Drug and Chemical Toxicology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/idct20

Cytoprotective effects of creosote bush (*Larrea tridentata*) and Southern live oak (*Quercus virginiana*) extracts against toxicity induced by venom of the black-tailed rattlesnake (*Crotalus ornatus*)

Luis Fernando Plenge-Tellechea , Sergio Acosta-Lara , Joaquín Rodrigo-García , Emilio Álvarez-Parrilla , David Meléndez-Martínez , Ana Gatica-Colima & Jorge A. Sierra-Fonseca

To cite this article: Luis Fernando Plenge-Tellechea , Sergio Acosta-Lara , Joaquín Rodrigo-García , Emilio Álvarez-Parrilla , David Meléndez-Martínez , Ana Gatica-Colima & Jorge A. Sierra-Fonseca (2020): Cytoprotective effects of creosote bush (*Larrea tridentata*) and Southern live oak (*Quercus virginiana*) extracts against toxicity induced by venom of the black-tailed rattlesnake (*Crotalus ornatus*), Drug and Chemical Toxicology, DOI: 10.1080/01480545.2020.1856864

To link to this article: https://doi.org/10.1080/01480545.2020.1856864

Published online: 09 Dec 2020.

DRUG AND CHEMICAL TOXICOLOGY
https://doi.org/10.1080/01480545.2020.1856864

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Cytoprotective effects of creosote bush (*Larrea tridentata*) and Southern live oak (*Quercus virginiana*) extracts against toxicity induced by venom of the black-tailed rattlesnake (*Crotalus ornatus*)

Luis Fernando Plenge-Tellechea[a], Sergio Acosta-Lara[a], Joaquín Rodrigo-García[b], Emilio Álvarez-Parrilla[a], David Meléndez-Martínez[a]*, Ana Gatica-Colima[a] and Jorge A. Sierra-Fonseca[a]

[a]Departamento de Ciencias Químico-Biológicas, Universidad Autónoma de Ciudad Juárez, Ciudad Juárez, México; [b]Departamento de Ciencias de la Salud, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, Chihuahua, México

ABSTRACT

The venom of *Crotalus ornatus* (v*Co*) poses a threat to human health, as it contains a mixture of toxins that can cause cytotoxic, necrotic, and hemolytic effects. The present study assessed methanolic and acetone extracts from leaves and flowers of *Larrea tridentata*, as well as the bark of *Quercus virginiana* as potential suppressors of the toxic effects of v*Co in vitro*. The content of total phenols, flavonoids, and tannins of the plant extracts were quantified for the suppression of v*Co* cytotoxicity in two cell culture models, human lymphocytes and porcine aortic endothelial (PAE) cells. Extracts from *Q. virginiana* displayed a greater concentration of total phenols, flavonoids, and tannins. Co-incubation of lymphocytes and PAE cells with fixed concentrations of v*Co* and plant extracts resulted in decreased v*Co*-induced cytotoxicity. A 24-hour co-incubation of lymphocytes with v*Co* (2.36 ± 0.17 μg/mL) and 0.5 μg/mL of methanolic leaf extract from *L. tridentata* (LLM) significantly suppressed the venom-induced cytotoxicity by $37.33 \pm 8.33\%$. Similarly, the LLM extract (4 μg/mL) caused a significant decrease in v*Co* cytotoxicity after 24 hours in PAE cells. In contrast, while the acetone extract of *Q. virginiana* bark (QA) suppressed cytotoxicity by $29.20 \pm 3.51\%$ ($p < 0.001$) in lymphocytes, it failed to protect PAE cells against v*Co* after 24 hours. In PAE cells, a shorter 4-hour co-incubation showed significant suppression of cytotoxicity with both extracts. Our results collectively suggest that LLM and QA possess cytoprotective properties against the *in vitro* toxic effects of v*Co*, and thus establish extracts from these plants as potential therapeutic interventions against *Crotalus* envenomation.

ARTICLE HISTORY
Received 18 September 2020
Revised 18 November 2020
Accepted 22 November 2020

KEYWORDS
Snake venom; phytochemicals; cytotoxicity; cytoprotection; crotalids; plant extracts

Introduction

Snakebite envenomation is known to affect millions of people worldwide, and since it is a significant cause of morbidity and mortality in many regions of the world, it has been recently recognized by the World Health Organization as a neglected tropical disease (Gutiérrez *et al.* 2011, Avau *et al.* 2016, Habib and Brown 2018). In North America, the large majority of snakebite reports correspond to crotalids, including members on the genus *Crotalus* (Ruha *et al.* 2017). The venom of members of this genus is characterized by the presence of hydrolytic enzymes, which are capable of producing a plethora of life-threatening effects, including neurotoxicity, myotoxicity, hemotoxicity, necrosis, and anticoagulation, with the severity of these toxic properties depending on factors such as age, species, and geographic location (Alape-Girón *et al.* 2008, Martins *et al.* 2014). In this regard, *Crotalus ornatus* (synonym of *Crotalus molossus molossus,* Anderson and Greenbaum 2012), commonly known as the black-tailed rattlesnake, produces a venom characterized by a complex combination of proteins such as nucleases, metalloproteinases, serine proteases, disintegrins, myotoxins, phospholipases, cysteine-rich secretory proteins (CRiSPs), and highly toxic L-amino acid oxidases (Mackessy 2010, Meléndez *et al.* 2014, Khedrinia *et al.* 2018). The damage caused by the venom includes hemorrhage, proteolysis, inhibition of platelet aggregation, fibrinolysis, neurovascular injury, among other deleterious effects (Sánchez *et al.* 2001, Zúñiga Carrasco and Caro Lozano 2013, Meléndez *et al.* 2014, Mourão de Moura *et al.* 2014, Borja *et al.* 2018).

Use of antivenom remains the treatment of choice for snakebite envenomation and while it is usually effective, it faces many limitations, such as prompt administration, risk of adverse immunological reactions, poor efficacy on local symptoms, and limited availability in underdeveloped regions (Gutiérrez *et al.* 2011, León *et al.* 2013). Therefore, in an effort to find alternative treatments against snakebite envenomation, the search for potential antidotes has turned to plant-derived extracts (Fanoudi *et al.* 2020). It has been found that extracts prepared from a variety of plant tissues contain

CONTACT Luis Fernando Plenge-Tellechea fplenge@uacj.mx Departamento de Ciencias Químico-Biológicas, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez. Anillo envolvente del PRONAF y Estocolmo s/n, Ciudad Juárez 32310, México
*Present address: Centro de Biotecnología FEMSA, Tecnológico de Monterrey, Monterrey, México.

phytochemicals and secondary metabolites with protective or neutralizing capabilities against snake venom, hence their potential use as pharmacological interventions (Panfoli *et al*. 2010, Gupta and Peshin 2012, Singh *et al*. 2017). For instance, an ethanolic extract prepared from the India-native species *Cordia macleodii* Hook (Boraginaceae), was found to inhibit the hemorrhagic effect of *Naja* spp. venom in rats. This ethnomedicinal plant species is also known for its high content of phenols, terpenoids, saponins, oils, and flavonoids (Bhide *et al*. 2011, Soni and Bodakhe 2014). The methanolic extract of *Leucas aspera* (Lamiaceae), another Indian species commonly used for its antipyretic properties, abolished the hemolytic properties of *Naja naja* venom (Prajapati *et al*. 2010, Gopi *et al*. 2014). *Quercus infectoria* (Fagaceae), an oak species native to Southern Europe and the Middle East known to have anti-microbial (Mustafa *et al*. 2018) and anti-fungal properties (Baharuddin *et al*. 2015), has been shown to neutralize the effects of crude *Naja* sp. venom (Shabbir *et al*. 2014), while extracts from *Bellucia dichotoma* (Melastomataceae) have been proven to inhibit the effects of *Bothrops jararaca* venom (Mourão de Moura *et al*. 2014). For *Crotalus* spp., it has been reported that extracts prepared from the callus of the wingleaf soapberry, *Sapindus saponaria* (Sapindaceae) displayed anti-ophidic effects on the activity of *C. durissus terrificus* and other non-crotalid vipers (da Silva *et al*. 2012). Also, extracts from *Renealmia alpina* (Zingiberaceae) a flowering plant that is commonly used to treat snakebites in Colombia, has been shown to neutralize enzymatic components present in the venom of *C. durissus cumanensis* (Patiño *et al*. 2013).

Two plant species with distribution in Northern Mexico are *Larrea tridentata* (Zygophyllaceae), commonly known as creosote bush, and the southern live oak, *Quercus virginiana* (Fagaceae), both of them being known for their high content of bioactive molecules, including tannins and flavonoids (Lira-Saldívar 2003, Osorio *et al*. 2010, Moreno *et al*. 2011), as well as their antioxidant properties (Skouta *et al*. 2018). *Q. infectoria* (Fagaceae) has been shown to inhibit necrosis-inducing factors from the venom of *Naja kaouthia,* (Pithayanukul *et al*. 2005), as well as neutralizing phospholipase, hyaluronidase, and L-amino acid oxidase activities present in the venom of *N. naja kauothia* (Leanpolchareanchai *et al*. 2009). Similarly, *L. tridentata* has been reported to have multiple beneficial effects, such as anti-fungal and anti-viral properties, as well as antioxidant capabilities due to its high content of polyphenol compounds (Cho *et al*. 2003, Peñuelas-Rubio *et al*. 2017, Peralta *et al*. 2018). The presence of beneficial phytochemical properties makes these plants ideal candidates for potential intervention strategies against the toxic effects of the venom of *C. ornatus*, a species typically found in rocky terrains in northern México (Anderson and Greenbaum 2012). Given the health threat posed by *C. ornatus* envenomation, the urgent need for novel antidotes, and the potential cytoprotective effects found in extracts prepared from plants of the Chihuahuan desert in Northern Mexico, this study aimed at evaluating the potential *in vitro* antivenom properties of extracts prepared from *L. tridentata* and *Q. virginiana* tissues, against the toxicity exerted by venom of *C. ornatus* (v*Co*).

Materials and methods

Venom

Samples of v*Co* were obtained from captive specimens maintained at the herpetarium of the Universidad Autónoma de Ciudad Juárez (UACJ). Venom samples were lyophilized and stored at −20 °C until used. The experimental protocol for use of animals in our research was approved by the Ethics Review Committee at UACJ (protocol # CIBE-2017–1-46).

Plant material

Specimens of *L. tridentata* (N 31° 39′ 20.392″, W 106° 29′ 29.546″) and *Q. virginiana* (N 31° 44′ 46.31″, W 106° 26′ 36.646″) were collected randomly during the month of October of 2016 in the suburban zone of Ciudad Juárez, México. Proper specimen identification of the collected plants was carried out with the assistance of the Herbarium at Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, with accompanying identification numbers (001510 for *L. tridentata* and 001262 for *Q. virginiana*). Samples of *L. tridentata* were split into two different groups: one with leaves only (LL), and another one with both leaves and flowers (LF). For *Q. virginiana* samples (Q), bark pieces were collected, and traces of wood were removed. Plant tissue from both species was subjected to dehydration by incubation in an oven at 50 °C for 48 hours, followed by storage in a dry and dark cabinet (Ventura *et al*. 2008, Aguilar *et al*. 2012).

Preparation of plant extracts

LL and LF samples of *L. tridentata*, as well as bark samples of *Q. virginiana* were triturated mechanically and further subdivided into subgroups of 3 g each: LLA, LFA, and QA for extraction with acetone, and LLM, LFM, and QM for methanolic extraction. Samples were placed into 50 mL conical tubes, and 20 mL of either 70% (v/v) acetone or 70% (v/v) methanol were added. Samples were sonicated on ice for 30 min, followed by centrifugation at 4000 rpm for 15 min. Supernatants were recovered, and solvent removal was performed with a rotary evaporator at 45 °C. Samples were then lyophilized and stored at −20 °C protected from light (Pérez *et al*. 2013).

Determination of total phenols

Total phenolic content was determined by a previously described method (Moreno-Escamilla *et al*. 2015). A 250 µL aliquot of plant extract (2 mg/mL) was mixed with 1 L of 7.5% Na_2CO_3 and incubated for 3 min, followed by the addition of 1.250 L of Folin–Ciocalteu reagent (1:10 v/v). The mixtures were then incubated for 15 min at 50 °C, and allowed to cool down at room temperature. Absorbance was then read at 760 nm, with gallic acid being used as standard. Results are expressed as mg of gallic acid equivalent per g of dry weight.

Determination of total flavonoids

Total flavonoid content was determined by a previously described method (Moreno-Escamilla *et al*. 2015), using catechin as standard. A 250 μL aliquot of plant extract (2 mg/mL) was mixed with 2 mL water followed by the addition of 125 μL of 5% $NaNO_2$. The mixtures were incubated for 5 min at room temperature, followed by the addition of 125 μL of 10% $AlCl_3$ and incubated for another 3 min. Subsequently, 2 mL of 0.5 M NaOH were added, and the mixtures were incubated for 30 min at room temperature. Absorbance was measured at 510 nm, and the results are expressed as mg of catechin equivalent per g of dry weight.

Determination of condensed tannin content

Condensed tannins were determined by the method of Li *et al*. (1996), using catechin as standard. A 250 μL aliquot of plant extract (2 mg/mL) was mixed with 50 μL of 2% DMACA reagent dissolved in methanol and acidified with 6 M HCl. The reactions were incubated for 20 min at room temperature and protected from light. Absorbance was read at 640 nm and results expressed as mg of catechin equivalent per g of dry weight.

Isolation of human lymphocytes and cell culture

Isolation of peripheral blood human lymphocytes was conducted as previously described (de la Rosa *et al*. 2001). Blood samples were obtained from healthy volunteer donors and collected in EDTA tubes, followed by isolation by differential centrifugation with the Percoll® gradient. Isolated lymphocytes were suspended in 1 mL of McCoy's 5 A modified medium, supplemented with sodium bicarbonate and 10% fetal bovine serum (FBS). The lymphocytes were grown in the supplemented medium with 10% (FBS) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Gibco™) and maintained in an incubator at 37 °C in a humidified atmosphere. Porcine aortic endothelial (PAE) cells (available from ATCC) were kindly provided by Dr. M. Miranda (UTEP), and grown in 25 cm^2 tissue culture flasks in Ham's F12 medium supplemented with 10% (FBS) and 100 μg/mL gentamycin, and maintained in a CO_2 incubator at 37 °C in a humidified atmosphere.

Cytotoxicity measurement using human lymphocytes

Lymphocyte viability was determined with the Evans blue exclusion method, using a Neubauer chamber. Immediately after isolation, 1.5 million cells were inoculated into a culture tube containing 5 mL of McCoy's 5 A modified medium in order to assess cell viability against different venom and plant extract concentrations. The various plant extracts and the v*Co* were tested on lymphocytes at different concentrations, prepared from a stock solution of 1 mg/mL in deionized water (dH_2O). Three control cultures were employed: vehicle only, one with snake venom (0.5, 1, 2, 4, and 6 μg/mL in dH_2O), and another without any additives. Cultures were incubated in a CO_2 incubator at 37 °C in a humidified atmosphere, for 72 h, conducting cell counts through the Evans blue method every 24 h. The results are displayed as percentage viability, with the no additive culture serving as 100% viability and the control with venom only representing 0% viability. The cytotoxic concentration 50% (CC_{50}) for the v*Co* and plant extracts were determined by this method.

Cytotoxicity measurement in PAE cells

Cytotoxicity of snake venom and plant extracts in PAE cells was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, following a previously described method (Slaninová *et al*. 2012). Briefly, 30 000 cells/well were seeded on 96 well plates and grown for 24 h. After undergoing different treatments with v*Co* (5, 10, 15, 20 and 25 μg/mL) and plant extracts (2, 4, 6, 8 and 10 μg/mL), 20 μL of the MTT reagent (2 mg/mL in PBS) were added to the culture media, and cells were then incubated for 120 min. The media was then carefully removed, and the formazan crystals were dissolved in 200 μL of acidified isopropanol, and absorbance was read at 540 nm using a microplate spectrophotometer. Viability measures were taken at several time points (2, 4, 8, 10, 24 and 48 h) for each treatment.

Statistical analysis

All assays were conducted three independent times in triplicate and analyzed using one-way ANOVA followed by Tukey's *post-hoc* test. In all cases, a $p < 0.05$ value was determined to be statically significant. Statistical analysis was conducted by means of the InfoStat and GraphPad Prism software packages.

Results

Quantification of phenolic compounds

The content of phenolic compounds was shown to differ among the extracts from *L. tridentata* and *Q. virginiana*, with the QA extract showing the highest amount of phenolic compounds. Table 1 shows the results from phenolic quantification of all the analyzed plant extracts.

Determination of CC_{50} of vCo and plant extracts

Before studying the potential cytoprotective effects of the plant extracts against v*Co*, the cytotoxic properties of each component individually were analyzed on both human lymphocytes and PAE cells, and the CC_{50} values for each mixture were determined. The cytotoxicity caused by v*Co* and *L. tridentata* and *Q. virginiana* extracts was shown to be dose-dependent, and the effects were also different depending on the cell type. CC_{50} values for all tested components on both cell types are shown on Table 2.

Human lymphocytes were revealed to be highly sensitive to the cytotoxic effects of v*Co*, displaying a CC_{50} value of 2.36 ± 0.17 μg/mL, and a v*Co* concentration of 6 μg/mL

causing ~80% of cellular death (Table 2, Figure 1(A)). Cultured lymphocytes were also affected by the presence of plant extracts, given that similar CC_{50} values were displayed by the cells in response to the different plant preparations (Table 2, Figure 1(B–G)). PAE cells also displayed sensitivity to v*Co* exposure, and the CC_{50} was found to be 3.32 ± 0.06 μg/mL (Table 2, Figure 2(A)). In the case of the different plant extracts, the response of PAE cells was found to be more variable and toxicity levels were lower in general (Table 2, Figure 2(B–G)).

Table 1. Quantification of phenolic compounds found in extracts from *L. tridentata* and *Q. virginiana*.

Extract	Total phenols mg EAG/g PS	Total flavonoids mg EC/g PS	Condensed tannins
LLM	1.217 ± 0.007	0.850 ± 0.189	0.014 ± 0.018
LLA	0.949 ± 0.020	0.358 ± 0.129	0.357 ± 0.130
LFM	0.951 ± 0.005	0.570 ± 0.176	0.340 ± 0.099
LFA	0.879 ± 0.023	0.326 ± 0.143	0.611 ± 0.088
QM	1.161 ± 0.013	0.622 ± 0.197	0.475 ± 0.054
QA	1.741 ± 0.016	1.062 ± 0.164	0.770 ± 0.154

Table 2. CC_{50} values for vCo and plant extracts on lymphocytes and PAE cells.

Extract	Lymphocytes CC_{50} (μg/mL)	PAE cells CC_{50} (μg/mL)
v*Co*	2.36 ± 0.17	3.32 ± 0.06
LLM	1.15 ± 0.13	7.78 ± 0.32
LLA	1.36 ± 0.19	9.17 ± 0.34
LFM	1.27 ± 0.12	7.81 ± 0.30
LFA	1.01 ± 0.17	5.92 ± 0.18
QM	1.49 ± 0.2	6.41 ± 0.34
QA	1.03 ± 0.16	8.54 ± 0.27

Modulation of vCo cytotoxicity by L. tridentata *and* Q. virginiana *extracts in human lymphocytes and PAE cells*

In order to examine the potential *in vitro* antivenom effects of our different plant extracts, human lymphocytes and PAE cells were co-incubated with the CC_{50} of v*Co* and a fixed concentration of plant extracts, and compared these cytotoxicity values to those obtained from incubations with v*Co* only. The selected plant extract concentrations were lower than the CC_{50} values displayed by human lymphocytes and PAE cells (Table 2). This was done to avoid higher cytotoxicity and to more clearly delineate a potential cytoprotective effect against the toxic effects of v*Co*. The selected concentrations were 0.5 μg/mL and 4 μg/mL, for human lymphocytes and PAE cells, respectively, and they were kept constant in all co-incubation experiments.

In the case of human lymphocytes co-incubated with v*Co* (2.36 ± 0.17 μg/mL) and 0.5 μg/mL of the LLM, LLA, QM, and QA extracts, statistically significant differences in cytotoxicity when compared with lymphocytes incubated with v*Co* alone were reported (Table 3). At 24 h, all extracts tested displayed significant differences in providing cytoprotection ($p < 0.05$), excepting the LFA extract ($p = 0.05$) (Tables 4 and 3,

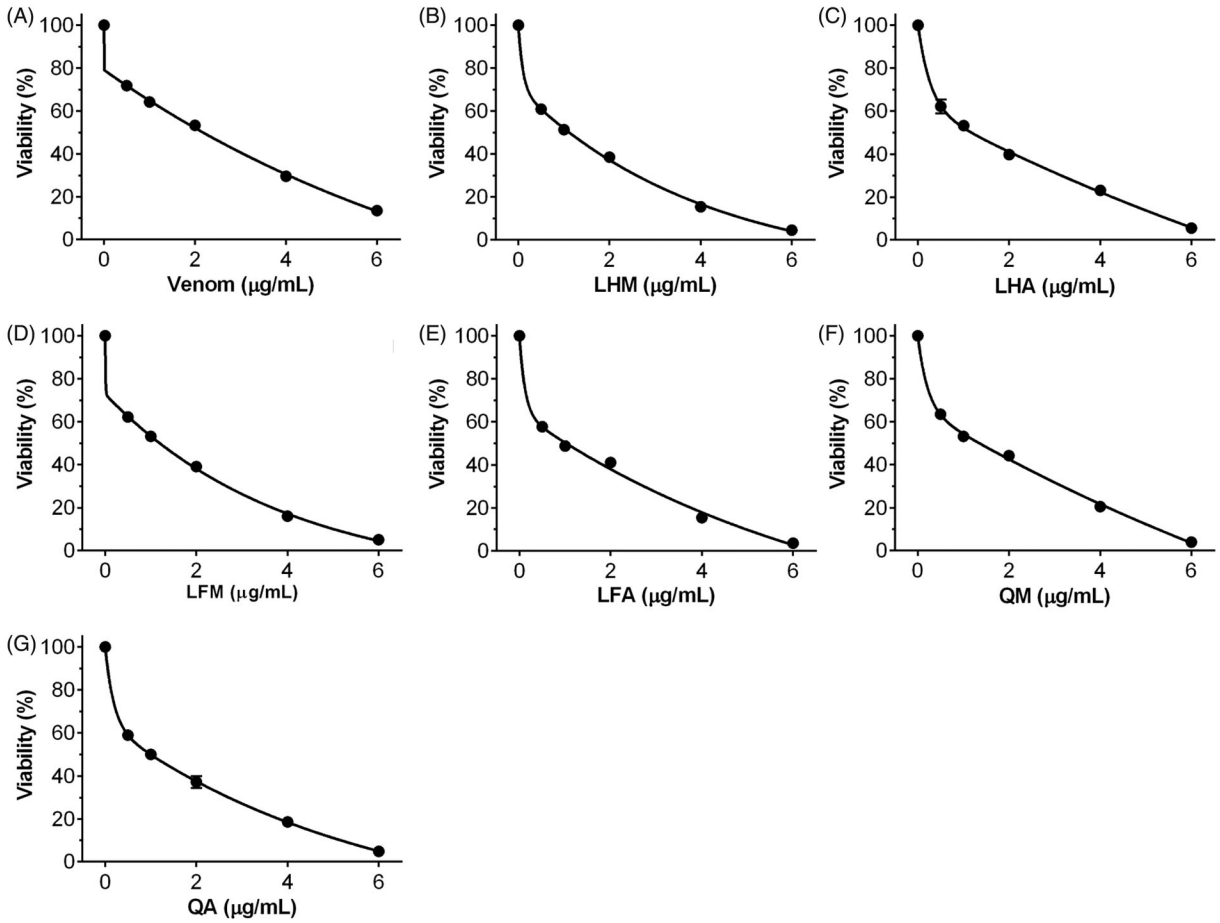

Figure 1. Determination of CC_{50} values of v*Co* and plant extracts on human lymphocytes. Cells were seeded on culture tubes and incubated with increasing concentrations of v*Co* (A) or plant extracts (B-G), for 72 h, taking viability measurements every 24 h through the Evans blue assay.

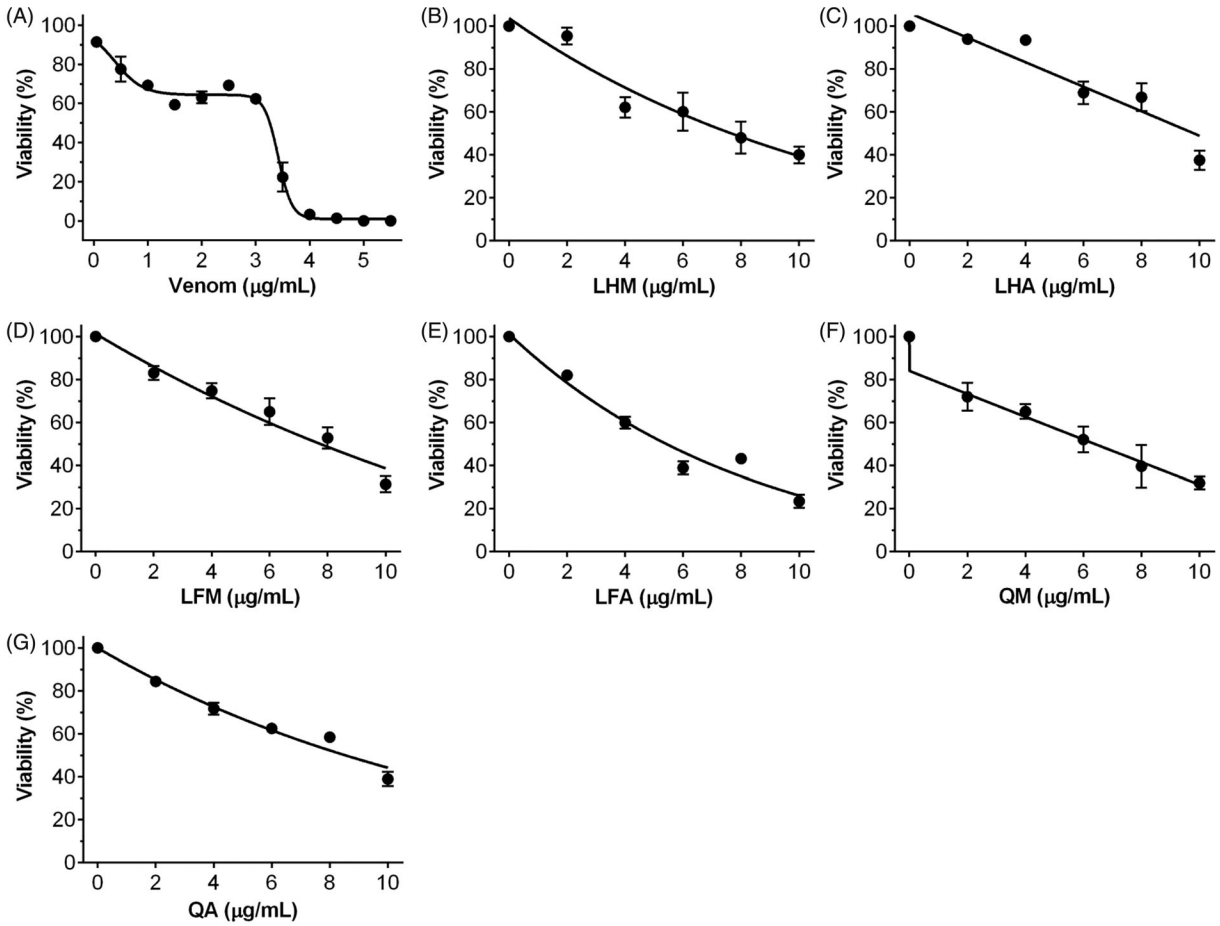

Figure 2. Determination of CC_{50} values of v*Co* and plant extracts on PAE cells. Cells were seeded on 96-well plates and incubated for 24 h at 37 °C, followed by incubation with increasing concentrations of v*Co* (A) or plant extracts (B-G). Cells were further incubated for 24 h to determine the CC_{50} for each component through the MTT assay.

Table 3. P values resulting from comparing human lymphocytes incubated with v*Co* alone versus lymphocytes with v*Co* and individual plant extracts.

Time/extract	24h	48h	72h
LLM	*<0.001*	*0.012*	0.430
LLA	*<0.001*	*0.041*	0.648
LFM	*0.010*	0.221	0.883
LFA	*0.050*	0.688	0.960
QM	*<0.001*	*0.049*	0.337
QA	*<0.001*	*0.039*	0.552

Table 4. Cytotoxicity inhibition of v*Co* by *L. tridentata* y *Q. virginiana* extracts on human lymphocytes.

	Inhibition (%)		
Extract	24 h	48 h	72 h
LLM	37.33 ± 8.33	11.59 ± 3.48	3.59 ± 0.29
LLA	22.48 ± 3.39	8.64 ± 3.40	2.35 ± 0.18
LFM	15.94 ± 4.05	5.60 ± 3.29	1.25 ± 0.23
LFA	11.81 ± 3.58	2.08 ± 3.40	0.12 ± 0.09
QM	25.63 ± 1.46	7.08 ± 3.40	3.24 ± 0.30
QA	29.20 ± 3.51	8.55 ± 3.19	2.69 ± 0.22

Figure 3). At 48 h, only LFM and LFA were not found to be significant, whereas co-incubation with the same mixture of v*Co* and plant extracts after 72 h did not show any significant differences. Unlike cultured lymphocytes, PAE cells reproduce over time in culture. For this reason, the cytotoxic activity of v*Co* in the presence of plant extracts was measured on shorter periods of time, starting at 2 h. The results showed that the LLM and QA extracts exerted a significant inhibitory effect against v*Co* in PAE cells (Tables 5 and 6, Figure 4). The only additional time point with significant cytoprotection against v*Co* in PAE cells was that of LLM at 24 h.

Discussion

Extracts prepared from a variety of plants have been known to decrease the toxicity of snake venoms, hence their potential use as pharmacological interventions (Panfoli *et al*. 2010). For instance, an ethanolic extract prepared from *Cordia macleodii* (Boraginaceae) was found to inhibit the hemorrhagic effect of *Naja* spp. venom in rats (Soni and Bodakhe 2014), while the methanolic extract of *Leucas aspera* (Lamiaceae) abolished the hemolytic properties of *Naja naja* venom (Gopi *et al*. 2014). The presence of tannins and flavonoids in these extracts confers them with the ability to chelate metal ions and inhibit venom enzymes, which can contribute to neutralize the deleterious effects of snake venom. Importantly, the plant extracts used in this study also display the presence of similar phytochemicals, which can constitute a contributing factor to the observed cytoprotective effects against v*Co* (Martínez *et al*. 2002, Patiño *et al*. 2013).

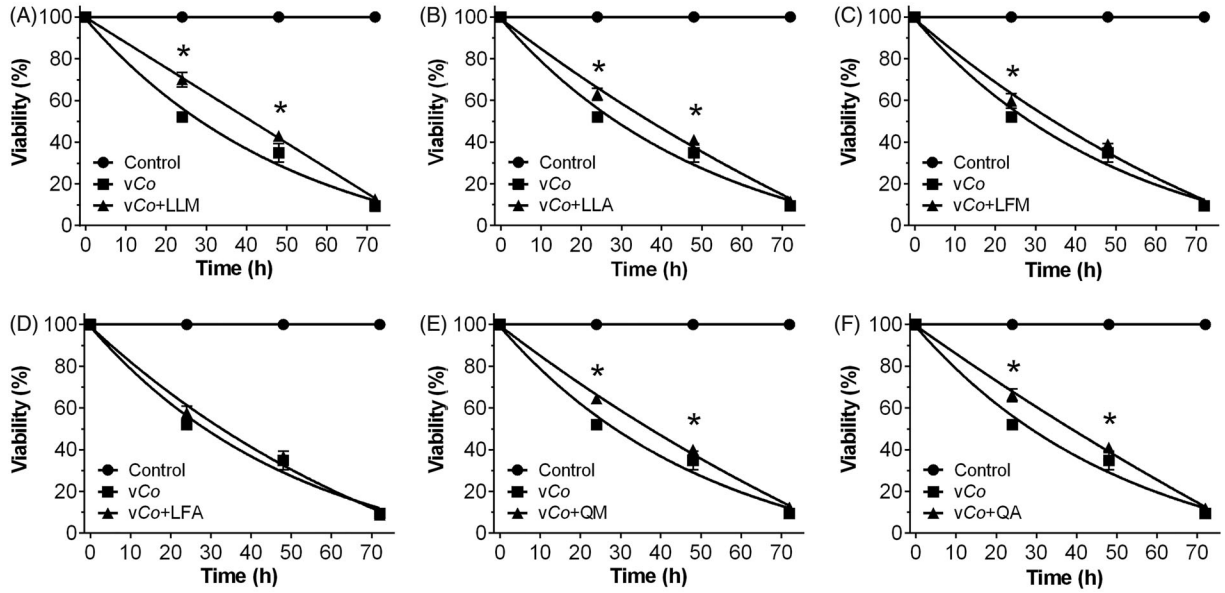

Figure 3. Modulation of v*Co* cytotoxicity by plant extracts on human lymphocytes. Human lymphocytes were exposed to a fixed concentration of v*Co* (CC_{50} 2.36 ± 0.23 μg/mL) alone (■) or in combination with a fixed concentration (0.5 μg/mL) of the plant extracts (▲). Human lymphocytes without additives served as control (●). Viability measures were taken every 24 h up until reaching 72 h via the Evans blue assay. (A) v*Co*. (B) LLM extract. (C) LLA extract. (D) LFM extract. (E) LFA extract. (F) QM extract. (G) QM extract.

Table 5. Cytotoxicity inhibition of v*Co* by *L. tridentata* y *Q. virginiana* extracts on PAE cells.

	Inhibition (%)					
Extract	2 h	4 h	8 h	10 h	24 h	72 h
LLM	49.64 ± 10.37	44.49 ± 3.59	30.29 ± 8.06	54.59 ± 7.88	25.89 ± 4.82	3.53 ± 0.73
LLA	34.97 ± 9.94	38.85 ± 9.94	29.82 ± 7.93	52.84 ± 6.11	23.27 ± 7.39	5.61 ± 1.91
LFM	38.11 ± 15.61	31.84 ± 7.11	22.45 ± 7.52	46.24 ± 10.39	12.97 ± 2.44	1.30 ± 0.62
LFA	19.50 ± 12.40	24.10 ± 5.76	17.95 ± 6.61	39.77 ± 1.83	6.04 ± 2.55	2.03 ± 0.78
QM	40.73 ± 17.91	35.39 ± 7.07	20.82 ± 7.41	5.61 ± 1.91	44.76 ± 3.81	3.04 ± 0.84
QA	50.64 ± 13.27	41.47 ± 6.40	32.93 ± 11.58	47.58 ± 2.81	15.13 ± 3.78	4.88 ± 1.45

Table 6. *P* values resulting from comparing PAE cells incubated with v*Co* alone versus cells with v*Co* and individual plant extracts.

Time/extract	2h	4h	8h	10h	24h	48h
LLM	0.116	0.011	0.193	0.179	0.024	0.751
LLA	0.311	0.332	0.252	0.247	0.201	0.690
LFM	0.407	0.387	0.480	0.462	0.078	0.977
LFA	0.424	0.408	0.645	0.645	0.696	0.887
QM	0.119	0.074	0.477	0.452	0.476	0.780
QA	0.212	0.041	0.325	0.371	0.347	0.700

Our data shows that the acetone bark extract of *Q. virginiana* prepared in this study is a promising candidate to decrease the toxic effects of v*Co* venom *in vitro*. Interestingly, analysis of its phytochemical composition showed that the QA extract possesses a greater content of flavonoids and tannins than those displayed by the other *Q. virginiana* and *L tridentata* extracts (QM, LLA, LLM, LFM, LFA) analyzed in this study (Table 1). Extracts from another *Quercus* species have been reported to display anti-ophidic properties, as *Q. infectoria* was previously shown to inhibit the activity of multiple components from the venom of the monocellate cobra, *Naja naja kaouthia* (Leanpolchareanchai *et al*. 2009). Interestingly, *Quercus* species, in this case *Q. infectoria* and *Q. iberica* (Fagaceae), are known to be rich in flavonoid, tannins, and low molecular weight phenolic compounds, and these phytochemicals have been demonstrated to possess high antioxidant activity that contributes to inhibit enzymes present in snake venom (Lira-Saldívar 2003, Alaniia *et al*. 2013).

The *L. tridentata* LLM extract was also shown to have cytoprotective effects against v*Co* venom, as shown by the cytotoxicity inhibition assay. Our phytochemical analysis revealed that the LLM extract possesses similar levels of flavonoid content to those seen in the QA extract. Interestingly, the phytochemical apigenin, which has been previously used in the small-molecule design of inhibitors of snake venom metalloproteases, is a known component of *L. tridentata* (Lira-Saldívar 2003, Srinivasa *et al*. 2014), and therefore could be a contributor in the cytotoxicity inhibition induced by our extract. Nevertheless, the phytochemical cytoprotective activity of *L. tridentata* extracts could also be limited in our assays by the presence of phenolic lignans, which have been previously shown to have cytotoxic effects in cultured cells (Lambert *et al*. 2005). A recent study found that ethanolic and aqueous extracts prepared from *L. tridentata* specimens collected from an adjacent geographic region to our study, did not exert significant cytotoxicity in cultured HS27 human fibroblasts at concentrations up to 120 μg/mL. However, when assayed *in vitro*, these extracts did exhibit efficient antioxidant properties related to their high phenolic content (Skouta *et al*. 2018).

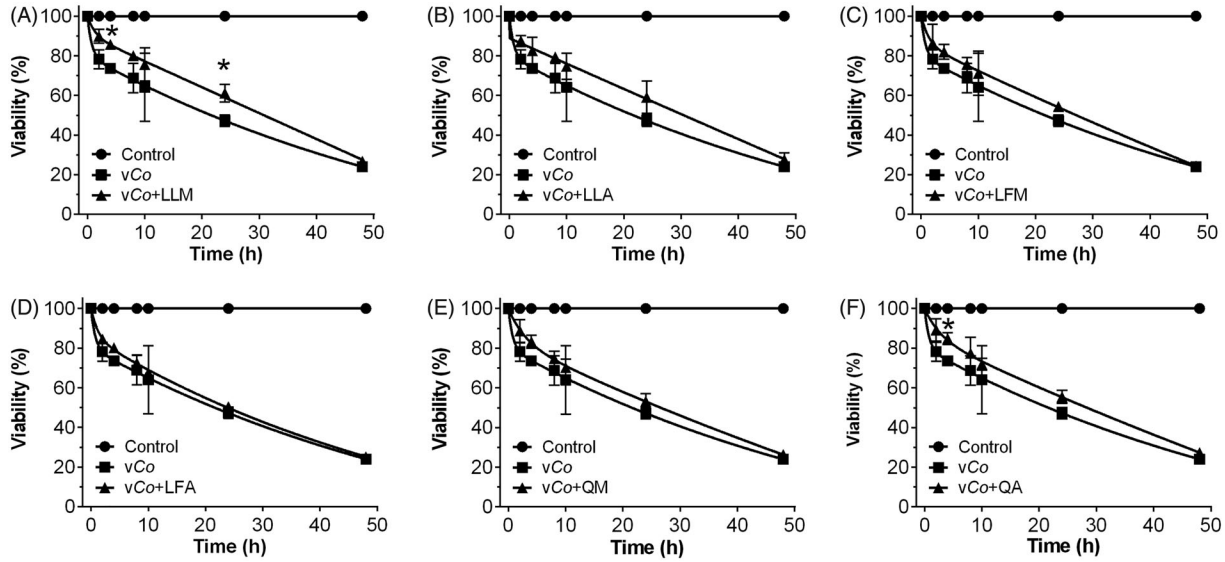

Figure 4. Modulation of v*Co* cytotoxicity by plant extracts on PAE cells. PAE cells were exposed to a fixed concentration of v*Co* (CC_{50} 3.32 ± 0.06 μg/mL) alone (■) or in combination with a fixed concentration (4 μg/mL) of the plant extracts (▲). PAE cells without additives served as control (●). Viability measures were taken at different time points up until reaching 48 h via the MTT assay. (A) v*Co* (B) LLM extract. (C) LLA extract. (D) LFM extract. (E) LFA extract. (F) QM extract. (G) QM extract.

Significant cytotoxicity was observed in both human lymphocytes and PAE cells exposed to different doses of v*Co* (Figures 1 and 2). This is not surprising, given that snake venom toxins have been shown to act synergistically or individually to exert cytotoxic effects on multiple cell lines (Kakanj *et al*. 2015). For instance, the phospholipase A2 molecules present in the venom of *Crotalus molossus nigrescens* (a species closely related to *C. ornatus*) have been reported to cause lymphocyte lysis (Meléndez-Martínez *et al*. 2017a), which leads to complete loss of cellular integrity (Harris and Scott-Davey 2013). Furthermore, in this same study (Meléndez-Martínez *et al*. 2017a), the venom of *C. molossus nigrescens* displayed a CC_{50} of 1.42 ± 0.16 μg/mL (12 h) on leucocytes, a value that is in line with the one of 2.36 ± 0.17 μg/mL found in the present study. In addition, human erythrocytes exposed to the venom of *Crotalus molossus nigrescens* undergo hemolysis as a result of membrane oxidative damage (Meléndez-Martínez *et al*. 2017b). Furthermore, other toxins such as peptide hydrolases, proteases, endopeptidases, and proteinases have been shown to damage membrane-bound proteins, thus mediating venom-induced morphological alterations (Vyas *et al*. 2013) . Also, in our previous study (Meléndez-Martínez *et al*. 2017a), a CC_{50} value of 3.42 ± 0.06 μg/mL was obtained for PAE cells, which is very similar to the one observed in the present study (3.32 ± 0.06 μg/mL, Table 2). Unlike lymphocytes, PAE cells have a high growth rate in culture, which could explain the higher cytotoxic concentration values observed for both v*Co* and the plant extracts (Table 1). This is supported by a previous study using cultured skeletal muscle cells, where it was found that the venom of *B. alternatus* and *B. diporus* displayed CC_{50} values of 16.5 μg/mL and 14.4 μg/mL, respectively (Bustillo *et al*. 2009).

Conclusion

Significant cytotoxicity as a result of v*Co* exposure was observed in cultured human lymphocytes and PAE cells. The methanolic extract of *L. tridentata* leafs and acetone extract of *Q. virginiana* bark, which were found to be enriched in phenolic compounds, displayed anti-venom properties by significantly decreasing cell death caused by v*Co* in both cell types. Furthermore, the cytoprotective effects of the plant extracts were found to be dependent on cell type and exposure time, as highlighted by the significant decreases in v*Co*-induced cytotoxicity observed during shorter co-incubation times in PAE cells. The results presented here thus serve to establish these two plant species as suppressors of the *in vitro* cytotoxic effects caused by v*Co*, warranting further studies to analyze potential *in vivo* cytoprotective effects.

Acknowledgements

This project was partially funded by Proyecto de Ciencia Básica from CONACyT (#256009). We are grateful to UACJ authorities and members of the laboratories of Chemistry (T-116), Molecular Biology and Biochemistry (T-216), and Academy of Biochemistry at UACJ. We also wish to thank Dr. Coyolxauhqui Figueroa Batalla from the Herbario at UACJ, for providing technical assistance to properly identify the collected plant specimens.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The present research was financially supported by Consejo Nacional de Ciencia y Tecnología.

ORCID

Luis Fernando Plenge-Tellechea http://orcid.org/0000-0002-1619-5004
Sergio Acosta-Lara http://orcid.org/0000-0001-5232-1242
Joaquín Rodrigo-García http://orcid.org/0000-0002-0997-5811

Emilio Álvarez-Parrilla http://orcid.org/0000-0002-6162-8139
David Meléndez-Martínez http://orcid.org/0000-0003-3643-2668
Ana Gatica-Colima http://orcid.org/0000-0002-6117-1327
Jorge A. Sierra-Fonseca http://orcid.org/0000-0003-3256-3257

References

Aguilar, J., *et al.*, 2012. Extraction and evaluation of condensed tannins from bark of eleven species of trees from Costa Rica. *Revista Tecnología en Marcha*, 5 (4), 15–22.

Alaniia, M., *et al.*, 2013. Study of antioxidant activity of phenolic compounds from some species of Georgian flora. *Georgian Medical News*, 222 (222), 69–72.

Alape-Girón, A., *et al.*, 2008. Snake venomics of the lancehead pitviper *Bothrops asper*: geographic, individual, and ontogenetic variations. *Journal of Proteome Research*, 7 (8), 3556–3571.

Anderson, C., and Greenbaum, E., 2012. Phylogeography of northern populations of the black-tailed rattlesnake (*Crotalus molossus* Baird and Girard, 1853), with the revalidation of *C. ornatus* Hallowell, 1854. *Herpetological Monographs*, 26 (1), 19–57.

Avau, B., *et al.*, 2016. The treatment of snake bites in a first aid setting: a systematic review. *PLoS Neglected Tropical Diseases*, 10 (10), e0005079.

Baharuddin, N.S., Abdullah, H., and Abdul Wahab, W.N., 2015. Anti-candida activity of *Quercus infectoria* gall extracts against *Candida* species. *Journal of Pharmacy and Bioallied Sciences*, 7 (1), 15–20.

Bhide, B., *et al.*, 2011. Pharmacognostic evaluation of leaf of *Cordia macleodii*. Hook., An ethnomedicinally important plant. *Ayu*, 32 (2), 254–257.

Borja, M., *et al.*, 2018. Ontogenetic change in the venom of mexican black-tailed rattlesnakes (*Crotalus molossus nigrescens*). *Toxins*, 10 (12), 501.

Bustillo, S., *et al.*, 2009. Cytotoxicity and morphological analysis of cell death induced by Bothrops venoms from the northeast of Argentina. *Journal of Venomous Animals and Toxins including Tropical Diseases*, 15 (1), 167–174.

Cho, M.H., *et al.*, 2003. (+)-Larreatricin hydroxylase, an enantio-specific polyphenol oxidase from the creosote bush (Larrea tridentata). *Proceedings of the National Academy of Sciences of the United States of America*, 100 (19), 10641–10646.

da Silva, M., *et al.*, 2012. Anti-snake venom activities of extracts and fractions from callus cultures of *Sapindus saponaria*. *Pharmaceutical Biology*, 50 (3), 366–375.

de la Rosa, L.A., *et al.*, 2001. Modulation of cytosolic calcium levels of human lymphocytes by yessotoxin, a novel marine phycotoxin. *Biochemical Pharmacology*, 61 (7), 827–833.

Fanoudi, S., *et al.*, 2020. Milk thistle (*Silybum Marianum*) as an antidote or a protective agent against natural or chemical toxicities: a review. *Drug and Chemical Toxicology*, 43 (3), 240–254.

Gopi, K., Renu, K., and Jayaraman, G., 2014. Inhibition of *Naja naja* venom enzymes by the methanolic extract of *Leucas aspera* and its chemical profile by GC-MS. *Toxicology Reports*, 1, 667–673.

Gupta, Y.K., and Peshin, S.S., 2012. Do herbal medicines have potential for managing snake bite envenomation? *Toxicology International*, 19 (2), 89–99.

Gutiérrez, J.M., *et al.*, 2015. A call for incorporating social research in the global struggle against snakebite. *PLoS Neglected Tropical Diseases*, 9 (9), e0003960.

Gutiérrez, J.M., León, G., and Burnouf, T., 2011. Antivenoms for the treatment of snakebite envenomings: the road ahead. *Biologicals: Journal of the International Association of Biological Standardization*, 39 (3), 129–142.

Habib, A.G., and Brown, N.I., 2018. The snakebite problem and antivenom crisis from a health-economic perspective. *Toxicon: Official Journal of the International Society on Toxinology*, 150, 115–123.

Harris, J., and Scott-Davey, T., 2013. Secreted phospholipases A2 of snake venoms: effects on the peripheral neuromuscular system with comments on the role of phospholipases A2 in disorders of the CNS and their uses in industry. *Toxins*, 5 (12), 2533–2571.

Kakanj, M., *et al.*, 2015. Cytotoxic effect of Iranian Vipera lebetina snake venom on HUVEC cells. *Iranian Journal of Pharmaceutical Research*, 14 (Suppl), 109–114.

Khedrinia, M., Aryapour, H., and Mianabadi, M., 2018. Prediction of novel inhibitors for *Crotalus adamanteus* l-amino acid oxidase by repurposing FDA-approved drugs: a virtual screening and molecular dynamics simulation investigation. *Drug and Chemical Toxicology*, 31, 1–10.

Lambert, J., *et al.*, 2005. Cytotoxic lignans from *Larrea tridentata*. *Phytochemistry*, 66 (7), 811–815.

Leanpolchareanchai, J., Pithayanukul, P., and Bavovada, R., 2009. Anti-necrosis potential of polyphenols against snake venoms. *Immunopharmacology and Immunotoxicology*, 31 (4), 556–562.

León, G., *et al.*, 2013. Pathogenic mechanisms underlying adverse reactions induced by intravenous administration of snake antivenoms. *Toxicon: Official Journal of the International Society on Toxinology*, 76, 63–76.

Li, Y.G., Tanner, G., and Larkin, P., 1996. The DMACA–HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. *Journal of the Science of Food and Agriculture*, 70 (1), 89–101.

Lira-Saldívar, R.H., 2003. Estado actual del conocimiento sobre las propiedades biocidad de la gobernadora (*Larrea tridentata* (D.C.) Coville). *Revista Mexicana de Fitopatología*, 21 (2), 241–222.

Mackessy, S.P., ed. 2010. *Handbook of Venoms and Toxins of Reptiles*. Boca Raton, FL: CRC Press.

Martínez, S., González, J., and Culebras, J., M.J., 2002. Flavonoids: properties and anti-oxidizing action. *Nutricion Hospitalaria*, 17 (6), 271–278.

Martins, W., *et al.*, 2014. A novel Phospholipase A2 (D49) from the venom of the Crotalus oreganus abyssus (North American grand canyon rattlesnake). *BioMed Research International*, 2014, 654170.

Meléndez, D., *et al.*, 2014. Capillary damage in the area postrema by venom of the northern black-tailed rattlesnake (*Crotalus molossus molossus*). *Journal of Venom Research*, 5, 1–5.

Meléndez-Martínez, D., *et al.*, 2017a. *In vitro* hemotoxic, α-neurotoxic and vasculotoxic effects of the Mexican black-tailed rattlesnake (*Crotalus molossus nigrescens*) venom. *Journal of Venom Research*, 8, 1–8.

Meléndez-Martínez, D., *et al.*, 2017b. Rattlesnake *Crotalus molossus nigrescens* venom induces oxidative stress on human erythrocytes. *Journal of Venomous Animals and Toxins Including Tropical Diseases*, 23 (1), 24.

Moreno, S., *et al.*, 2011. Antifungal effect of gobernadora extracts (*Larrea tridentata* L.) on *in vitro* inhibition of *Aspergillus flavus* and *Penicillium* sp. *Polibotánica*, 32, 193–205.

Moreno-Escamilla, J.O., *et al.*, 2015. Effect of the smoking process and firewood type in the phytochemical content and antioxidant capacity of red Jalapeño pepper during its transformation to chipotle pepper. *Food Research International*, 76 (Pt 3), 654–660.

Mourão de Moura, V., *et al.*, 2014. A comparison of the ability of *Bellucia dichotoma* Cogn. (Melastomataceae) extract to inhibit the local effects of *Bothrops atrox* venom when pre-incubated and when used according to traditional methods. *Toxicon*, 85, 59–68.

Mustafa, H., Ismail, N., and Wahab, W., 2018. Anti-microbial activity of aqueous *Quercus infectoria* gall extract against pathogenic *Leptospira*. *The Malaysian Journal of Medical Sciences*, 25 (4), 42–50.

Osorio, E., *et al.*, 2010. Biological efficiency of polyphenolic extracts from pecan nuts shell (*Carya illinoensis*), pomegranate husk (*Punica granatum*) and creosote bush leaves (*Larrea tridentata* Cov.) against plant pathogenic fungi. *Industrial Crops and Products*, 31 (1), 153–157.

Panfoli, I., *et al.*, 2010. Inhibition of hemorragic snake venom components: old and new approaches. *Toxins*, 2 (4), 417–427.

Patiño, A., Benjumea, D., and Pereañez, J., 2013. Inhibition of venom serine proteinase and metalloproteinase activities by *Renealmia alpinia* (Zingiberaceae) extracts: comparison of wild and *in vitro* propagated plants. *Journal of Ethnopharmacology*, 149 (2), 590–596.

Peñuelas-Rubio, O., *et al.*, 2017. *Larrea tridentata* extracts as an ecological strategy against *Fusarium oxysporum radicis-lycopersici* in tomato plants under greenhouse conditions. *Revista Mexicana de Fitopatología*, 35 (3), 360–376.

Peralta, I., *et al.*, 2018. Food preservation by *Larrea divaricata* extract: participation of polyphenols. *Food Science & Nutrition*, 6 (5), 1269–1275.

Pérez, V., *et al.*, 2013. Extraction of phenolic compounds from lime peel (*Critrus limetta* Risso) and antioxidant activity determination. *Biotecnia*, 15 (3), 18–22.

Pithayanukul, P., *et al.*, 2005. Inhibition of *Naja kaouthia* venom activities by plant polyphenols. *Journal of Ethnopharmacology*, 97 (3), 527–533.

Prajapati, M.S., *et al.*, 2010. *Leucas aspera*: a review. *Pharmacognosy Reviews*, 4 (7), 85–87.

Ruha, A.M., *et al.*, and ToxIC Snakebite Study Group. 2017. The epidemiology, clinical course, and management of snakebites in the North American snakebite registry. *Journal of Medical Toxicology*, 13 (4), 309–320.

Sánchez, E., *et al.*, 2001. Partial characterization of a basic protein from *Crotalus ornatus* (Northern *Blacktail rattlesnake*) venom and production of a monoclonal antibody. *Toxicon*, 39 (4), 523–537.

Shabbir, A., *et al.*, 2014. Protective activity of medicinal plants and their isolated compounds against the toxic effects from the venom of *Naja* (cobra) species. *Journal of Ethnopharmacology*, 157, 222–227.

Singh, P., *et al.*, 2017. A review on venom enzymes neutralizing ability of secondary metabolites from medicinal plants. *Journal of Pharmacopuncture*, 20 (3), 173–178.

Skouta, R., *et al.*, 2018. Assessing the antioxidant properties of *Larrea tridentata* extract as a potential molecular therapy against oxidative stress. *Molecules*, 23 (7), 1826.

Slaninová, J., *et al.*, 2012. Toxicity study of antimicrobial peptides from wild bee venom and their analogs toward mammalian normal and cancer cells. *Peptides*, 33 (1), 18–26.

Soni, P., and Bodakhe, S., 2014. Antivenom potential of ethanolic extract of *Cordia macleodii* bark against *Naja* venom. *Asian Pacific Journal of Tropical Biomedidine*, 4 (Suppl 1), 449–454.

Srinivasa, V., *et al.*, 2014. Novel apigenin based small molecule that targets snake venom metalloproteases. *PLos One*, 9, e106364.

Ventura, J., *et al.*, 2008. Fungal biodegradation of tannins from Creosote Bush (*Larrea tridentata*) and Tar Bush (*Flourensia cernua*) for gallic and ellagic acid production. *Food Technology and Biotechnology*, 46 (2), 213–217.

Vyas, V. K., Brahmbhatt, K., and Parmar, U., 2013. Therapeutic potential of snake venom in cancer therapy: current perspectives. *Asian Pacific Journal of Tropical Biomedicine*, 3 (2), 156–162.

Zúñiga Carrasco, I.R., and Caro Lozano, J., 2013. Aspectos clínicos y epidemiológicos de la mordedura de serpiente en México. *Evidencia Médica e Investigación en Salud*, 6 (4), 125–136.