographic grade. Deionized water was purified by an E-pure water purification system (Thermo Scientific). As internal standard to establish the relative retention time, we used flavone (Sigma-Aldrich), analytical grade.

Plant material and extraction

Aerial parts of *Euphorbia furcillata* Kunth (coyote grass) were collected at a Mixtec region, Luz Nagore, Huajuapan de León, Oaxaca, Mexico, in March,

2016. *E. furcillata* K. was identified and voucher specimen was deposited (voucher in process) at the Herbarium, Oaxaca, Mexico.

The leaves of the plant were separated, washed whit water, dried to room temperature (25º C), and crushed in an industrial blender. Then, the powdered leaves (673 g) were subjected to successive maceration processes utilizing hexane (5.0 L), dichloromethane (3.4 L), ethyl acetate (4.5 L), and finally methanol (2.7 L) with three solvent changes every 72 h. After filtration, organic extracts were concentrated *in vacuum* at 40º C using a Rotavapor (Buchi® R-200) and the percentages of the yields obtained were as follows: Hexanic Extract (HE*Ef*): 2.6%; Dichloromethane Extract (DE*Ef*): 0.95%; Ethyl acetate Extract (EaE*Ef*): 3.65%, and Methanolic Extract (ME*Ef*), 5.24%, respectively.

Chromatographic fingerprints of E. furcillata extracts

An Agilent Technologies 1200-series HPLC system (Agilent, San Jose, CA, USA) with a quaternary pump and a UV-DAD detector equipped with a Zorbax Eclipse Plus C18 column (250 mm × 4.6 mm i.d., 5 µm, Agilent, USA) was employed. Chromatography was performed under gradient conditions with H_2O: MeOH with a mobile-phase flow rate of 1.5 mL/min and with the injection of 5 µL of the sample. The column was purged with the mobile phase for 10 min, followed by equilibration for 10 min. Total time required for sample analysis was 40 min. Spectral data were collected and plotted at detection wavelengths of 280 nm.

Pharmacological evaluation

Animals

Adult male Wistar rats (250–300 g bodyweight) were obtained from the Animal House of Centro de Investigaciones Regionales "Dr. Hideyo Noguchi" of the Universidad Autonóma De Yucatán (UADY), Mexico. Animals were housed in polycarbonate cages and maintained under standard laboratory conditions (12-h light/dark cycle, at a temperature of 25 ± 2º C, and with a humidity of 45–65%), and were fed with standard rodent diet and water *ad libitum*. All animal procedures were conducted in accordance to our Federal Regulations for Animal Experimentation and Care (SAGARPA, NOM-062-ZOO-1999, México) and approved by the Institutional Animal Care and Use Committee. All experiments were carried out using six animals per group. All study animals were euthanized by cervical dislocation after deep anesthesia with ether.

General procedures

Rats were euthanized by cervical dislocation and thoracic dissection was carried out to extract the thoracic aorta. The latter was cleaned from adjacent and connective tissue and then cut into strips 3 mm in length. In addition, for some aortic rings, the endothelium layer was gently removed by manual procedures. Then, the tissue sections were assembled using stainless steel hooks under an optimal tension of 3 g and were allowed to stabilize for 20 min in chambers at 37° C containing Krebs–Henseleit Solution (KHS; composition, mM: NaCl, 119; KCl, 4.6; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.5; $NaHCO_3$, 20; EDTA, 0.026, and glucose, 11.4; pH 7.4) constantly bubbled with an O_2:CO_2 (95:5) mixture. Tension changes were recorded by Grass-FT03 force transducers (Astro Med, West Warwick, RI, USA) connected to an MP150 analyzer (BIOPAC 4.1 Instruments, Santa Barbara, CA, USA) as described previously by Estrada-Soto *et al*. (2010).

After the stabilization period, sensibilization was carried out. The tissues were stimulated with Noradrenaline (NE [0.1 µM]) during 15 min, washed with fresh KHS, and allowed to stabilize for 15 min. This procedure was repeated three times. The absence of endothelium was confirmed by the lack of the relaxant response induced by carbachol (CCH [1 µM]) in the last contraction with NE prior to washing with fresh KHS to assess viability.

Vasorelaxant effect of extracts, controls, and vehicle on the contraction induced by NE

After sensibilization, tissues were allowed to stabilize for 20 min and then, these were contracted with NE [0.1 µM]. Extracts [3.03–1000 µg/mL], vehicle [100% final concentration] or positive controls (CCH for endothelium-intact aortic rings, E+ [0.303–100 µg/mL] or Nifedipine for endothelium-denuded aortic rings, E-, [3.89×10^{-5}–3.46 µg/mL]) were added to the chamber in cumulative concentrations [Concentration–Response Curves (CRC)]. The relaxant effect of the samples was determined by their ability to reduce the maximal vascular contraction induced by NE, comparing tissue tension before and after its addition.

Determination of the EaEEf mode of action

In order to establish the EaE*Ef* mode of action, the following experiments were conducted:

a) To establish a possible antagonism of adrenergic receptor or disruption of the NE pathway, the following procedures were performed on endothelium-denuded aortic rings. A cumulative NE-induced contraction [1.15×10^{-11} to 1.00×10^{-5} M] CRC was built and established as positive control (control CRC). In another experiment, aortic rings were pre-incubated with EaE*Ef* (median Effective Concentration [EC_{50} = 145 μg/mL]) for 15 min, and then, the CRC to NE-induced contraction was performed to compare the NE-induced contraction in the absence and presence of EaE*Ef*.

b) In order to know the role of endothelium-derived relaxing factors such as nitric oxide (NO) or rostacyclin (PGI_2), endothelium-intact aortic rings were pre-incubated with NG-nitro-L-arginine methyl ester (L-NAME, a nitric oxide synthase (NOS) inhibitor, [100 μM]) or indomethacin (a cyclooxygenase (COX) unspecific inhibitor, [10 μM]), respectively, for 15 min prior to contraction with NE [0.1 μM]. The relaxation CRC of EaE*Ef* [3.03 - 1000 μg/mL] was built as described in the vasorelaxant set of experiments. The maximal relaxing effect of EaE*Ef* was compared in the absence and presence of L-NAME or Indomethacin.

c) To establish the possible inhibition of the soluble guanylyl cyclase enzyme (sGC), endothelium-intact aortic rings were pre-incubated with 1-H-[1,2,4]-oxadiazolo-[4,3a]-quinoxalin-1-one (ODQ, an sGC inhibitor, [10 μM]) for 15 min prior to the contraction with NE [0.1 μM]. The relaxation CRC of EaE*Ef* [3.03 - 1000 μg/mL] was built as described previously. The maximal relaxing effect of EaE*Ef* was compared in the absence and presence of ODQ.

d) In order to know the role of K^+ channels in the extract-induced vasorelaxant effect, intact-endothelium aortic rings were pre-incubated with tetraethylammonium (TEA, a non-selective KCa^{2+} channel blocker [10 μM]) for 15 min prior to the contraction with NE [1 μM]. The relaxation CRC of EaE*Ef* [3.03 - 1000 μg/mL] was built as described previously. The maximal relaxing effect of EaE*Ef* was compared in the absence and presence of TEA.

e) To determine whether inhibition of extracellular Ca^{2+} influx was involved in the extract-induced vasorelaxation, the experiments were carried out in Ca^{2+}-free KHS. Endothelium-denuded aortic rings were washed with Ca^{2+}-free KHS containing KCl [80 mM] after sensibilization and were allowed to stabilize for 15 min. Then, a CRC for the $CaCl_2$-induced contraction was obtained in the absence of EaE*Ef* (control group). Once the maximal contraction was reached, tissue was washed with Ca^{2+}-free, KCl [80 mM] KHS and allowed to stabilize for 20 min. Finally, after a 15-min incubation with the EaE*Ef* [EC_{50}= 45 μg/mL], another CRC for the $CaCl_2$-induced contraction was obtained. The contractile effect induced by $CaCl_2$ was compared in the absence and presence of EaE*Ef*.

Data analysis

Results are expressed as the mean (n = 5) ± Standard Error of the Mean (S.E.M). Concentration–Response Curves (CRC) were plotted, and the experimental data from the CRC were adjusted by the nonlinear Hill equation with a curve-fitting program ORIGIN 8.0 and were calculated pharmacological parameters [efficacy (E_{max}) and median Effective Concentration (EC_{50}) values]. The statistical significance of differences between means was assessed by a one-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test; p values <0.05 (*p<0.05) were considered statistically significant (Bailey, 1995; Daniel, 2002).

RESULTS

Pharmacological Evaluation

Aerial parts of *Euphorbia furcillata* were subjected to extraction by maceration: the extract with the highest yield was the methanolic extract (ME*Ef*, 5.24%), followed by the Ethyl acetate Extract (EaE*Ef*, 3.65%), the hexanic extract (HE*Ef*, 2.6%), and the Dichloromethane Extract (DE*Ef*, 0.95%). HE*Ef*, DE*Ef*, EaE*Ef*, and ME*Ef* were evaluated using aortic rings pre-contracted with NE to determine their potential activity as vasorelaxant agents and their mode of action. Additionally, we determined the

chromatographic fingerprints of the *Euphorbia furcillata* extracts.

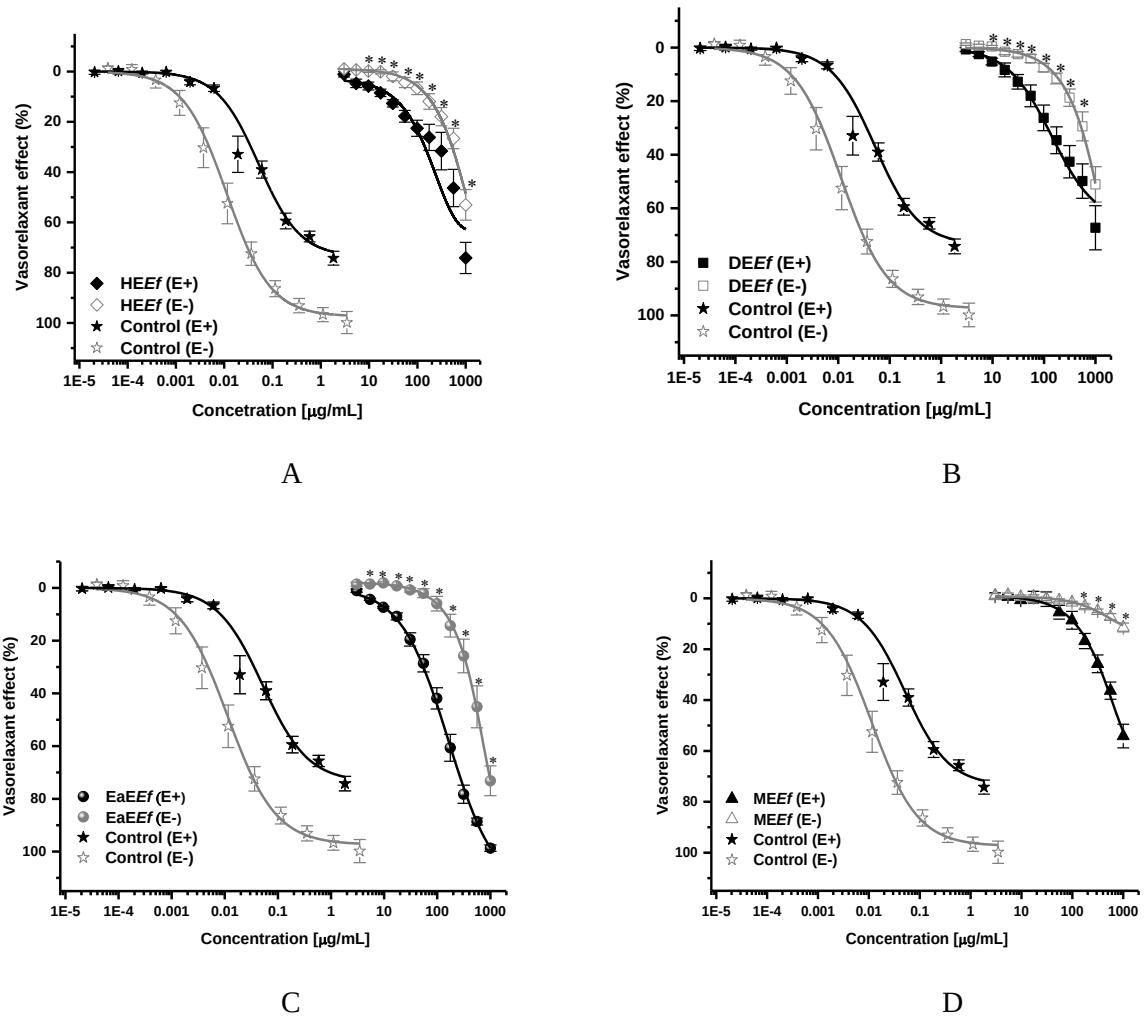


Figure No. 1
Concentration-response curves of vasorelaxant effect from *Euphorbia furcillata* K. extracts A) HE*Ef*, B) DE*Ef*, C) EaE*Ef*, and D) ME*Ef*. Results are expressed as the means ± SEM of six experiments, *$p<0.05$ represents significant difference compared with endothelium-absence aortic rings.

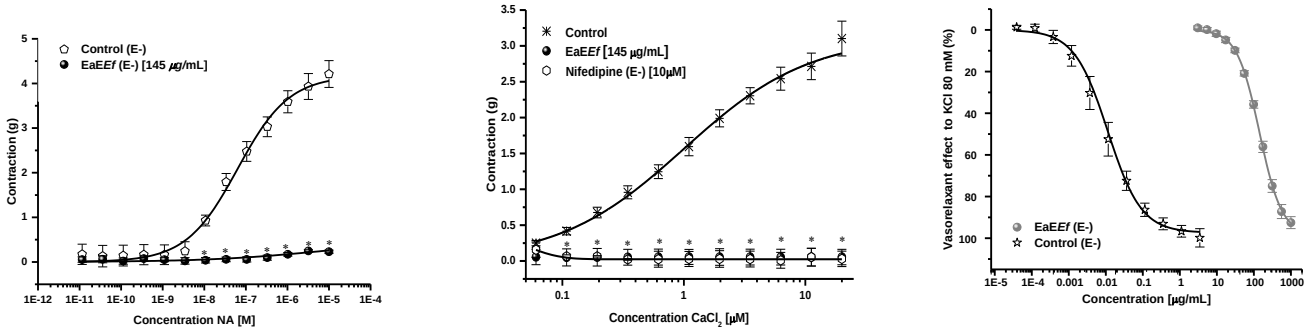
Results showed that EaE*Ef* was the most effective and potent extract (E_{max}= 98.69 ± 1.24%; EC_{50} = 145 µg/mL) (Figure No. 1C) in endothelium-intact vascular relaxation compared with HE*Ef* (E_{max} = 74.13 ± 6.21%; EC_{50} = 667.21 µg/mL) (Figure No. 1A), DE*Ef* (E_{max} = 67.27 ± 8.25%; EC_{50} = 473.06 µg/mL) (Figure No. 1B), and ME*Ef* (E_{max} = 54.16 ± 4.64%; EC_{50} = 909.51 µg/mL) (Figure No. 1D) and was also more effective than positive control (Carbachol: E_{max} = 75%) (Figure No. 1C). The vasorelaxant effect showed by four organic extracts was concentration- and partially endothelium-dependent (Figure No. 1A & 1D). Table No. 1 depicts the pharmacological parameters (E_{max} and EC_{50}) obtained for the extracts and positive controls employed. EaE*Ef* was more potent and efficient in both endothelium-intact and endothelium-denuded vascular relaxation in aorta ring assays, demonstrating a vasorelaxant effect that was partially endothelium-dependent; thus, its possible functional mode of action was assessed. To investigate the possible adrenergic receptors antagonism or the

disruption of the NE-Ca^{2+} intracellular increase pathway, endothelium-denuded aortic rings were pre-incubated with EaE*Ef* followed by a NE-induced contraction curve, and the maximal contractile effect induced by NE was compared in the absence (E_{max} = 4.17 ± 0.16 g, control curve) and presence of EaE*Ef*. As a result, during pre-incubation with the EaE*Ef* assay, the CRC showed significant reduction of NE-induced maximal contraction (E_{max} = 0.48 ± 0.46 g) (Figure No. 2A).

Table No. 1
Pharmacological parameters of vasorelaxant effect of *Euphorbia furcillata* Kunth extracts and controls

Extract	Aortic rings endothelium-intact (E+)		Aortic rings without-endothelium (E-)	
	E_{max} (%)	EC_{50} (μg/mL)	E_{max} (%)	EC_{50} (μg/mL)
Carbachol (Control, E+)	75±2.75	0.11±0.03	-	-
Nifedipine (control, E-)	-	-	100±4.38	0.012±0.014
HE*Ef*	74.13±6.21	610.90±6.81	53.02±6.09	979.14±6.09
DE*Ef*	67.27±8.25	473.06±6.22	51.07±6.60	957.56±6.60
EaE*Ef*	**98.69±1.24**	**145.06**±4.56	**73.16±5.66**	**614.62**±6.80
ME*Ef*	54.16±4.64	909.51±4.01	11.47±1.72	>1000*

E_{max}: maximum effect, EC_{50}: Effective concentration medium, HE*Ef*: Hexanic extract of *Euphorbia furcillata* Kunth, DE*Ef*: Dichloromethane extract of *Euphorbia furcillata* Kunth, EaE*Ef*: Ethyl acetate extract of *Euphorbia furcillata* Kunth, ME*Ef*: methanolic extract of *Euphorbia furcillata* Kunth.
***Parameter not determined, since the maximum effect was less than 50%**



A B C

Figure No. 2
Concentration-response curves (CRC) contraction to A) Norepinephrine and B) $CaCl_2$ C) CRC of EaE*Ef*-induced relaxation to KCl (80 mM)-induced contraction. *$p<0.05$ represents significant difference compared with control.

To assess whether Ca^{2+} channel blockade was involved in the vasorelaxant effect of EaE*Ef*, a contraction curve with $CaCl_2$ was obtained (control); the maximal contractile effect induced by $CaCl_2$ was compared in the absence and presence of EaE*Ef*. As result, the $CaCl_2$-induced contraction was totally abolished by pre-incubation with EaE*Ef* [145 μg/mL] such as nifedipine (positive control) (Figure No. 2B).

Moreover, the EaE*Ef* (3.03 - 1000 μg/mL**)** produced a significant vasorelaxant effect on the KCl (80 mM)-induced contraction (E_{max} = 92%) (Figure No. 2C).

The role of K^+ channels on the EaE*Ef*-induced vasorelaxant effect was assessed with TEA pre-incubation prior to contraction with NE; the relaxant effect of EaE*Ef* in the presence of TEA was not modified (data not shown). To identify the participation of NO-sGC-cGMP or PGI_2 –AC-cAMP pathways, aortic rings were pre-incubated with L-NAME and ODQ or Indomethacin, respectively, prior to contraction with NE. The EaE*Ef* relaxation CRC shifted to the right and decreased the maximum effect in the presence of L-NAME (E_{max} = 82.18%; EC_{50} = 428.80 μg/mL) and ODQ (E_{max} = 87.37%; EC_{50} = 266.16 μg/mL) in comparison to the control curve (E_{max} = 98.69 %; EC_{50} = 145 μg/mL) (Figure No. 3). While Indomethacin curve was shifted to the left (EC_{50} = 63.12 μg/mL) with respect to the control (EC_{50} = 145 μg/mL).

The fingerprint analysis of the methanolic extract (ME*Ef*) demonstrated a few signals around the 20-min mark in the chromatogram, identified as more polar according to the mobile phase elution (Figure No. 4A). The less polar extracts (EaE*Ef*, DE*Ef*, and HE*Ef*) showed the same signal in the chromatograms. The EaE*Ef* extract (Figure No. 4B) presented the majority of signals (11 signals) compared to the DE*Ef* (Figure No. 4C) and the HE*Ef* extract (Figure No. 4D). However, signals 15, 16, and 17 were found in the three of these. These signals were found more concentrated in the EaE*Ef* extract and at lowest concentration in the HE*Ef* extract. In the chromatographic profile, the signals were observed around the 28–35-min mark, with a relative relation time of 1.03–1.20 min compared to the flavone (Figure No. 4E) (Table No. 2).

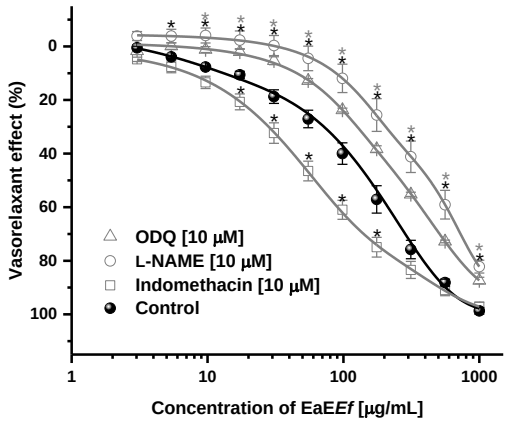


Figure No. 3
EaE*Ef*-induced relaxation concentration-response curves in presences of ODQ, L-NAME and Indomethacin. *$p<0.05$ represents significant difference compared with control

DISCUSSION

HE*Ef*, DE*Ef*, EaE*Ef*, and ME*Ef* were evaluated to determine their potential activity as vasorelaxant agents for the treatment of hypertension. The vasorelaxant effect exhibited by all organic extracts was concentration- and partially endothelium-dependent, the latter suggests that Endothelium-Derived Relaxing Factors (EDRF), such as Nitric Oxide (NO), Prostacyclin (PGI_2), and/or the Endothelium-Derived Hyperpolarizing Factor (EDHF), are involved in extract-induced relaxation (Hernández-Abreu *et al*., 2009), as well as in mechanisms related to smooth-muscle-cell activity, such as α_1-adrenoceptor antagonism, Ca^{2+} channel blockade, K^+ channel opening, cyclic nucleotide (cAMP or cGMP) synthesis, or the Calcium-Calmodulin complex (Ca^{2+} -CaM) inhibition (Huang & Ho, 1996; Zhang & Tan, 1998; Maciel *et al*., 2004; Zhu *et al*., 2007; Vergara-Galicia *et al*., 2010).

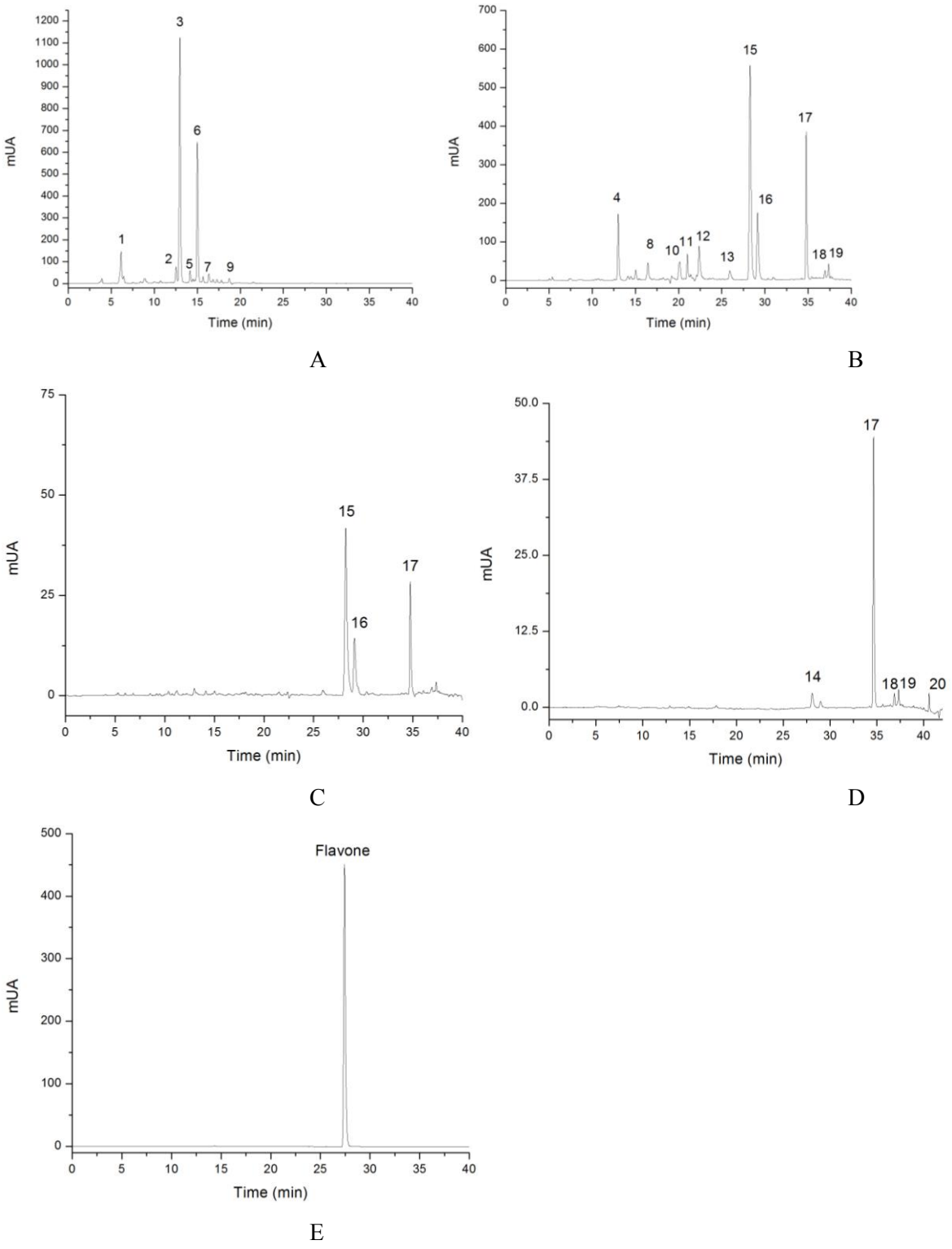


Figure No. 4
Fingerprint analyses of *Euphorbia furcillata* K. extracts A) ME*Ef* B) EaE*Ef* C) DE*Ef* D) HE*Ef* and E) Flavone as Internal Standard from leaves part at 280 nm

Table No. 2
Retention time in fingerprint analyses (n = 3) of *Euphorbia furcillata* Kunth

		Relative Retention Time							
		ME*Ef*		**EaE*Ef***		**DE*Ef***		**HE*Ef***	
Signal	Retention time (Rt)	Averages	RSD	Averages	RSD	Averages	RSD	Averages	RSD
1	6.148	0.224	0.452	N/D	N/D	N/D	N/D	N/D	N/D
2	12.543	0.457	1.203	N/D	N/D	N/D	N/D	N/D	N/D
3	12.970	0.475	0.803	N/D	N/D	N/D	N/D	N/D	N/D
4	13.008	N/D	N/D	0.473	0.241	N/D	N/D	N/D	N/D
5	14.156	0.515	0.596	N/D	N/D	N/D	N/D	N/D	N/D
6	15.007	0.545	0.701	N/D	N/D	N/D	N/D	N/D	N/D
7	16.341	0.592	0.590	N/D	N/D	N/D	N/D	N/D	N/D
8	16.447	N/D	N/D	0.604	0.695	N/D	N/D	N/D	N/D
9	18.711	0.685	0.498	N/D	N/D	N/D	N/D	N/D	N/D
10	20.128	N/D	N/D	**0.738**	**0.485**	N/D	N/D	N/D	N/D
11	21.016	N/D	N/D	**0.766**	**0.718**	N/D	N/D	N/D	N/D
12	22.369	N/D	N/D	**0.817**	**0.449**	N/D	N/D	N/D	N/D
13	25.928	N/D	N/D	**0.944**	**0.490**	N/D	N/D	N/D	N/D
Flavone	27.427	1.000	0.000	1.000	0.000	1.000	0.000	1.000	0.000
14	28.089	N/D	N/D	N/D	N/D	N/D	N/D	1.024	0.247
15	28.277	N/D	N/D	**1.033**	**0.458**	1.034	0.591	N/D	N/D
16	29.145	N/D	N/D	**1.063**	**0.471**	1.066	0.472	N/D	N/D
17	34.775	N/D	N/D	**1.270**	**0.211**	1.277	0.402	1.277	0.522
18	36.940	N/D	N/D	**1.347**	**0.409**	N/D	N/D	1.347	0.422
19	37.362	N/D	N/D	**1.363**	**0.334**	N/D	N/D	1.362	0.224
20	40.572	N/D	N/D	N/D	N/D	N/D	N/D	1.473	0.370

N/D: Not Detectable, RSD: Relative standard desviation, HE*Ef*: Hexanic extract of *Euphorbia furcillata* Kunth, DE*Ef*: Dichloromethane extract of *Euphorbia furcillata* Kunth, EaEE*f*: Ethyl acetate extract of *Euphorbia furcillata* Kunth, ME*Ef*: methanolic extract of *Euphorbia furcillata* Kunth.

Because EaE*Ef* was the more potent and efficient extract thus; its possible functional mode of action was assessed. Pre-incubation with the EaE*Ef* assay, the CRC showed significant reduction of NE-induced maximal contraction suggesting non-competitive antagonism of adrenergic receptor or NE-induced contraction pathway disruption. NE contraction is mediated by Ca^{2+} intracellular increase. This initial transient increase in cytosolic Ca^{2+} originates from Ca^{2+} release from intracellular stores (sarcoplasmic reticulum), while the latter increase arises from the extracellular space through Ca^{2+} channel receptors (Hilgers & Webb, 2005). In this context, the contraction induced by $CaCl_2$- was totally abolished by preincubation with EaE*Ef* also producing a significant vasorelaxant effect on the KCl-induced depolarization, this behavior suggests blockade of L-type Ca^{2+} channels in smooth-muscle-cell membrane (Hilgers & Webb, 2005). Changes in the intracellular Ca^{2+} concentration and membrane

depolarization stimulate large-conductance Ca^{2+}-activated K^+ channels (BKCa), which are thought to play an important role in maintaining the membrane potential of vascular smooth muscle cells (Ko *et al*., 2008). The role of K^+ channels on the EaE*Ef*-induced vasorelaxant effect were assessed with TEA (a non-selective KCa^{2+} channel blocker) but the effect was not modified in the presence of TEA, suggesting that there is no opening of potassium channels in the vasorelaxant mode of action. On the other hand, EaE*Ef* exhibited an endothelium-dependent effect, based on which we investigated the participation of EDRF such as NO or PGI_2. To identify the participation of NO-sGC-cGMP or PGI_2 –AC-cAMP pathways, aortic rings were preincubated with L-NAME (a NOS inhibitor) or ODQ (an sGC inhibitor) or Indomethacin (COX inhibitor) respectively; the EaE*Ef* relaxation CRC shifted to the right and decreased the maximum effect in the presence of L-NAME and ODQ. The latter suggests that NO production and the subsequent cGMP increase might be involved in EaE*Ef*-induced relaxation. Physiological actions of NO are mediated by activation of the sGC and the consequent increase in the concentration of cGMP. Cyclic GMP activates Protein Kinase G (PKG), preventing calcium influx through voltage-dependent calcium channels and calcium release mediated by Inositol 1,4,5-triphosphate (IP_3) receptors (IP_3R) in vascular smooth muscle (Moncada *et al*., 1988; Ignarro, 1991). Finally relaxation-CRC in the presence of Indomethacin was shifted to the left indicating that EaE*Ef* relaxant activity was synergized, but this effect may be attributed to the synthesis inhibition of contractile endogenous prostanoids, such as PGH_2 (Aboulafia, 1976; Furci *et al*. 1991). The fact that the vasorelaxant effect of EaE*Ef* was diminished by L-NAME and ODQ and the fact that EaEEf, as well as its efficiency on KCl-80-induced depolarization abolished $CaCl_2$-contraction; suggest NO production and calcium channel blockade as the vasorelaxant mode of action of EaE*Ef*.

In the UltraViolet (UV) spectrum of ME*Ef*, a highest signal of around 280–360 nm was observed, corresponding to flavonoid or polyphenolic compounds. According to Noori *et al*., the main flavonoids isolated from phytochemical studies performed on 17 *Euphorbia* species were rutin, quercetin, kaempferol, and myricetin, all of these eluting before the flavone (ISTD), indicating that they are more polar than this compound (Noori *et al.*, 2009).

In the chromatographic profile of EaE*Ef* suggest that these signals possess a high capacity factor in reverse- phase chromatography. According to Bicchi *et al*., some diterpenoids were isolated from *Euphorbia* seed oil, such as ingenol, hydroxylathyrol, and epoxylathyrol. In this work, a chromatographic profile of a caper spurge extract was reported that, on comparison to the chromatographic profile of EaE*Ef*, exhibited similar signals and the same UV spectrum (Bicchi *et al*., 2001). On the other hand, Wu *et al*. (2009) reported that *Euphorbia* species have many uses in folk medicine that are attributed to diterpenoid constituents, especially those with abietane, tigliane, and ingenane skeletons..Some of the isolated compounds present singular characteristics, such as the conjugations presents in different rings of the structure, such as helioscopinolides, and epoxy, hydroxyl, and ketone derivatives from abietane skeleton. Tian *et al*. (2011) report there have been isolated from Ethyl acetate extract of different *Euphorbiaceae* mainly lathyrane diterpenoids showed significant vasorelaxant activities against phenylephrine-induced vasoconstriction, the evaluation of vasorelaxant activity result from its vasodilatory effects directly upon vascular smoot muscle with relation rates of 48% to 53% at 10^{-6} M concentration (Tian *et al*., 2011; Tian *et al.,* 2013). All of these structures possess low polarity and eluted in the final section of the chromatogram (signals 15, 16, and 17) with high retention times.

The signals related to the pharmacological effect were those with lowest polarity and highest retention time. Naturally occurring diterpenes exert several biological activities such as anti-inflammatory action, antimicrobial and antispasmodic activities. Several diterpenes have demonstrated pronounced cardiovascular effects such as positive inotropic responses, activation of adenylate cyclase (forskolin), vasorelaxant properties (eleganolone and 14-deoxyandrographolide) and inhibition of smooth muscle contraction by blocking L-type calcium channels (marrubenol) (Tirapelli *et al*., 2008). As well, Bruneton (2001), reports antihypertensive properties of labd-8 (17) -en-15-oic acid and of forskolin, Ulubelen *et al*. (2002), demostrated cardiovascular activities of abietane diterpenes (Bruneton, 2001; Ulubelen *et al.*, 2002) and Hipólito *et al*. (2009), reports ability of diterpene ent-8(14),15-pimaradien-3beta-ol (PA-3beta-ol) to

induce vascular relaxation through extracellular Ca^{2+} influx blockade and by the activation of NO-cGMP pathway. Based in this, cardiovascular pharmacological effect showed by EaE*Ef* could be relate with diterpenoids molecules presents in *E. furcillata*

CONCLUSIONS

To our knowledge, we have reported, for the first time, the cardiovascular pharmacological effect and chromatographic profiles of *Euphorbia furcillata* Kunth extracts. Our results, suggest that the vasorelaxant activity presented by the EaE*Ef* extract could be attributed to diterpenoids molecules with abietane, tigliane, and ingenane skeletons whose effect involve NO production and calcium channel blockade as vasorelaxant mode. Our results contribute to pharmacological evidence of *E. furcillata* specie; however, it must be submitted to further *in vivo* studies to identify its antihypertensive effect.

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