REVISTA DE Biología Tropical

Antimicrobial activity of four *Valeriana* (Caprifoliaceae) species endemic to the Venezuelan Andes

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Received 11-II-2018. Corrected 23-V-2018. Accepted 25-VI-2018.

Abstract: *Valeriana* L. genus is represented in Venezuela by 16 species, 9 of these are endemic of Venezuelan Andes growing in high mountains at 2 800 masl. In this investigation, four species were analyzed in order to determine the main secondary metabolites and antimicrobial activity of extracts obtained from aerial parts of *Valeriana parviflora*, *V. rosaliana*, *V. triplinervis* and *V. phylicoides*. Alkaloids, flavonoids, tannins, sterols, triterpenoids and saponins were qualitatively observed in all methanolic extracts tested. The color intensity or a precipitate formation was used as analytical response to these tests. Antimicrobial activity was evaluated against *Gram* positive, *Gram* negative bacterial strains and yeast, using disc diffusion method. *N*-hexane extracts of *V. triplinervis* and *V. rosaliana* showed the highest efficiency against *Staphylococcus aureus,* exhibiting inhibition zones of 16 mm and 15 mm; MIC (Minimal Inhibition Concentration) values were observed at 116 mg/mL and 150 mg/mL, respectively. Dichloromethane and methanolic extracts of *V. triplinervis* and methanolic extract of *V. rosaliana* showed a rather moderate activity (MIC between 200 to 316 mg/ml) but a very weak antibacterial activity was observed in *V. phylicoides* and *V. parviflora* extracts (MIC > 420 mg/mL). None of the extracts assayed in this investigation showed any activity against *Candida albicans* and *Candida krusei*. Statistical analysis showed no significant differences on the different polarity extracts assayed with respect to antimicrobial activity against *S. aureus* ($P > 0.10$), however it was observed significant differences between the *Valeriana* species analyzed ($P < 0.10$) in relation to the minimal inhibitory concentration (MIC). Rev. Biol. Trop. 66(3): 1282-1289. Epub 2018 September 01.

Key words: *Valeriana*; antimicrobial activity; phytochemical; Venezuela.

Valeriana genus (Caprifoliaceae, *subf*. Valerianoideae) comprises about 281 species distributed around the world being a representative genus for South America Andes (Bell & Dologhue, 2005). In Venezuela, 16 species have been reported, nine of these are endemic from the Andean Paramo (Xena, 1993). Regarding traditional medicine, *Valeriana* species are widely used by its sedative, hypnotic, anxiolytic and antidepressant properties. Thus, many phytomedicinal products based on *Valeriana officinalis* L. extracts or powder are commercialized as capsules, tablets and tinctures, especially from the roots of this species (Patoĉka & Jakl, 2010). Due to the frequent use of these commercial products, *V. officinalis*, has been subject to numerous investigations in order to establish the chemical composition and pharmacological activity, particularly in central nervous system. Valepotriate sesquiterpenes and other compounds present in essential oil obtained from *V. officinalis* might also be

responsible for this activity (Houghton, 1988; Houghton, 1998; Miyasaka & Soares 2006; Celis, Rincón, Guerrero, 2007). Recently, a group of researchers reported that antidementia activity attributed to sesquiterpenes and monoterpenes isolated from *V. officinalis* var. *latiofolia* mediate its action probably through acetylcholinesterase inhibition (Chen et al., 2016). Neuroprotective activity of *V. wallichii* and *V. jatamansi* with effect against Parkinson´s disease has also been observed (Sridharan et al., 2015; Tan et al., 2016).

Cytotoxic, antioxidant, antispasmodic, anthelmintic and antibacterial activity were also detected on extracts obtained from some *Valeriana* species (Gilani, Khan, Jabeen, Subhan, & Ghafar, 2005; Sati, Khulbe, & Joshi, 2011; Potdar, Lole, & Patil, 2011; Bhatt et al., 2012; Aydin, Dikmen, & Kismali, 2016). Present investigation; aim the study of four endemic species, *Valeriana parviflora* (Trevis) Höck; *V. rosaliana* F.G.Mey.; *V. triplinervis* (Turcz.) Briq., and *V. phylicoides* (Turcz.) Briq., with restricted distribution in Mérida and Táchira states from Venezuela. These four species are shrubs that grow in high mountains at 2 800 masl, have a strong odour, characteristic of this genus, and are used by local people as insect repellent, to aid sleep alterations and stomach disorders. Previously, our research group reported the chemical composition of *V. parviflora* essential oil collected in two different season of the year (rainy and dry). Linalool (11.9 %) and eugenol (8.9 %) were the major constituents in dry season, while o-xylol (16.2 %) and 3-methyl isovaleric acid (10.6 %) were the main compounds observed in the oil obtained in rainy season (Fernández, Rondón, Rojas, Morales, & Rojas-Fermin, 2015).

The aim of this investigation is to determine qualitatively the presence of several secondary metabolites and to evaluate antimicrobial activity against *Gram* positive, *Gram* negative bacterial strain and yeasts of four *Valeriana* species. To the best of our knowledge there are no previous reports on this matter, thus, results are considered a contribution to the natural products investigation.

MATERIALS AND METHODS

Plant material: Fresh aerial parts of four endemic *Valeriana* L. species were collected from the Venezuelan Andes. *Valeriana parviflora* (Trevir) Hoeck (February 2015; Páramo Piedras Blancas, Mérida, 8°51'31'' N - 71°57'06'' W); *V. phylicoides* (Turcz.) Briq (June 2015; Páramo El Rosal, Táchira, 8°0'36.72'' N & 71°58'53.76'' W); *V. triplinervis* Turcz (October 2015; Sierra Nevada de Santo Domingo, Mérida, 8°48'6'' N & 70°49'40'' W) and *V. rosaliana* Meyer (May 2012, La Fría, Táchira; 8°06'52.4'' N - 71°54'57.6'' W). Botanical identification was carried out by Ing. Luis Enrique Gámez, Herbarium MER, Faculty of Forestry and Environmental Sciences, University of Los Andes. Voucher specimens were deposited under the following codes: *V. parviflora* (MERR 02); *V. phylicoides* (986) and *V. rosaliana* (MERR 01) in Herbarium MERF, Faculty of Pharmacy and Bioanalysis, University of Los Andes. *V. triplinervis* (945458) was deposited at Herbarium MER, Faculty of Forestry and Environmental Sciences.

Extract preparation: Fresh aerial parts of *Valeriana parviflora* (400 g), *V. rosaliana* (640 g), *V phylicoides* (100 g) and *V. triplinervis* (350 g) were dried using a stove at 40 °C for 3 days, and then pulverized. Crude plant extracts were prepared by maceration at room temperature using 1 L of each different polar solvents of increasing polarity [*n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate and methanol (MeOH)]. Solvents used for extractions were obtained from Merck. All extracts were filtered through a cotton plug followed by Whatman filter paper number 1 and concentrated under reduced pressure at 40 °C using rotary evaporator. Dried extracts were kept under refrigeration at 4 ºC until phytochemical and microbiological analyses were performed.

Phytochemical Screening: Crude extracts were phytochemically evaluated to determine the presence of chemical constituents using

standard procedures described by Harbone (Harbone, 1973).

Antimicrobial method: The microorganisms and yeast used for the antimicrobial method were *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25992), *Klebsiella pneumoniae* (ATCC 23357), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (DCCB 385) and *Candida krusei* (ATCC 6258).

The antimicrobial activity was carried out according to the disc diffusion assay described by (Velasco et al., 2007). The strains were maintained in agar conservation at room temperature. Each bacterial inoculum was incubated in 2.5 mL Müeller-Hinton broth (BBLTM®) at 37 ºC for 18 hours. The bacterial inoculum was diluted in sterile 0.85 % saline to obtain turbidity visually comparable to McFarland Nº 0.5 standard (10^{6-8} CFU/mL). Every inoculum was spread over plates containing Müeller-Hinton agar. Paper filter discs (6 mm) saturated with 20 µL of every extract (*n*-hexane, dichloromethane, ethyl acetate and methanol) of *Valeriana parviflora*, *V. rosaliana*, *V. triplinervis* and *V. phylicoides* diluted 1:10 ratio with the same solvents used for each extract, were placed over the plates. These were preincubated at 4 ºC for 18 h and finally incubated at 37 ºC for 16-18 h. Antifungal activity was also evaluated following the disc diffusion methodology described by National Committee for Clinical Laboratory Standards (NCCLS, 2004). Twenty mL Müeller-Hinton agar (BBLTM®) supplemented with glucose (2 %, w/v) and methylene blue (0.5 µg/mL) were mixed with 1 mL of each yeast inoculum and turbidity was adjusted to McFarland Nº 1 ($3x10^8$ CFU/mL) standard. The content of Petri dishes was allowed to solidify at room temperature and sterile control was also prepared. The inhibitory zone around the disc was measured and expressed in mm. A positive control was also assayed to check the sensitivity of the tested organisms using the following antibiotics: Oxacillin® (10 µg), Vancomycin® (30 µg), Tobramycin® (30 µg), Aztreonam® (10 µg), Cefepime® (75 µg), Ceftazidime® (30 µg), Fluconazole® (100 µg) and Voriconazole® (400 µg/mL). A negative control was also included in the test using a filter paper disc saturated with *n*-hexane, dichloromethane, ethyl acetate and methanol to discard any activity of these solvents against the microorganism assayed. The experiments were repeated at least twice. MIC was determined by dilution of each extract in *n*-hexane, dichloromethane, ethyl acetate and methanol by pipetting 10 µL of each dilution onto a filter paper disc. Dilutions of the extracts within a concentration range of 100-900 mg/mL were also carried out. Minimal inhibitory concentration was performed only on samples that showed growth inhibition and was defined as the lowest concentration that inhibited the visible bacterial growth (NCCLS, 2004).

Statistical analysis: One-way analysis of variance (ANOVA) was carried out to determine whether there is significant difference for antimicrobial activity either on the extracts or within the *Valeriana* species under investigation. In event of finding any significant difference, further analysis will be performed by using the Duncan´s multiple ranges test. Significance level has been established at $\alpha = 0.10$.

RESULTS

Phytochemical screening: In order to qualitatively identify the presence of main chemical constituents present in *Valeriana* L., species from Venezuelan Andes, a screening of crude methanolic extracts obtained from four *Valeriana* species were carried out. Results (Table 1) showed mainly presence of alkaloids, flavonoids, sterols, triterpenoids, saponins and tannins.

Antimicrobial activity: Antimicrobial activity was performed for *n*-hexane, dichloromethane, ethyl acetate and methanol extracts of *Valeriana parviflora*, *V. rosaliana*, *V. triplinervis* and *V. phylicoides* against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*,

TABLE 1
Phytochemical screening of methanolic extracts obtained from four *Valeriana* L. species endemic of Venezuelan Andes

Sample	Alkaloids			Flavonoids		Sterols and triterpenes			Saponis	Tannins
	DR	WR	MR	NaOH	PR	KR	L-B R	SR	FR	$FeCl_3$
V. parviflora	+++	++	++	+++	+++	++	+++	+++	+++	+++
V. rosaliana	+++	++	++	++	++	++	+++	+++	+	+++
V triplinervis	+++	++	++	+++	++	++	+++	+++	+	+++
V. phylicoide	+++	++	++	++	+++	+	+++	+++	+	+++

+: less abundant; ++: medium; +++: high abundance; -: absence. DR: Dragendorff reagent; WR: Wagner reagent; MR: Mayer reagent; NaOH 10% solution; Pews reagent; KR: Komarowsky reagent; LB R: Lieberman- Bouchard reagent; SR: Salkowky reagent; FR: Foam reagent; $FeCl_3$ 5 % solution.

Pseudomonas aeruginosa, *Candida albicans* and *Candida krusei*. All extracts showed effectiveness against only *Gram* positive bacterial strain, *S. aureus*. Results are shown in table 2 and table 3. Statistical analysis showed no significant differences on the different polarity extracts assayed with respect to antimicrobial activity against *S. aureus* ($P > 0.10$).

DISCUSSION

Nearly 150 different compounds have been found in *Valeriana* genus (Backlun & Moritz, 1998). Details on some of these compounds are given in various phytochemical investigations. For instance, phenolic compounds such as tannins and flavonoids isolated as linarin and hesperidine with activity on the Central Nervous System has been identified from some *Valeriana* species (Marder et al., 2003; Fernández, Wasowski, Paladini, & Marde, 2004). It is important to state that, these components are observed in all samples analyzed, in present investigation, by using $FeCl_3$ 5 %, NaOH 10 % and Pew´s reactive, respectively. Furthermore, *Valeriana* genus is recognized mainly for the presence of alkaloids (pyridine derivatives) and terpenoids (iridoids-monoterpenes and valepotriates) as the major chemical constituents found on extracts of these species (Jiang, Zhang, Liu & Fang, 2007; Zhou, Fang, Gong, Duan, & Liu, 2009; Patoĉka & Jakl, 2010). Regarding alkaloids, actinidine, chatinine, valerianine and valerene, are the most common isolated from this genus. Investigations have pointed these alkaloids as responsible for the psychoactivity observed in *Valeriana officinalis* L., traditionally used to treat sleeping disorders and anxiety. Researchers believe that, those alkaloids may act through an interaction with GABA and benzodiazepine receptors (Miyasaka & Soares, 2006). In present study, abundant presence of alkaloids were observed in all samples assayed, thus, these species are considered interesting for further investigations related to the sleep inducer effect. Other secondary metabolites present in *Valeriana* extracts are saponins, triterpenes and steroids (Backlun & Moritz, 1998).

Regarding antibacterial activity, according to results (Table 2 and Table 3), all extracts assayed showed growth inhibition against *S. aureus*, which is responsible for a number of serious human infectious diseases (Tong et al., 2015; Blomfeldt, Eskesen, Aamot, Leegaard, & BiØrnholt, 2016) with MIC values ranging between 150 to 500 mg/mL. The most active extract was *n*-hexane from *V. triplinervis* showing the highest inhibition zone (16 mm), MIC value of 116 mg/mL, followed by *V. rosaliana* (15 mm); with 150 mg/mL; while the lowest activity was observed for *V. parviflora* and *V. phylicoides* (inhibition zone < 11 mm).

Furthermore, dichloromethane extract from *V. triplinervis* showed an inhibition zone of 15 mm with MIC value of 200 mg/mL. However, same solvent extracts from *V. rosaliana* and *V. phylicoides* exhibited a rather poor activity against *S. aureus* while *V. parviflora* showed no activity at all. Ethyl acetate extracts exhibited the lowest antibacterial activity

TABLE 2
Antimicrobial activity of the four *Valeriana* crude extracts, endemic species of Venezuelan Andes

Microorganism	Inhibition Zone (mm)																Inhibition Zone (mm)							
	V. parviflora				*V. rosaliana*				*V. phylicoides*				*V. triplinervis*				Reference compounds							
	HE	DE	EAE	ME	HE	DE	EAE	ME	HE	DE	EAE	ME	HE	DE	EAE	ME	OX	VA	T	AZT	FEP	CAZ	FL	VOR
S. aureus ATCC (25923)	8	-	-	11	15	8	8	12	7	8	8	9	16	15	-	14	22							
E. faecalis ATCC (29212)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		23						
E. coli ATCC (25922)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			20					
K. pneumoniae ATCC (23357)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				33				
P. aeruginosa ATCC (27853)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-					36	31		
Candida albicans DCCB 385	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-							36	
Candida krusei ATCC 6258	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-								22

- : no activity found; HE: *n*-Hexane extract; DE: Dichloromethane extract; EAE: Ethyl Acetate Extract; ME: Methanol extract OX: Oxacillin® (10 µg); VA: Vancomycin® (30 µg); T: Tobramycin® (30 µg); AZT: Aztreonam® (10 µg); FEP: Cefepime® (75 µg); CAZ: Ceftazidime® (30 µg). FL: Fluconazole® (100 µg) y VOR: Voriconazole® (400 µg/mL). *Inhibition Zone, diameter measured in mm, disc diameter 6 mm, average of two consecutive assays.

TABLE 3
Minimal inhibitory concentration of four *Valeriana* species crude extracts

Microorganism	*V. parviflora*				*V. rosaliana*				*V. phylicoides*				*V. triplinervis*			
S. aureus ATCC 25923	HE	DE	EAE	ME	HE	DE	EAE	ME	HE	DE	EAE	ME	HE	DE	EAE	ME
	500	-	-	500	150	500	460	300	480	440	420	500	116	200	-	316

- : no activity found; HE: *n*-Hexane extract; DE: Dichloromethane extract; EAE: Ethyl Acetate Extract; ME: Methanol, MIC: Minimal inhibition concentration (mg/mL).

against *S. aureus* since only *V. rosaliana* and *V. phylicoides* extracts showed activity with MIC values over 400 mg/mL. Finally, methanol extract of *V. triplinervis* showed an inhibition zone of 14 mm, at MIC of 316 mg/mL. None of the extracts assayed showed any activity against the two yeasts tested.

According to statistical analysis, significant difference was observed between the four *Valeriana* species analyzed ($P < 0.10$) with respect to the minimal inhibitory concentration (MIC) values against *S. aureus*. Furthermore, Duncan´s multiple ranges test probed that two groups, not clearly separately, are formed within these species; *parviflora-phylicoides* and *triplinervis-rosaliana,* being the second group that exhibited the best antibacterial activity against *S. aureus*.

Previous investigations on antimicrobial activity of *Valeriana* species have revealed that methanolic and aqueous extracts show the strongest activity, suggesting that polar compounds might be responsible for such activity (Sati et al., 2011). Additionally, essential oils isolated from different *Valeriana* species have shown significant activity against a wide spectrum of bacterial strains (Tzakou, Couladis, Pavlovic, & Sokovic, 2004; Wang et al., 2010). This activity may be attributed to secoiridoids and secoiridoids glucosides frequently found in *Valeriana* species that have exhibited antimicrobial properties (Nadinic et al., 2002), as well as to flavonoids and tannins presents in the extracts (Table 1).

Evidence on the presence of secondary metabolites such as alkaloids, flavonoids, tannins, steroids, terpenoids (valepotriates and baldrinal) have been observed in *Valeriana parviflora*, *V. rosaliana*, *V. triplinervis* and *V. phylicoides* extracts. Regarding antibacterial activity, all extracts showed effectiveness against only Gram-positive bacterial strain, *S. aureus*, which is responsible for a number of serious human infectious diseases. This investigation, aims to provide value scientific information that might be useful for further studies on the therapeutic potential of these species, growing at Venezuelan Andes.

ACKNOWLEDGMENTS

Authors wish to thank CDCHT, Universidad de Los Andes (FA-528-12-08) and FONACIT (2013001838) for the financial support.

RESUMEN

Actividad antimicrobiana de cuatro especies de *Valeriana* (Caprifoliaceae) endémicas de los Andes venezolanos. El género *Valeriana*, está representado en Venezuela por 16 especies, 9 de las cuales son endémicas de Los Andes y crecen en las altas montañas a 2 800 msnm. En esta investigación cuatro especies fueron analizadas para determinar los principales metabolitos secundarios y la actividad antimicrobiana de los extractos obtenidos de las partes aéreas de *Valeriana parviflora*, *V. rosaliana*, *V. triplinervis* y *V. phylicoides*. Compuestos como alcaloides, flavonoides, taninos, estroles, triterpenos y saponinas fueron detectados cualitativamente en todos los extractos metanólicos ensayados. La intensidad del color o la formación de un precipitado se tomaron como respuesta positiva para estos análisis. Actividad antimicrobiana fue evaluada frente a bacterias Gram positivas, Gram negativas y levaduras, usando el método de difusión en discos. Los extractos en *n*-hexano de *V. triplinervis* y *V. rosaliana* mostraron la mayor eficiencia frente a *Staphylococcus aureus,* mostrando zonas de inhibición de 16 mm y 15 mm con valores de CIM (Concentración Inhibitoria Mínima) observados a 116 mg/mL y 150 mg/mL, respectivamente. Los extractos con diclorometano y metanol de *V. triplinervis* y metanol de *V. rosaliana* revelaron moderada actividad (CIM entre 200 y 316 mg/ml), mientras actividad muy leve se observó en los extractos de *V. phylicoides* y *V. parviflora* (CIM > 420 mg/mL). Ninguno de los extractos ensayados mostraron actividad frente a *Candida albicans* y *Candida krusei*. Los análisis estadísticos mostraron que no hay diferencia significativa entre los solventes de diferentes polaridades con relación a la actividad antimicrobiana frente a *S. aureus* ($P > 0.10$), sin embargo, sí se observó diferencia significativa ($P < 0.10$), entre las especies de *Valeriana* ensayadas con respecto a la concentración inhibitoria mínima (CIM).

Palabras clave: *Valeriana*; actividad antimicrobiana; fitoquímica; Venezuela.

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