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Journal of Ethnopharmacology 141 (2012) 964–974

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

A comparison on the metabolic profiling of the Mexican anxiolytic and sedative plant *Galphimia glauca* four years later

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ARTICLE INFO

Article history:
Received 11 November 2011
Received in revised form 7 March 2012
Accepted 18 March 2012
Available online 27 March 2012

Keywords:
Galphimia glauca
Metabolic profiling
Sedative
Anxiolytic
Anti-inflammatory
Triterpenes

ABSTRACT

Ethnopharmacological relevance: Galphimia glauca has a long traditional use, and continues to be used in Mexico as a natural tranquilizer for the treatment of Central Nervous System disorders as well as for other illnesses.
Aim of the study: In 2005 the initial use of metabolic profiling to populations of *Galphimia glauca* resulted in two of the six collected populations being producers for galphimines, the markers for sedative and anxiolytic activities. The aim of this investigation was to confirm the previously established metabolic profile, as well as the previous *in vivo* results on mice. Additionally in this study we wanted to investigate potential anti-inflammatory properties.
Materials and methods: Four years later, we collected samples in the five localities designated for the first-stage investigation in 2005, and in two new locations. Metabolic profiling was carried out by means of ^{1}H NMR spectroscopy and multivariate data analysis applied to crude extracts from wild plant specimens. HPLC analysis was performed to confirm and quantify the presence of galphimines. Two neuropharmacological *in vivo* assays on mice were employed to study anxiolytic (elevated plus maze test) and sedative (sodium pentobarbital-induced hypnosis model) activities in the extracts. Anti-inflammatory activity was determined by using the tetradecanoylphorbol acetate-induced mouse ear inflammation model (TPA).
Results and conclusions: The results for the 2009 collected species were similar to the 2005 collection, confirming the metabolic profiles and that galphimines are consistent good markers for CNS activity. Galloylquinic acid levels varied between the years without, as of yet, known effects. *In vivo* anti-inflammatory activity was similar for all plants and thus not linked with galphimines, requiring further studies to identify the active compound(s). Areas of collection affect neuropharmacological activities but not anti-inflammatory action.

1. Introduction

Galphimia glauca (Cav.) Kuntze is a member of the tropical and sub-tropical Malpighiaceae family that grows throughout most of México and is frequently called "calderona amarilla" (Rzedowski and Equihua, 1987; Argueta-Villamar, 1994). This plant has a wide popular use as a tranquilizer for anxiety (Estrada, 1985, 1990), as well as for a variety of other ailments (Diaz, 1976; Linares et al., 1994).

Since prehispanic times *Galphimia glauca* has been used to lower fever, and during the colonial period Francisco Hernandez also described its use to help women in labour, and those with indigestion and dysentery (Hernández, 1959). Maximino Martinez, who gathered information for the Mexican medicinal herbarium during 1920–1950, reported its employment as an emollient for injuries (Martínez, 1969). Its documented uses for asthma, allergies, diarrhoea, dysentery, gastroenteritis and malaria (Diaz, 1976), as well as for heart pain (Linares et al., 1994) also exist. Additional documentation includes treatment for wounds, pimples, postpartum pain, uterus inflammation as well as inflammations in general (Gómez and Chong, 1985; Ortíz, 1986).

Even though *Galphimia glauca* is widely distributed in Mexico, scientific investigation on this species has been limited to populations growing in specific localities. Alcoholic extract of *Galphimia glauca* showed antiallergic activity in rodents due to tetragalloylquinic acid and other compounds (Neszmélyi et al., 1993).

Abbreviations: MC, Cuernavaca, Morelos; MT, Tepoztlan, Morelos; GM, Doctor Mora, Guanajuato; JG, Guadalajara, Jalisco; QJ, Jalpan, Queretaro; CT, Tuxtla Gutierrez, Chiapas; MM, Miacatlan, Morelos.

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doi:10.1016/j.jep.2012.03.033

Fig. 1. Map of the Mexico with sample collection locations for the year 2009.

Methanolic extracts inhibit acute bronchial reactions to allergens and platelet-activating factor (PAF) in guinea pigs (Dorsch et al., 1992).

By extensive phytochemical and pharmacological analysis conducted in specimens growing in the state of Guanajuato (GM), a family of nine bioactive nor-secofriedelanes designated as galphimines, was identified (Cardoso-Taketa et al., 2004). The sedative activity of galphimines has been demonstrated using *in vitro* models (Prieto-Gómez et al., 2003) and the anxiolytic action of galphimines A, B and E were documented in mice (Herrera-Ruiz et al., 2006). Moreover, in a clinical study performed in patients with general anxiety, the anxiolytic effect from standardized preparations of *Galphimia glauca* administered to the patients, was clearly demonstrated (Herrera-Arellano et al., 2007).

In our previous study conducted to investigate the metabolic profiling of *Galphimia glauca* using ^{1}H NMR spectroscopy and multivariate data analysis, the overall results showed the benefits of using this *in silico* analysis to discriminate populations. Due to the clear differences in metabolic profiles and to the fact that the different populations showed differential level of activity, it was possible to identify the compounds correlating with activity, by using multivariate analysis. In the mentioned study samples were collected in six different locations (MC, MT, MS, GM, JG and QJ) in September 2005. By using Principal Component Analysis (PCA) applied to ^{1}H NMR spectra, the results showed that sedative high activity coincides with the presence of galphimines. Two populations (GM and QJ) out of six were high in these compounds. This information was confirmed with HPLC analysis. Partial least square regression discriminant analysis (PLS-DA) confirmed a correlation with the sedative and anxiolytic responses displayed by the extracts (Cardoso-Taketa et al., 2008). The differences in the chemical profiles exhibited between populations of *Galphimia glauca* that grow in different regions of the country, could possibly be related to geographical, climatic or soil conditions. The purpose of the present work was to determine, after four-years, the consistency of the chemical profiles obtained for populations of *Galphimia glauca* that were collected in the same localities during the first study. Two new populations were incorporated for the first time. In a similar way to the first study, metabolic profiling of crude extracts was carried out using ^{1}H NMR spectroscopy and multivariate data analysis techniques. Further targeted chemical identification and quantification of galphimines were conducted by HPLC analysis, and bioassays for sedative and anxiolytic action were also performed. For the

Table 1
General data for *Galphimia glauca* populations collected in 2005 (A) and in 2009 (B).

Population	Voucher	Locality	Date and time of collection	Position	Altitude (meters)	Ecosystem
(A)						
GM	25728	Doctor Mora, Guanajuato	July 31 10.00–11.00 h	W101°18.05 N21°08.06	2120	Semi-dry secondary vegetation
MS	25729	San Andres de la cal, Morelos	August 11 13.00–14.00 h	W99°07.39 N18°58.24	1662	Seasonal dry tropical forest secondary vegetation
MT	25730	Tepoztlan, Morelos	August 11 15.00–16.00 h	W99°02.05 N18°53.06	1700	Seasonal–dry tropical forest secondary vegetation
MC	25731	Cuernavaca, Morelos	August 12 11.00 –12.00 h	W99°10.26 N19°00.35	2204	Pine oak forest
JG	25732	Zapopan, Jalisco	August 26 15.00–16.00 h	W103°29.38 N20°48.08	1585	Seasonal–dry tropical forest secondary vegetation
QJ	25733	Jalpan de Serra, Queretaro	September 12 13.00–14.00 h	W 109°19.30 N20°25.30	1548	Pine oak forest
(B)						
GM	15189	Doctor Mora, Guanajuato	September 16 12.00–13.00 h	W100°19.22 N21°08.74	2120	Semi-dry secondary vegetation
MT	15485	Tepoztlan, Morelos	September 6 12.00–13.00 h	W99°06.97 N18°59.35	1700	Seasonal–dry tropical forest secondary vegetation
MC	15011	Cuernavaca, Morelos	September 10 13.00–14.00 h	W99°13.48 N18°58.91	2204	Pine oak forest
MM	15426	Miacatlan, Morelos	September 25 14.00–15.00 h pm	W99°21.75 N18°45.57	1004	Seasonal–dry tropical forest secondary vegetation
JG	15014	Zapopan, Jalisco	September 20 14.00–15.00 h	W103°19.95 N20°40.67	1585	Seasonal–dry tropical forest secondary vegetation
CT	15421	Tuxtla Gutierrez, Chiapas	September 28 11.00–14.00 h	W93°05.95 N16°49.52	559	Pine oak forest
QJ	15018	Jalpan de Serra, Querétaro	September 15 15.00–16.00 h	W99°28.57 N21°28.50	1548	Pine oak forest

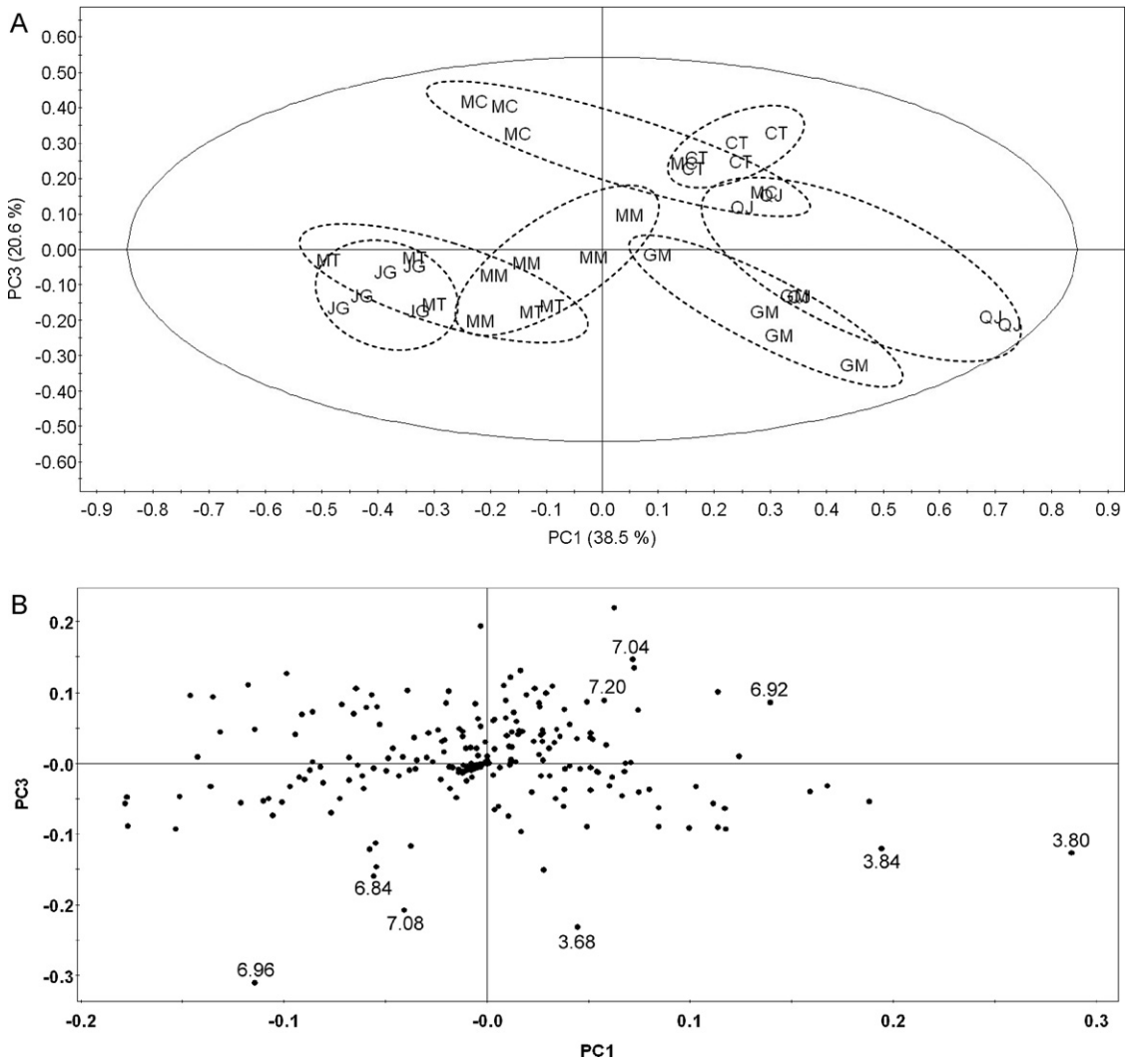

Fig. 2. Score (**A**) and loading (**B**) plots of principal component analysis (PC-1 *vs*. PC-3) of crude extracts from Dr. Mora, Guanajuato (GM samples); Jalpan, Queretaro (QJ samples); Tepoztlan, Morelos (MT samples); Miacatlan, Morelos (MM samples); Cuernavaca, Morelos (MC samples); Guadalajara, Jalisco (JG samples) and Tuxtla Gutierrez, Chiapas (CT samples). Replicates represent separate extractions from individual plants (35 in total).

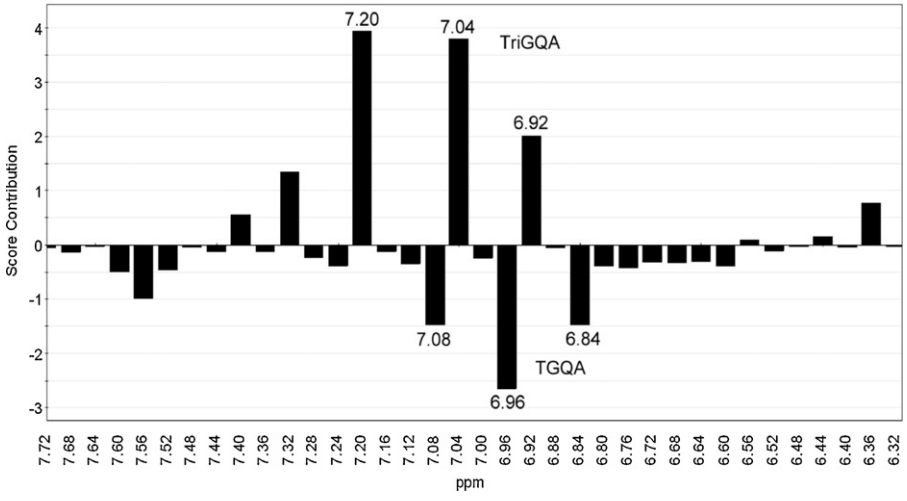

Fig. 3. Loading plot contribution of MC samples to PC3. TGQA: 1,3,4,5-tetra-O-galloylquinic acid; TriGQA: 3,4,5-tri-O-galloylquinic acid.

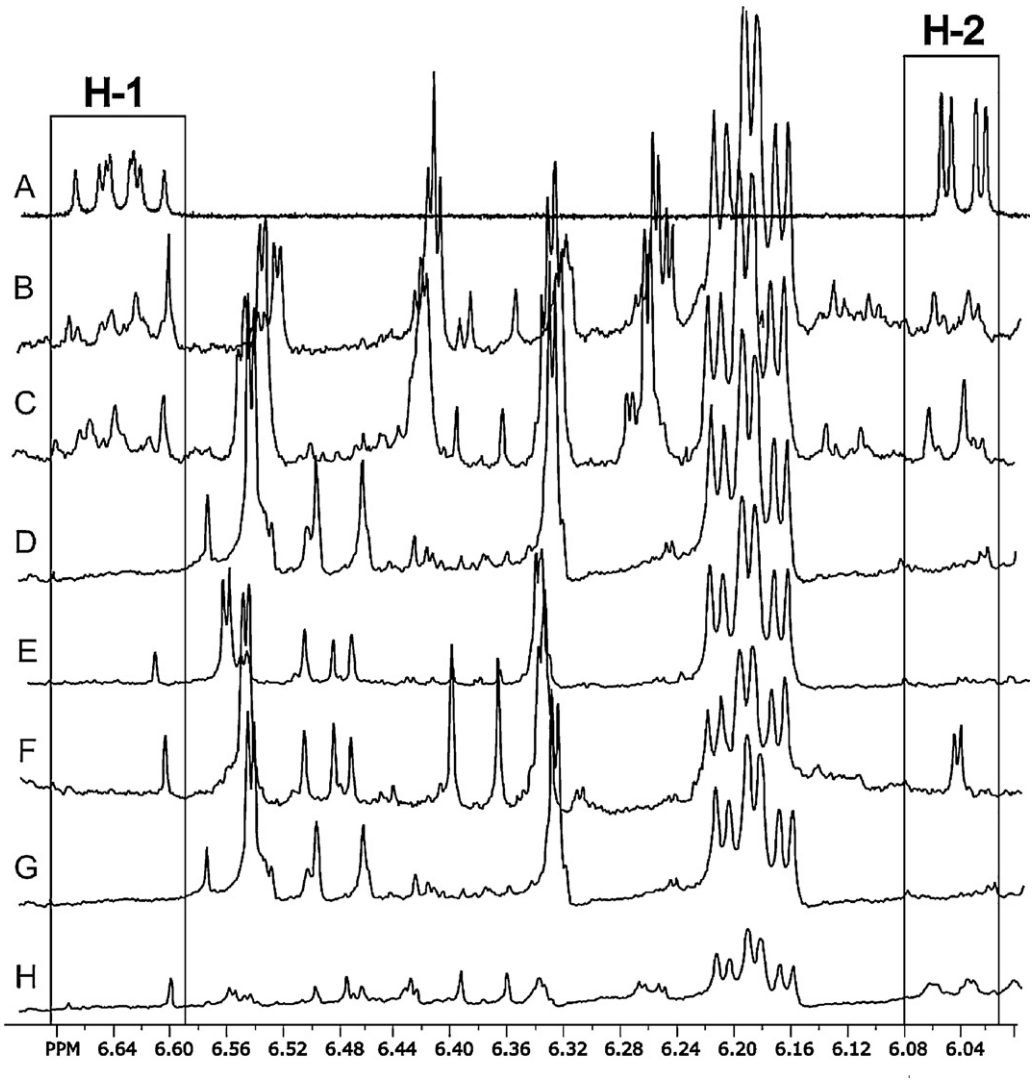

Fig. 4. ^{1}H NMR spectra of (A) resonances for vinylic protons H-1 and H-2 of an HPLC peak containing the diastereomeric pair of galphimines B (1) and F (5); (B) MeOH crude extract from one individual collected in Jalpan, Queretaro (QJ sample) showing the resonances for H-1 (δ = 6.59–6.67) and H-2 (δ = 6.02–6.07) for the galphimine series, (C) MeOH crude extract from one individual collected in Dr Mora, Guanajuato (GM sample) showing the resonances for H-1 (δ = 6.59–6.67) and H-2 (δ = 6.02–6.07) for the galphimineseries; (D), (E), (F), (G) and (H) MeOH crude extracts from individuals collected in Tepoztlan, Morelos (MT sample), Jalisco Guadalajara (JG sample), Tuxtla Guiterrez, Chiapas (CT sample), Miacatlan, Morelos (MM and Cuernavaca, Morelos (MC sample) respectively, without signals for galphimines.

first time, anti-inflammatory activity was determined in the populations of *Galphimia glauca* by using the tetradecanoylphorbol acetate-induced mouse ear inflammation model (TPA).

2. Materials and methods

2.1. Plant material

Plant material was collected in the summer of 2009 (August and September) from seven different locations in Mexico (Fig. 1). Specimens were deposited at the HUMO Herbarium, CEAMISH (Centro de Educacion Ambiental e Investigacion Sierra de Huautla), UAEM, after authentication by Rolando Ramirez. Details for plant specimens collected in both 2005 (Cardoso-Taketa et al., 2008) and that collected in 2009 are presented in Table 1A and B, respectively. In both investigations the samples (leaves) were dried for 3–4 days in a cool and dry place without direct sunlight. The samples were then pulverized by mortar and pestle and kept at −20 °C until used for ^{1}H NMR, HPLC, TLC and different bioassays. Up to this point the treatment of both collections was similar although collection A was thus processed and recorded in 2005 and B in 2009.

2.2. General procedures

^{1}H NMR spectra from extracts of *Galphimia glauca* were recorded using a 600 MHz Bruker Avance spectrometer. NMR experiments were controlled with a Bruker XWINNMR version 3.1 software, whereas all other operations were controlled with a Bruker HyStar version 2.3 or 3.2 software. The HPLC determinations were performed on an instrument of Waters, and consisted of a Waters 600E multisolvent delivery system, equipped with Waters W996 diode array detector (216 nm), and a Waters 717 plus autosampler. Equipment control, processing, data acquisition, and management of chromatographic information, were carried out by Millennium 32 software program (Waters Corporation). Principle component analyses and partial least square regression discriminant analyses were performed using SIMCA-P 11.0 software (Umetrics). Pareto

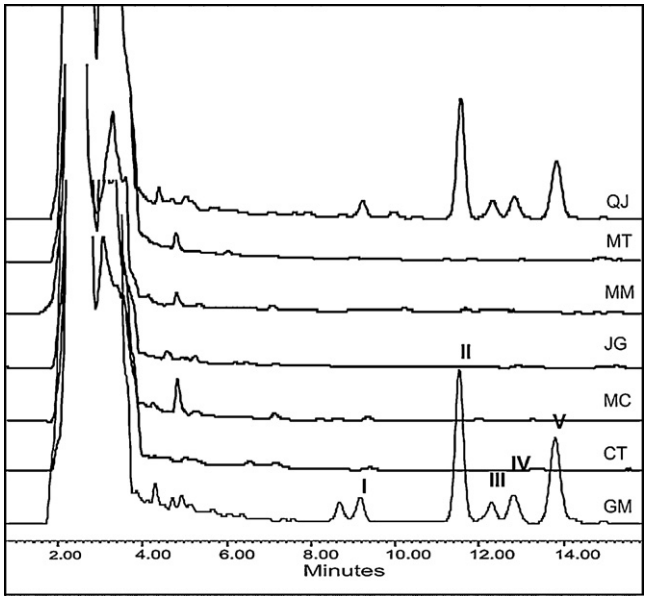

Fig. 5. Reversed phase HPLC separations of the sedative triterpenes crude extracts of *Galphimia glauca* obtained from seven different locations QJ, MT, MM, JG, MC, CT and GM. Peak assignments: I, compounds **3** and **7** (t_R = 9.10 min); II, compounds **1** and **5** (t_R = 11.54); III, compound **9** (t_R = 12.33 min); IV, compounds **4** and **8** (t_R = 12.87 min); peak V, compounds **2** and **6** (t_R = 13.90 min).

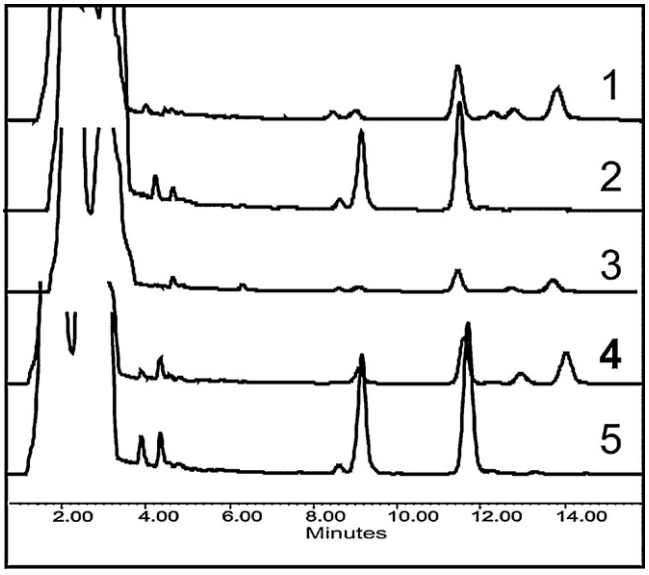

Fig. 6. Reverse phase HPLC analysis of 5 different individual collected from Jalpan, Queretaro (QJ samples) showing a different level of accumulation of galphimines. In sample 2 and 5, peaks I and II are showing a comparatively higher concentration of the corresponding galphimines.

and unit variance methods were applied to PCA and PLS-DA, respectively.

2.3. Generation of ^{1}H NMR profiles

To obtain a broad range of different polarity compounds, a previously published method using a deuterated protonic solvent was employed (Cardoso-Taketa et al., 2008). Leaves were dried in a fresh place without direct sunlight. Samples were pulverized by mortar and pestle, and 50 mg of each sample (35) were placed in fresh individual Eppendorf tubes (2.0 mL). Then, 750 μL of CD_3OD was added and the mixture vortexed for 30 s. To each tube, 750 μL of D_2O with KH_2PO_4 (1.23%, w/w) containing 0.05% (w/w) TMSP (trimethylsilanepropionic acid sodium salt) was added. After vortexing for 30 s and sonicating for 10 min, tubes were centrifuged at 3000 rpm for 10 min. The supernatants were transferred into fresh Eppendorf tubes (2 μL) and re-centrifuged for 5 min. Then, 800 μL of the extracted mixture from each tube was transferred to individual NMR tubes. For the NMR measurements pH of D_2O was adjusted to 6.0 with the addition of 1 M NaOD solution.

2.4. Generation of HPLC profiles

Pulverized dried leaves material (100 mg) for each sample was mixed with 1 mL MeOH. Each sample was vortexed for 2 min, sonicated for 15 min and then centrifuged at 10,000 rpm for 15 min. The supernatant was removed and the material residue containing the pellet was re-centrifuged (5×). All five supernatants were combined and directly injected (20 μL) into the HPLC. Quantification studies were performed using a Symmetry C18 column (Waters; 5 μm, 4.6 mm × 250 mm), an isocratic elution with CH_3CN–H_2O (1:1), a flow rate of 0.7 mL/min, and 20 μL of the sample injection. Calibration curves were constructed using pure galphimine B obtained as previously described. The procedure was followed as described by Cardoso-Taketa et al. (2008).

2.5. Neuropharmacological assays

Male ICR mice was used and experiments were conducted according to the laws and institutional guidelines approved by the CEIB-UAEM Animal Experimentation Committee (approval No. CBEA 05-7-10) under the Mexican guidelines NOM-062-ZOO-199. Animals (40–45) were housed in groups of 7 for at least 1 week prior to the experiments. The animals were conditioned to a 12 h light/dark cycle, at a temperature of 22 ± 1 °C. Food and water were available *ad libitium*. Methanolic extracts were prepared for each sample under vortex (2 min), sonication (15 min) and centrifugation (5 min) at 5000 rpm. This procedure was repeated for 3 times. All the supernatants were combined, filtered and evaporated. Two neuropharmacological models; the elevated plus maze and the sodium pentobarbital-induced hypnosis model, were employed as described by Cruz et al. (1994) and by Marder et al. (2005). Animals were grouped in one of the following lots. Group 1: negative controls, which received intraperitoneally (*i.p.*) 0.75 mL/g of vehicle (8% of Tween 80 Sigma in saline); group 2: positive controls which received *i.p.* 0.1 mg/100 g diazepam (Relazepam from Pisa Lab); groups 3–9: experimental animals which received *i.p* 50 mg/100 g of crude extracts from plants samples (seven plant populations) dissolved in the same vehicle used for controls. Following injections, the animals were subjected to the "elevated plus maze test", and after administering subsequent doses of pentobarbital, to the "pentobarbital-induced hypnosis model". Results were scored in relation to the number of observations: for vehicle (8) diazepam (7), GM (6), MT (5), MC (5), MM (5), JG (5), CT (5) and QJ (5) in both pharmacological tests. Each group consisted of ≥5 mice.

2.6. Anti-inflammatory assay

Tetradecanoylphorbol acetate (TPA)-induced mouse ear inflammation test was performed according to a previously reported protocol (Rao et al., 1993). This test is one of the most common assays to measure anti-inflammatory activity from crude plant extracts. Male BALB/c mice were employed (25–30 g). Animals were maintained in adequate living conditions (transparent acrylic box at constant temperature of 24 °C, with a light photo period of 12/12 h and of light/darkness, and with water and food element *ad*

libitum. A total of 45 mice were employed. Under general anesthesia by sodium pentobarbital (3.5 mg/kg, intraperitoneally), 10 μL of TPA in ethanol (0.25 mg/mL) was applied to the right ear. After 10 min of incubation, 3 mg of plant samples in ethanol were applied in the same ear. The left ear (negative control) received only 10 μL of ethanol, and 20 μL of indometacin was used as a positive control. After 4 h of incubation with TPA, animals were anesthetized with ether, and sacrificed by cervical dislocation. Both ears (7 mm each) were taken as samples for further measurement. Increase in the weight of the right ear in respect to the left was considered oedema. The inhibition of oedema was calculated by the formula:

$$\%\ \text{of inhibition} = \left[\frac{C-E}{C}\right] \times 100$$

where C is oedema of control group (treated with TPA) and E is oedema of experimental group (TPA with plant sample).

2.7. Statistical analysis

Statistical analysis was done using the program SAS 9.1 (SAS Institute Inc.). Data were converted and presented as mean ± S.D. Further analysis was done by using one-way ANOVA, followed by Dunnett's *t*-test. A significant difference was obtained among groups where the *p* value was lower than 0.05.

3. Results and discussion

The dimensionality of the ^{1}H NMR spectra dataset from *Galphimia glauca* samples that were harvested in seven locations of Mexico was reduced by using principal component analysis (PCA), resulting in a clear discrimination between the accessions. The score plot (PC1 *vs*. PC3) diagram in Fig. 2A shows that these two principal components clearly separate GM and QJ populations from the rest. This finding was in accordance with the previous work where we demonstrated a metabolic divergence between the sedative active populations (GM and QJ) and the other inactive samples depending on the presence of galphimines and of 1,3,4,5-tetra-O-galloylquinic acid (Cardoso-Taketa et al., 2008). As it was shown in the previous investigation, in the present study the CT population was also observed to cluster with GM and QJ samples. The position displayed by CT samples on PC1 score plot was not connected with C-27 methyl ester protons signals from galphimines as observed with GM and QJ samples, because galphimines were absent in CT samples. However, the loading plots for CTs showed that aromatic signals ranging from 6.56 to 6.92 ppm were connected with the positive value in the PC1 discrimination (Fig. 2B).

Although the first two principal components PC1 *vs.* PC2 score plot showed the best clustering in the former study, at this time, PC1 *vs*. PC3 score plot displayed a better discrimination between the samples. However, in both cases, the loading plots revealed that the proton signals of 1,3,4,5-tetra-O-galloylquinic acid at δ 6.84, 7.08 and 6.96 were the mean features for the positive values in PC2, in contrast with the negative values produced in PC3. Now, PC3 displayed a better clustering of the accessions and less dispersion as compared to PC2.

To verify the influence of 1,3,4,5-tetra-O-galloylquinic acid concentration in all of the samples, the integration of the ^{1}H NMR signals for the galloyl moieties was performed in order to compare the relative quantity of this acid in each of the extracts. In this inspection, the following values were found (in times) in relation to the CT (value of 1.0): GM (2.12), JG (2.10), MM (1.86), MT (1.70), QJ (1.30), and MC (1.20). CT and MC samples presented the lowest levels of 1,3,4,5-tetra-O-galloylquinic acid, in contrast to the two times higher concentrations observed for GM and JG samples. The presence of this acid is directly correlated with the clustering at the negative mode to PC3, as it is shown in the loading plot of Fig. 2B,

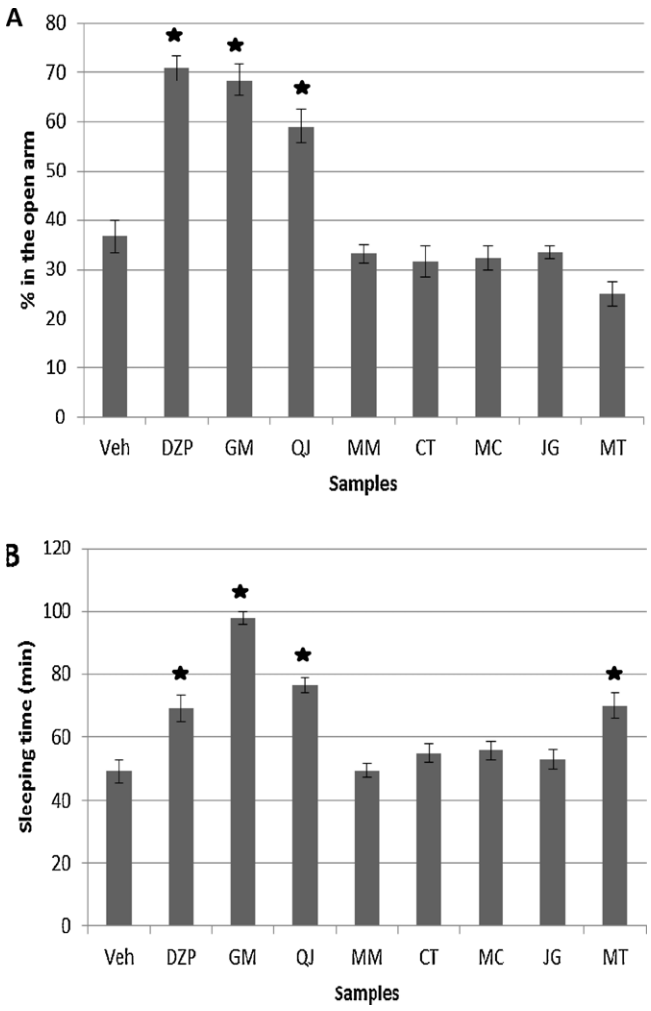

Fig. 7. (**A**) Elevated plus maze model calculating the percentage of time spend in the open arms (after 25 min of *i.p.* injection of vehicle and crude samples), and (**B**) sodium pentobarbital-induced hypnosis model (after 30 min *i.p.* injection of vehicle and crude samples), total sleeping time measured between disappearance and reappearance of the righting reflex. Crude extracts (50 mg/100 g) of *Galphimia glauca* (GM, QJ, MM, CT, MC, JG, and MT) were injected, control vehicle (0.75 mL/100 g at 8% Tween 80 in saline) and diazepam (0.1 mg/100 g). $^*p < 0.05$ on the ANOVA followed by *post hoc* Dunnett's *t*-test (mean ± S.D.).

evidencing that the resonances of the galloyl moieties are decisive for the PCA discrimination.

Thus the concentrations of the tetra-galloylquinic acid derivatives play an important role in the score plots distribution observed in the former and in the current study. Furthermore, MC samples show ^{1}H NMR signals at δ 6.92, 7.04 and 7.20 corresponding to the 3,4,5-tri-O-galloylquinic acid structure, while the rest of the samples show exclusively the tetra-galloylquinic acid derivative. The loading plot of PC3 (Fig. 3) explains that the tri-derivative signals correspond with a positive score in PC3, whereas the signals from the tetra-derivative correlate with negative score in PC3.

The signals for the galphimines C-27 methyl ester protons, ranging between δ 3.68 and 3.88, were the main diagnostic resonances for the positive scores in PC1, as it was determined in the previous work.

Results from ^{1}H NMR of plant extracts are shown in Fig. 4, where the presence of galphimines in the samples from GM and QJ is confirmed. Samples from other localities (MM, MC, MT, JG and CT) do not show galphimines signals in the NMR spectra when compared with the spectra of the references galphimines B and F. These results strongly correlate with HPLC analysis (Fig. 5), where only

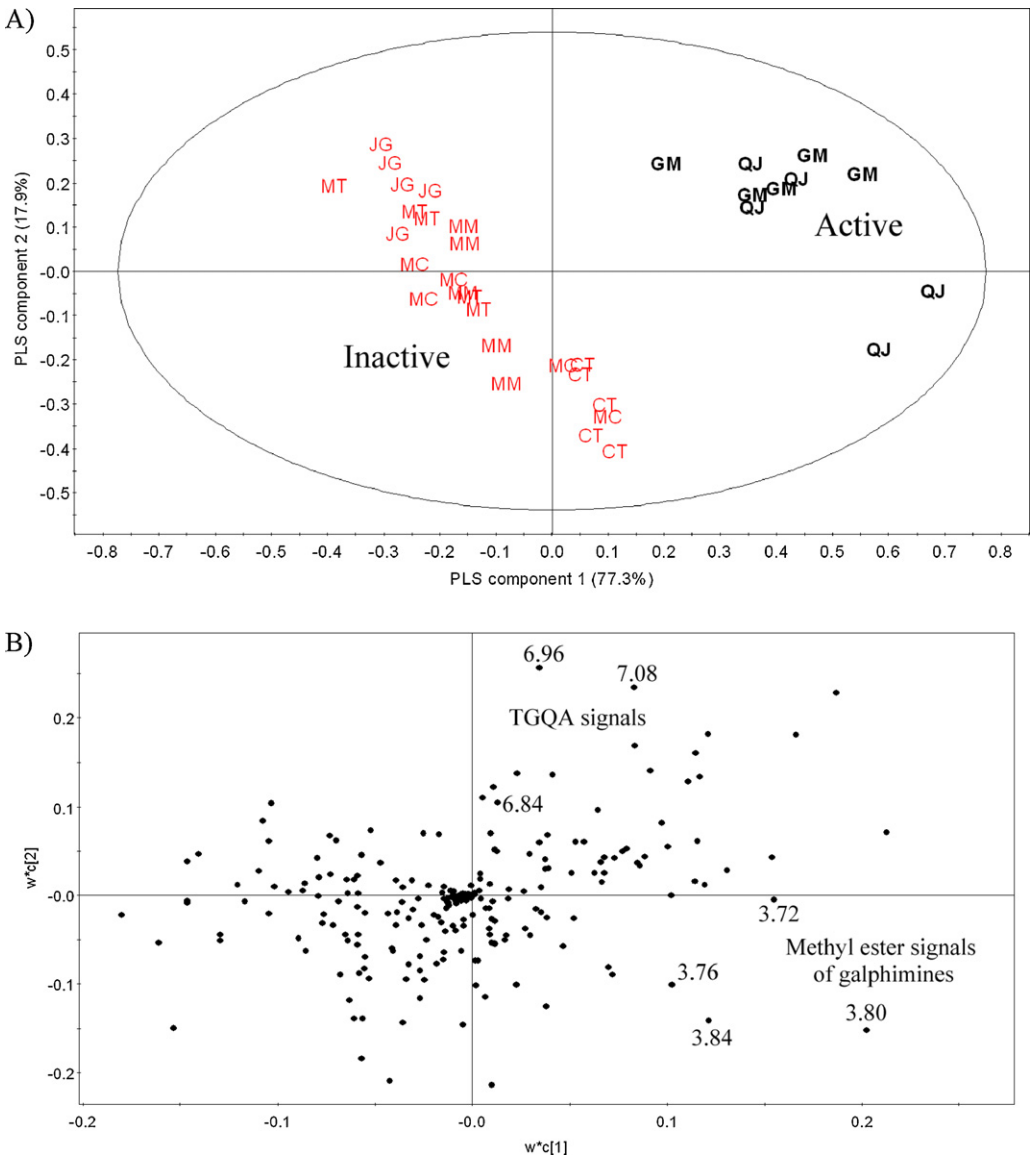

Fig. 8. Score (**A**) and loading (**B**) plots of PLS-DA models (PLS-1 *vs.* PLS-2) based on ^{1}H NMR resonances and anxiolytic activity of *Galphimia glauca* crude extracts classified in two classes: active and inactive extracts. Active (>59.1%) extracts from GM and QJ. Inactive extracts (<33.6%) from MT, MS, MC, JG, MM and CT. TGQA: 1,3,4,5-tetra-O-galloylquinic acid.

populations from GM and QJ showed 5 peaks that corresponded to galphimines, identified by retention time and co-elution experiments.

Total galphimines accumulation for GM and QJ populations was recorded by means of HPLC (mean values of 7.21 mg/g DW and 6.19 mg/g DW, respectively) (Table 2), and was comparable to those values obtained in the first-stage study (6.58 mg/g DW and 5.66 mg/g DW, respectively). For GM samples, the most abundant galphimines were GB + GF (peak II), and comparable to GE + GG (peak V) with mean values of 2.83 and 2.52 mg/g, respectively (Table 2). In the GM populations of our former study, these galphimines were also the most abundant (3.18 and 2.41 mg/g for peak II and peak V, respectively). In the case of the QJ population the concentration of galphimines present in peak II were found high (mean values 2.56 mg/g) when compared with other galphimines, while those mean values of peak V were very low (Table 2). Moreover, variation on the accumulation of different galphimines among the QJ population was observed among individuals, as it is shown in Fig. 6. In our previous investigation, galphimines present in peak V were the most abundant ones after those present in peak II (Cardoso-Taketa et al., 2008). This situation may reflect differences in plant growth and defense conditions. It is interesting to note that harvesting of QJ samples for collection 2005 as well as for 2009, were performed in the same month of the year and at a similar time of the day (Table 1A and B).

Pharmacological activities (sedative and anxiolytic) using two neuropharmacological assays were measured for all accessions. The elevated plus maze test is one of the most utilized models to determine anxiolytic activity from active substances (Gonzalez and File, 1997). The total time which mice spend in open arms (insecure) and in closed arms (more secure), was measured in a calm and noise-less environment.

Fig. 7A shows a significant difference between animals injected with vehicle and those injected with diazepam (vehicle 36.80%; diazepam 70.80%). Of those injected with the crude extracts, only the mice treated with GM and QJ spent a higher percentage of time in the open arms [GM 68; QJ 59; MM 33.20; CT 31.6; MC 32.40; JG 33.60; MT 25%. $F = 38.27$, $p < 0.05$]. Fig. 7B shows that GM, QJ and MT crude extract samples manifested significant differences in terms of their levels of sedation (in min), by an enhancement in

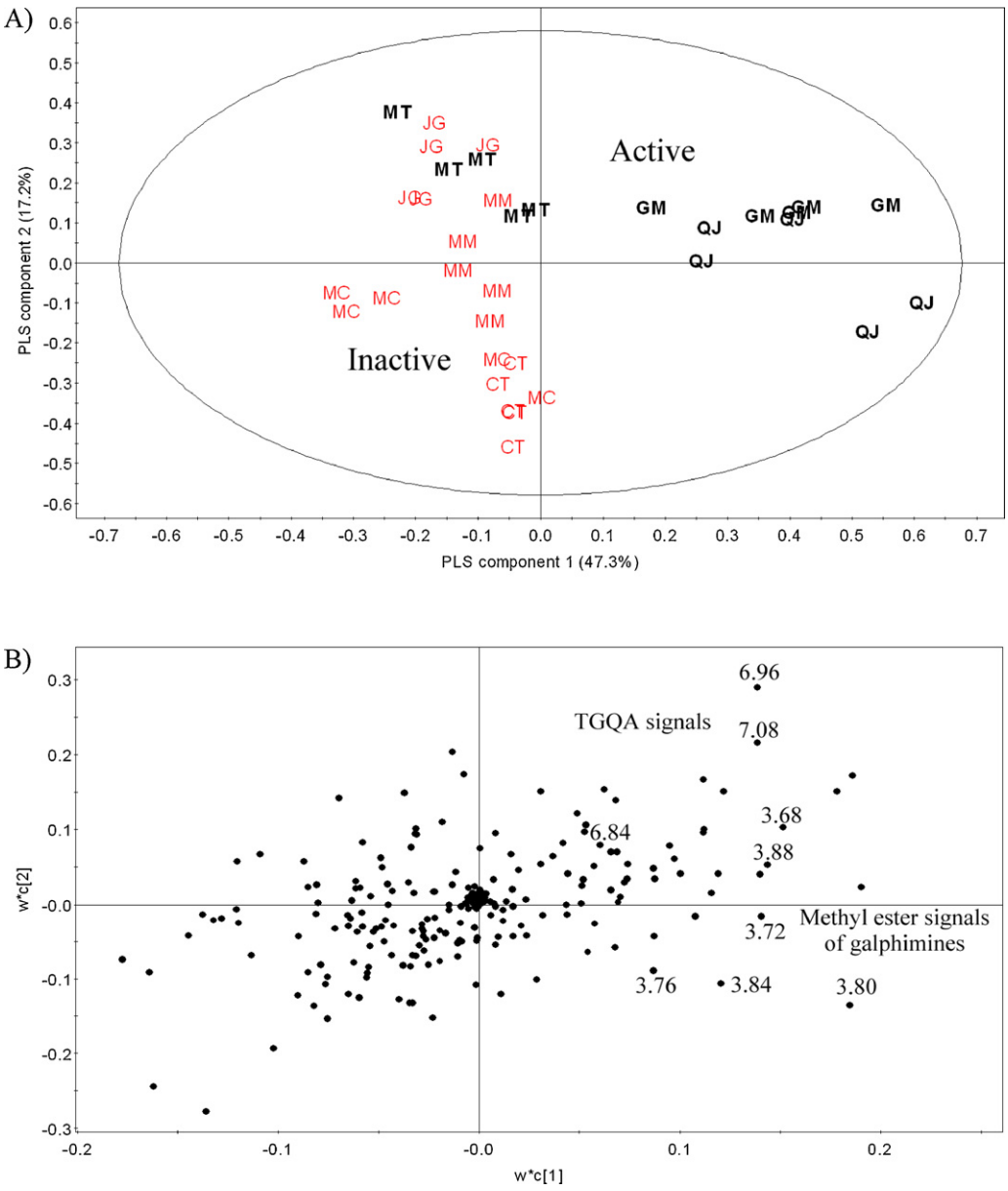

Fig. 9. Score (**A**) and loading **(B**) plots of PLS-DA models (PLS-1 *vs.* PLS-2) based on ^{1}H NMR resonances and sedative activity of *Galphimia glauca* crude extracts classified in two classes: active and inactive extracts. Active (>70.0 min) extracts from GM, QJ and MT (low active). Inactive extracts (<55.6 min) from MS, MC, JG, MM and CT. TGQA: 1,3,4,5-tetra-O-galloylquinic acid.

Table 2
Accumulation of galphimines (mg/g DW) determined by HPLC in leaves obtained from *Galphimia glauca* individuals growing in Dr. Mora, Guanajuato (GM) and Jalpan, Queretaro (QJ).

Samples	Galphimines (mg/g)					
	Peak I (GA + GH)	Peak II (GB + GF)	Peak III (GC)	Peak IV (GD + GI)	Peak V (GE + GG)	Total Galphimines
GM	0.65	2.79	0.76	0.88	1.84	6.92
GM	0.72	4.05	0.41	1.64	4.00	10.82
GM	0.43	3.12	0.37	1.01	2.02	6.95
GM	–	2.37	0.37	1.04	2.19	5.97
GM	0.36	1.82	–	0.97	2.27	5.42
Mean	0.43	2.83	0.38	1.1	2.52	7.21
QJ	0.62	2.5	0.65	0.8	1.92	6.49
QJ	1.93	2.6	0.33	–	–	4.86
QJ	0.42	0.88	0.40	–	0.68	2.38
QJ	0.92	2.4	0.32	0.69	2.14	6.47
QJ	3.33	4.46	0.39	0.35	–	8.53
Mean	1.44	2.56	0.41	0.36	0.94	6.19

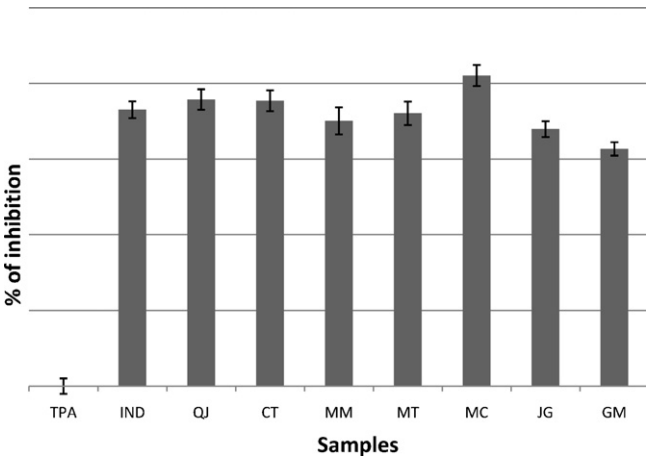

Fig. 10. Anti-inflammatory activity of *Galphimia glauca* crude extracts collected from QJ (73.77%), CT (75.44%), MM (70.11%), MT (72.11%), MC (82.11%), JG (67.91%) and GM (62.68%). Control vehicle (TPA 0.25 mg/mL) and indometacin (IND) with 73.05% of inhibition.

sedation as shown by longer duration of sleeping time. When the sodium pentobarbital-induced hypnosis test was applied [vehicle 49; diazepam 69.20; GM 98; QJ 76.60; MM 49.4; CT 54.8; MC 55.60; JG 52.8; MT 70 min, $F = 33.92$, $p < 0.05$].

These effects may be explained by the presence of galphimines in these plant samples. Even though MT samples also showed a weak sedative-like activity, it is clear by HPLC and NMR analyses that they do not contain galphimines, so this activity may be due to some other compound which will be subject for further investigation. There was neither sedative nor anxiolytic activity observed in samples from the new localities CT and MM. Overall results from these assays support our previous published data.

Partial least square regression modeling-discriminant analysis (PLS-DA) was used as a supervised approach to maximize the separation between the active and inactive classes correlated to the neuropharmacological activities and metabolic profiling. For the elevated plus model activity, samples were divided into active (>59.1%) and inactive (<33.6%); and for the sodium pentobarbital-induced hypnosis model, two classes including active (>70.0 min) and inactive (<55.6 min) accessions were established, according to the statistical differences calculated by Dunnett's test. It can be seen from Fig. 8 that, for the anxiolytic activity, a clear discrimination between anxiolytic and inactive samples (Fig. 8A) is supported by the robust model with variance (Q^2) and cross-validated variance (R^2) of 0.94 and 0.98, respectively, in agreement that a value of $Q^2 > 0.5$ is acceptable as good (Bailey et al., 2004). Loading plot of PLS-DA for the relationship between the two dataset (metabolites resonances and anxiolytic activity), indicated the influence of methyl ester signals of galphimines as the major set of resonances (δ 3.72–3.85) responsible for the PLS component 1 separation (Fig. 8B). The contribution of 1,3,4,5-tetra-O-galloylquinic acid signals at δ 6.84, 6.96 and 7.08 causes the segregation of the samples for the PLS component 2 towards a positive values for JG, MT, GM, and QJ (samples with high content of this galloylquinic acid).

Galphimines are clearly associated with the sedative activity as shown in PLS-DA (Fig. 9A and B), where the signals at δ 3.68–3.84 from C-27 methyl ester protons contributed to a clustering in the PLS component 1 (Fig. 9B). The low values of $Q^2 = 0.47$ and $R^2 = 0.64$ for the PLS-DA calculated by the supervised correlation with the sedative activity indicated that this model is not robust enough to separate the low active group MT from the inactive, or to generate a particular cluster for the half-active groups (Fig. 9A).

Anti-inflammatory activity displayed by the crude extracts of the collected populations is shown in Fig. 10. It is evident that this activity is not correlated neither with PCA nor with neuropharmacological data, since all populations have a high level of anti-inflammatory activity as compared to the control indometacin. This may suggest that other chemical compounds are responsible to this activity.

Fig. 11 shows the score plots from *Galphimia glauca* samples collected during the summer season of two different years: 2005 (A) and 2009 (B). In order to more strictly compare the metabolic profiles obtained for both years, only those sample populations that were common for the two studies were selected. Thus, the ^{1}H NMR data from samples GM, QJ, MT, JG and MC were statistically processed to cross their PC1 with PC2 values, resulting in the score plots of the years 2005 and 2009. To carry out this comparison, PC2 was used instead of PC3, since PC2 presented a better clustering in the analysis performed with samples collected in the former study. In contrast, PC1 discrimination, mainly produced by the presence of the signals for the galphimine C-27 methyl ester protons, was important in both years, and segregated the neuropharmacologically active populations GM and QJ from the inactives ones.

Visual inspection of the galloylquinic acid ^{1}H NMR spectra allowed to determine the relative concentration of this acid in all samples (Fig. 12). The influence of galloylquinic acid proton resonances on PC2 is shown by the fact that, in the 2005 study, it provoked negative values to QJ and JG samples, both with the highest galloylquinic acid content; GM and MC presented positive values on PC2 and corresponding with the lowest galloylquinic acid content. In a different way, in the 2009 collection, the highest amount of galloylquinic acid was observed in GM, QJ, and JG groups; and the lowest amount was found in MC samples.

The changing in the galloylquinic acid content for the plant material collected in 2005 and 2009 explains the different metabolic profiles produced by PC2. For the 2009 study, a positive value on PC2 scale was correlated to samples with a high content of galloylquinic acid, the contrary was observed in the 2005 sample collections. It is noteworthy to emphasize how a change in the synthesis of a unique metabolite can drastically modify the metabolic profiling on the score plot graphic.

Sample classes from 2005 analysis were tightly grouped together within their classes, whereas the samples from 2009 appeared much more diffuse. This finding might be attributed to changes in the general plant metabolism due to ecological pressures in which the plants are exposed, as climatic variation and insect attacks, among other factors.

This investigation corroborates the efficiency of the ^{1}H NMR and multivariate data analysis using PCA to discriminate *Galphimia glauca* plants with anxiolytic and sedative action from others with anti-inflammatory activity. It is obvious that many metabolites have a more or less influence on PCs loading plots. However, the presence of galphimines and galloylquinic acid derivatives play a decisive role in this analysis. Comparison between collections made in the years 2005 and 2009 allowed to observe that the galphimine content in both years remains quite the same in GM and QJ populations. In the opposite way, the content of the galloylquinic acid derivatives varies and its synthesis seems to be more sensitive to environmental factors. Further studies must be performed in order to confirm these findings using *Galphimia glauca* cultivars under controlled growth conditions. For the first time, ethnobotanical use of this plant in different locations of Mexico as an anti-inflammatory medicine, is supported by *in vivo* testing using animal models.

Importantly, this study shows that for the Central Nervous System activity, metabolic profiling can be employed for quality control by using the galphimines signals as markers. Both NMR and HPLC analysis can be used for this purpose.

The importance of a non-targeted metabolic profiling of medicinal plants when studying pharmacological effects is clearly

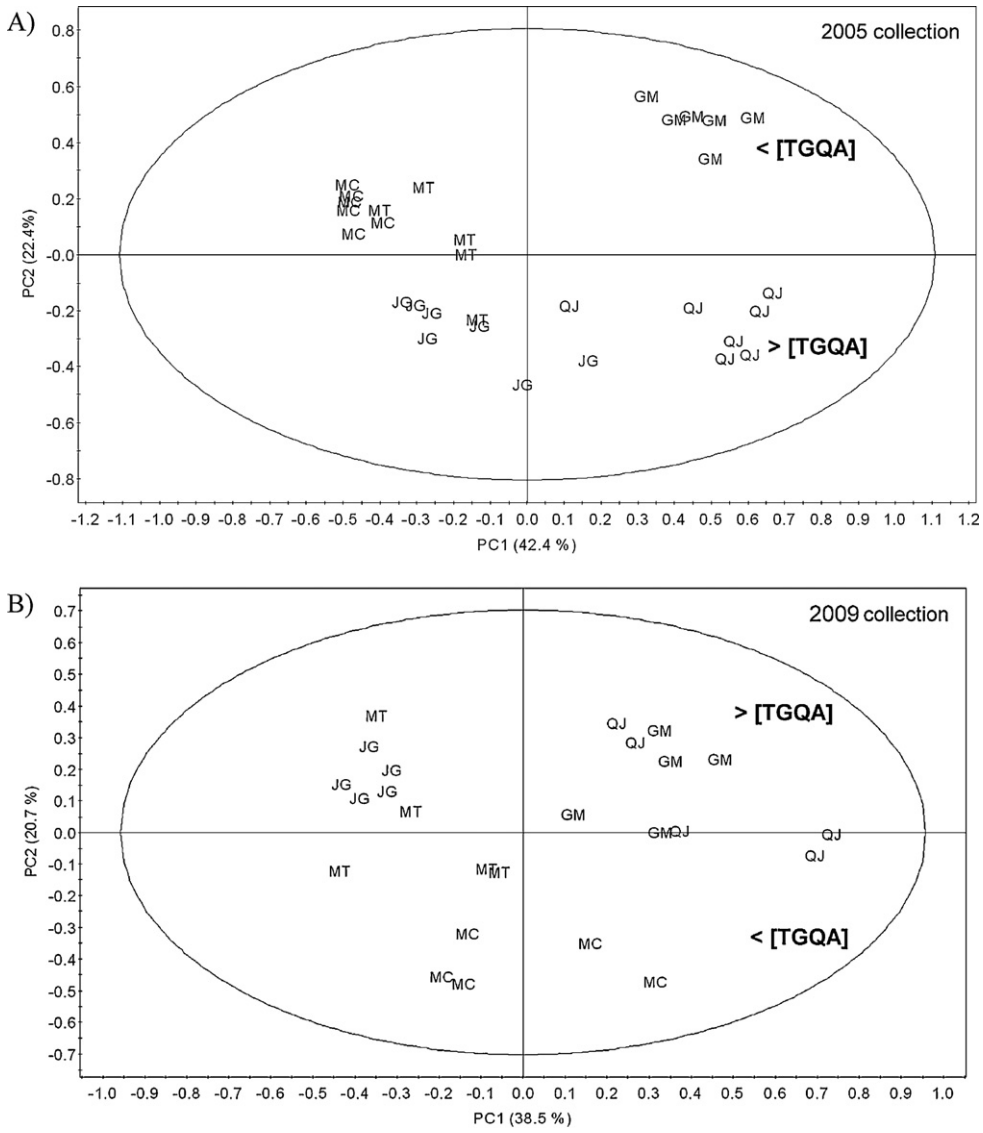

Fig. 11. Score plots for sample collections during the years 2005 (**A**) and 2009 (**B**) of principal component analysis (PC1 *vs.* PC2) of crude extracts from Dr. Mora, Guanajuato (GM sample); Jalpan, Queretaro (QJ sample); Tepoztlan, Morelos (MT sample); Cuernavaca, Morelos (MC sample); and Guadalajara, Jalisco (JG sample). [TGQA]: concentration of 1,3,4,5-tetra-O-galloylquinic acid.

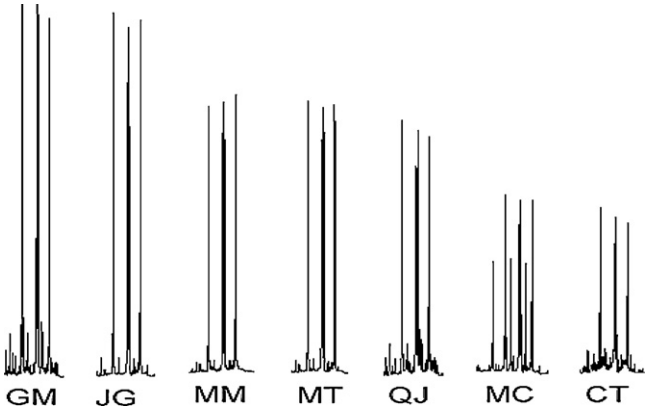

Fig. 12. ^{1}H NMR detail corresponding to signals from 1,3,4,5-tetra-O-galloylquinic acid at δ 6.84, 6.95, 6.96 and 7.08 presented in GM, JG, MM, MT, QJ, MC and CT samples from 2009 collection. Chemical shifts at δ 6.91, 7.03 and 7.20 from 3,4,5-tri-O-galloylquinic acid structure were observed only in MC samples. Signals intensities were fit according to the reference TMSP.

demonstrated in this study. The different traditional uses reported for this plant thus ask for further studies of the various chemotypes and their biological activity. Particularly correlating reported local uses with the chemotypes would be of interest.

It is notable that where the plant grows in the collected populations is very important for CNS activity but not for the anti-inflammatory effect, and greatly affects quality control for its effective use pharmacologically. Tracking the growth location of plants proves to be very important.

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