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Toxicity, antimicrobial activity and detection of xanthones in *Gentianella nevadensis*

Doris Hidalgo Báez[1]*, Maribel Quintero[1], Beatriz Nieves[2], Carmen de los Ríos[1], Quilianio Contreras[1] and Javier Estrada[3]

[1]Departamento de Química, Facultad de Ciencias.
[2]Escuela de Bioanálisis, Facultad de Farmacia. [3]Centro Jardín Botánico, Facultad de Ciencias, Universidad de Los Andes. Mérida, Venezuela

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Abstract

The species *Gentianella nevadensis* (Gilg) Weaver & Rudenberg (Gentianaceae), is known as "dictamo real" in Mérida where a legend is found which speaks about its rejuvenation attribute. A decoction or an alcoholic beverage of the roots and aerial parts of *G. nevadensis* is used as a tonic, analgesic, to regulate the circulation, as an aphrodisiac and as a stomachic. This plant was screened for toxicity using the brine shrimp: *Artemia salina* bioassay and antimicrobial activity. The detection of xanthones was also carried out by HPLC coupled with a UV photodiode-array. All crude extracts except the ethanol ones showed a low toxicity against *Artemia salina*. The highest toxicities were observed in some fractions of dichloromethane and ethyl acetate extracts. Ethyl acetate extract and its fractions were the only ones with antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis*. Xanthones were detected in dichloromethane and ethyl acetate extracts and their fractions. Bellidifolin and desmethylbellidifolin were isolated, both have been reported as inhibitors of MAO. The results of this study suggest that *G. nevadensis* could be a possible antidepressive and anti-infectious agent.

Keywords: Antibacterial activity; ethnopharmacology; Gentianaceae; *Gentianella nevadensis*; toxicity; xanthones.

Toxicidad, actividad antibacteriana y detección de xantonas en *Gentianella nevadensis*

Resumen

La especie *Gentianella nevadensis* (Gilg) Weaver & Rudenberg (Gentianaceae), se conoce como "díctamo real" en Mérida, donde existe la leyenda sobre sus atributos rejuvenecedores. Una decocción o maceración en aguardiente, de las raices y partes aéreas de *G. nevadensis* se usa como un tónico, analgésico, para regular la circulación, como un afrodisiaco y un estomacal. A esta planta se le evaluó su toxicidad utilizando el bioensayo de *Artemia salina*, y su actividad antibacteriana. La detección de xantonas se llevó a cabo por HPLC acoplado con un detector de fotodiodo. Todos los extractos crudos, excepto el de etanol, mostraron una baja toxicidad contra *Artemia salina*. La mayor toxicidad se observó en algunas fracciones de los extractos de diclorometano y acetato de etilo. El extracto de acetato de etilo y sus fracciones fueron las úni-

* To whom correspondence should be addressed.

cas que mostraron actividad antibacteriana contra *Staphylococcus aureus* y *Bacillus subtilis*. En los extractos de diclorometano y acetato de etilo, y sus fracciones, se detectaron xantonas. Se aislaron la bellidifolina y la desmetilbellidifolina, ambas son reportadas como inhibidoras de la MAO. Los resultados de este estudio sugieren que *G. nevadensis* podría ser un posible agente antidepresivo y anti-infeccioso.

Palabras clave: Actividad antibacteriana; etnofarmacología; Gentianaceae; *Gentianella nevadensis*; toxicidad; xantonas.

Introduction

Our interest in the phytochemical study of medicinal plants of the Andes region at Mérida-Venezuela led us to carry out an ethnobotanical evaluation of these plants.

Based on ethnomedical information *Gentianella nevadensis* (Gild) Weaver & Rudenberg (Gentianaceae) was selected, known from the Páramos of Venezuelan Andes, Oriental Cordillera of Colombia to the North of Ecuador (1), this is a very variable species in size, type of branch, size and form of the leaves and size and colour of the flowers. Because of this variety we consider it is necessary to report a detailed description of the specimen collected for this study.

Gentianella Moench in Engler Bot. Jahrb. 54 (beibl. 118): 4-89 (1916) is a subcosmopolitan genus that comprises about 250-300 species, perhaps the centre of diversity is in the Andean region (2). Several authors have been studying these species in some South America countries but there is not a monograph of the complete genus.

Placed in the family Gentianaceae Jussieu, *Gentianella* was formerly included in *Gentiana L.* The principal exomorphological features of this genus, are: Calyx without a membrane in the adaxial surface; corolla without interlobular plicas, lobules 5-9 nerved and nectaries in the base of the tube and versatile anthers (3).

In Venezuela three species have been reported: *Gentianella nevadensis* (Gilg) Weaver & Rudenberg, *G. corymbosa* (HBK.) Weaver & Rudenberg and *G. viridis* (Griseb.) Weaver & Rudenberg, all of them in the Andean States of Mérida, Táchira and Trujillo, collected at Páramo vegetation, at altitudes between 3.000-4.000 m.

The following description was made considering only the specimens collected in the Mérida State, including the voucher specimen.

Gentianella nevadensis (Gilg) Weaver & Rudenberg, J. Arnold Arbor. 56(2): 215 (1975).

Gentiana nevadensis Gilg in Engler Bot. Jahrb. 22: 313 (1896) [Basionym].

Herb monocarpic, biennial, tap-rooted. Stems forming a tuft; the central erect and flowering, the lateral ones decumbent and may be flowering, it seems to be stoloniferous. 10-15 cm long, quadrangular or terete in section, ca. 0.5 mm in diameter, narrowly 4-winged; internodes longer than leaves upwards, shorter to the base, glabrous. Leaves opposite decussate, sessiles, lanceolate to narrowly elliptic or narrow oblanceolate, psudospathulate toward the base of the stems, 12-20 x 1.5-6 mm, apices acute or obtuse, base subsheathing toward the inflorescence and pseudopetiolate to the base of the stem, lamina smooth or papillous and rough when dried. Inflorescence in a terminal dichasium more or less dense, bracteate, 3-6 flowers. Calyx campanulate, 0.6-1.5 cm long, glabrous, lobules 3-nerved, 4-7 mm long, 1.5-2.5 mm broad at the base, acute to obtuse, margin hyaline, Corolla campanulate, whitish or violaceous, 1.1-1.5 x 0.7 cm; lobules 6.5- 11 x 4.5, elliptic to obovate; nectaries like small bags at the

base of the tube, 0.3 mm diameter or inconspicuous. Anthers elliptic, in the voucher specimen bluish, 1-1.2 x 0.4-0.6 mm; filaments 5-8 x 0.4-0.5 mm. Ovary long stipitated, ginophore 2-4.5 mm long; ovary oblong, 4.5-6 x 2 mm, smooth or rugose, green or bluish tinged in the upper third; stigmas 2, distinct, ca. 0.5 mm.

In order to screen biologically active compounds, antimicrobial and brine shrimp toxicity assays were selected. Xanthones are common phenolics compounds occurring in Gentianaceae. Their activity as inhibitors of monoamine oxidase (MAO) and their characteristic as chemotaxonomic markers (4,5) led us to investigate their occurrence in *G. nevadensis*.

We report here the antibacterial activity, brine shrimp toxicity, detection of xanthones in extracts and fractions, and identification of two isolated xanthones. The results of this work give information which will be useful to continue our investigation in this species.

Materials and Methods

Ethnobotanical evaluation

The ethnobotanical evaluation was accomplished in the herbolaria of the popular markets of Mérida city. It was conducted by mean of interviews with herbalists and a review of the literature. The interviews had been carried out following a pre-printed form designed to record the information. The local names, uses, plant part used, mode of preparation and methods of its administration, were recorded. *Gentianella nevadensis* as well as all the medicinal plants of the popular markets are resold by vendor, therefore a field work was done in the locality of the plants.

Plant collection

A sample of *G. nevadensis* was collected in each herbolarium and all the samples are deposited (DH & MQ2H1) in the Herbarium MERC of the Centro Jardín Botánico, Facultad de Ciencias, Universidad de Los Andes. Another samples were collected by J. Estrada, J.C. Gaviria and D. Hidalgo during the field work carried out in the Páramo de Los Conejos, where Omar Quintero collects *G. nevadensis* and distributes it in many market herbolaria. A voucher specimen (Estrada, J., Gaviria, J.C. & Hidalgo, D. 946) is kept at the Herbarium MERC. The taxonomic identity was established by Dr. Javier Estrada, Centro Jardín Botánico, Universidad de Los Andes.

Preparation and fractionation of the extracts

The dried and powdered whole plant (952.7 g) was extracted with solvents of different polarities, using reflux at 40°C for 1 h. The extracts were obtained with hexane (12.16 g), dichloromethane (19.33 g), ethyl acetate (49.1 g), ethanol (200.98 g) and aqueous ethanol (1:1) (30.01 g).

The dichloromethane extract was fractionated over a silica gel vacuum column using hexane as solvent followed by gradient addition of ethyl acetate up to 100% and MeOH : H2O (50%). Seven collective fractions were obtained after TLC analysis. The ethyl acetate extract (20.0 g) was fractionated under the same conditions as described for the CH_2Cl_2 extract. After TLC analysis eleven fractions were collected. A precipitate was formed in fraction 5. This precipitate (0.6 g) was separated through a silica gel vacuum column and one of the sub-fractions was separated by TLC in silica gel plates. Bellidifolin (15.0 mg) and desmethylbellidifolin (10.0 mg) were isolated. Their structures were established by comparation of the spectral data (UV, ^{1}H and ^{13}C NMR) with those reported in the literature (6-9).

Detections of xanthones

A rapid qualitative survey of xanthones in the crude extracts and their fractions can

be done using liquid chromatography with UV photodiode-array detection (10-12).

The extracts of CH_2Cl_2 and EtOAc, and their fractions as well as the ethanol extract were analyzed by HPLC on a µ-bondapak C18 column (10 µm, 3.9×300 mm, Waters) at a flow rate of 0.75 or 1.0 mL/min using MeOH or MeOH : H2O (80%) as solvents. The instrument was equipped with a UV photodiode array detector.

Brine shrimp lethality bioassay

The toxicity was tested using the brine shrimp (Artemia salina) assay described by Meyer (13). All samples were prepared dissolving 20 mg of the sustance in 2 mL of the solvent. From this solution 500, 50 and 5µL were transferred to vials corresponding to the final concentrations of 1000, 100 and 10 µg/mL, respectively. After the solvent was evaporated 5 mL of sea water and 10 shrimps were added. DMSO was used when it was necessary. 24 h later the number of survivors nauplii was counted. Data analysis were done with Stephan's program (14) to determine LC_{50} values and 95% confidence intervals. All crude extracts and the fractions obtained from CH_2Cl_2 and EtOAc extracts were tested.

Antimicrobial assay

Antimicrobial evaluation of all crude extracts and the fractions obtained from CH_2Cl_2 and EtOAc extracts was performed using the disc-diffusion assay (15). The strains of microorganisms employed were: Staphylococcus aureus (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (Cepario de la Escuela de Bioanálisis, Universidad de Los Andes) and *Candida albicans* (clinical origin). Paper discs (10 mm) were impregnated with 25 µL of the sample solution (40 mg/mL) and wetted with the solvents used to prepare the solutions. The plates were kept at 4°C for 5 h to allow diffusion. Afterwards, samples were incubated at 37°C for 17 h. The zone of inhibition was measured in mm. All assays were done in duplicated.

Results

The results of ethnobotanical evaluation reveal that *Gentianella nevadensis* is known as "dictamo real" in the herbolaria of the popular markets of Mérida city. The interviews confirm that there is a legend which speaks about its rejuvenation attributes, that is also reported in the literature (16).

A decoction or an alcoholic beverage ("aguardiente" or wine) of the whole plant (7 g/L) is used as a tonic, analgesic, to regulate the circulation, as an aphrodisiac and as a stomachic. The recommended dosis is 100 mL once a day. Contraindications were not reported.

The crude extracts showed a median lethal concentration greater than 1000 ppm toward the brine shrimp. Only the EtOAc extract had antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis*. None of other microorganisms was affected. Xanthones were detected in CH_2Cl_2 and EtOAc extracts.

Bioassay-guided fractionation of CH_2Cl_2 extract indicated that the second and third fractions had the highest toxicity against *A. salina* (LC_{50} 31.11 ppm and 232.63 ppm, respectively). HPLC analysis revealed that these fractions have three identical xanthones. Fraction 4 (LC_{50} > 1000 ppm) has another three xanthones (Table 1).

The results of the median lethal concentrations against *A. salina*, antibacterial activity and detection of xanthones in ethyl acetate fractions are shown in Table 2. The lowest values of median lethal concentrations were found in the fractions 4, 5 and 6 (72.97, 216.33 and 746.79 ppm, respectively). A precipitate was formed in the fractions 5 and 6. These precipitates had LC_{50} lower than their original fractions (42.99 and 263.75 ppm, respectively). Xanthones

Table 1
Results of the median lethal concentrations for *Artemia salina* and detection of xanthones in dichloromethane fractions of *Gentianella nevadensis*

Fractions	LC_{50} (24h) ppm	Xanthones
1	> 1000	-
2	31.11	+
3	232.63	+
4	> 1000	+
5	> 1000	-
6	> 1000	-
7	> 1000	-

Table 2
Results of the median lethal concentration for *Artemia salina*, antibacterial activity and detection of xanthones in ethyl acetate fractions of *Gentianella nevadensis*

Fractions	LC_{50} (24 h) ppm	Zone of Inhibitions (mm)		Xanthones
		S. aureus	*B. subtilis*	
1	> 1000	*	*	-
2	> 1000	*	*	-
3	> 1000	13	15	+
4	72.97	16	20	+
5	216.33	14	16	+
6	746.79	11	17	+
7	> 1000	14	16	-
8	> 1000	16	16	-
9	> 1000	11	11	-
10	> 1000	*	*	-
11	> 1000	13	*	-

*Antibacterial activity was not observed.

were detected from the fraction 3 to 6. There was one xanthone which was found only in the fraction 3. One other xanthone occurred in the fraction 4 and it is also present in the fraction 5 besides another two xanthones, which were detected in the fraction 6 as well as in the precipitates of fractions 5 and 6. They were indentified as bellidifolin and desmethylbellidifolin. By comparation of chromatograms and UV spectra their presence was observed in the fractions 2 and 3 of CH_2Cl_2 extract.

The results of HPLC analysis suggest the presence of eight xanthones in *G. nevadensis*, two of them were isolated and identified. All these xanthones exhibit an absorption maximum between 274-278 nm in the UV spectra.

Discussion

The tonic, analgesic and stomachic uses attributed to *G. nevadensis* also have been assigned to some species of a closely related genus *Gentiana* (17-19). As the xanthones bellidifolin and desmethylbellidifolin have been isolated from other species of the genus *Gentianella*, their presence in the studied species corroborates its taxonomic identity. They are inhibitors of monoamine oxidase *in vitro* (20), therefore *Gentianella nevadensis* could be a possible antidepressive agent.

The activity against *Staphylococcus aureus* and *Bacillus subtilis* suggests that this species has antibacterial compounds, possibly effective for infectious disease. More experiments have to be done in order to give evidence on the antidepressives and antiinfectious properties suggested in the present work.

Two identified xanthones were present in all fractions which showed toxicity against *A. salina*, therefore, according to these results it may be concluded that the brine shrimp bioassay-guided fractionation was useful for isolating biologically active xanthones.

To our knowledge, this is the first report on ethnomedical information, biological activities and chemistry of *Gentianella nevadensis*.

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