Anxiolytic Effect of Natural Galphimines from *Galphimia glauca* and their Chemical Derivatives

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The anxiolytic effects of galphimine B (**1**), galphimine A (**2**), and galphimine E (**3**), natural nor-secofriedelanes isolated from *Galphimia glauca*, as well as derivatives obtained by acetylation (**4**), hydrogenation of the C-1/C-2 double bond (**5**), basic hydrolysis followed by hydrogenation of the C-1/C-2 double bond (**6**), and deacetylation (**7**) of galphimine E (**3**), were evaluated on ICR mice exposed to the elevated plus-maze test. This study also included the evaluation of a galphimines-rich fraction (GRF) with a known concentration of **1**−**3**, obtained from the dry leaves of *G. glauca*. Intraperitoneal administration of 15 mg/kg of **1**, **2**, **6**, and GRF (1 h before testing) caused an anxiolytic-like effect in the animals, increasing significantly ($p < 0.001$) the percentage of time of permanence and the number of crossings toward the open arms of the plus-maze. No activity was detected after administration of compounds **3**, **4**, **5**, and **7**. These results showed that GRF had activity similar to the most active pure galphimines (**1** and **2**) and that, like for the spasmolytic activity previously reported, the main determining factor responsible for the anxiolytic activity of the compounds was the presence of free hydroxyl groups at C-4, C-6, and C-7 and the presence of the double bond in the A ring.

Galphimia glauca, commonly known as "calderona amarilla", has been widely used in Mexican traditional medicine for the treatment of nervous hyperexcitability disorders. On the basis of the traditional medical use, it was demonstrated, in in vitro and in vivo models, that the crude extract from the aerial parts of *G. glauca* possesses sedative effects.[1] By means of a bioguided study, galphimine B (GB, **1**), a nor-seco triterpene to which the central nervous system (CNS) depressant effect was attributed, was identified.[2,3] It was also demonstrated that GB has a selective inhibiting effect on dopaminergic neurons of the ventral tegmental area (VTA) in rats, and through electrophysiologic techniques, it was proved that this effect does not result from the interaction with the GABAergic system.[3,4]

After the identification of GB, other compounds with similar chemical structure and with significant spasmolytic effect on the model of the isolated guinea-pig ileum have been isolated, and an SAR study on the natural galphimines (**1**−**3**) and semisynthetic analogues demonstrated that the main determining factor responsible for the spasmolytic activity of the compound studied is the presence of free hydroxyl groups at C-4, C-6, and C-7, as well as the double bond in the E ring.[5] Despite the significant progress in the analysis of the pharmacological effects produced by GB on the CNS, until now an anxiolytic effect produced by any of the compounds in *G. glauca* has not been demonstrated. Recently, the anxiolytic-like effect of the methanolic extract of *G. glauca* (standardized on GB content, 8.3 mg/g) was demonstrated by using the elevated plus-maze (EPM) test.[6] This extract increased significantly the number of entries as well as the time spent in the open arms of the EPM, indicating an anxiolytic-like effect.

Herein we characterize the anxiolytic-like effect produced by the natural galphimines, galphimine B (GB, **1**), galphimine A (GA, **2**) and galphimine E (GE, **3**), as well as the derivatives **4**−**7** obtained by chemical transformation of **3**, and a fraction rich in galphimines (GRF), obtained by fractionation of the MeOH extract, constituted by the mixture of natural galphimines **1**−**3** (in ratios of 2.3, 2.17, and 9.3%, respectively).

Results and Discussion

Animals treated with diazepam showed a significant increase in the percentage of time they spend in the open arms of the EPM, as well as an increase in the percentage of the number of entrances into the same arms. On the other hand, mice that received a dose of 2.0 mg/kg of picrotoxin showed a decrease of these parameters with regard to the group that received only the vehicle (Figure 1).

Administration of 15 mg/kg of galphimines **1**, **2**, **6**, and GRF caused a significant increase ($p < 0.001$) in the percentage of time that mice spend in the open arms (76.3, 68.3, 76.8, and 70.8%, respectively), related to the untreated group (42.7%). Another important parameter of this test is the percentage of number of crossings the animals make to the open arms; in this case, compounds **1**, **2**, **6**, and GRF induced a significant increase ($p < 0.001$) (72.5, 53.0, 61.8, and 60.7%, respectively) when compared with the control group (24.5%). The effect produced by these compounds was not significantly different from the effect produced by diazepam.

In regard to the structural facts of the active compounds, **2** and **6** have free hydroxyl groups at C-4, C-6, and C-7, while compound **1** has them at C-4 and C-7, and there were not significant differences in the activity of these three galphimines, despite the fact that **6** is devoid of the A ring and a saturated C-1/C-2 double bond. On the other hand, administration of 15 mg/kg i.p. of compounds **3**, **4**, **5**, and **7** did not induce changes, as related to the control group, but did show significant ($p < 0.001$) differences with the group that received diazepam. It seems that acetylation of hydroxyl groups (in compounds **3** and **4**) is detrimental to the anxiolytic effect (Figure 1). Likewise, the modification of the lactone ring to a tetrahydrofurano moiety (compound **7**) also generates a decrease in the effect. Surprisingly, when the hydrogenated derivative of galphimine E (compound **5**) was tested, it did not show any activity. However, the hydrogenated derivative of galphimine E (compound **6**), in which the lactone ring was opened, had a significant anxiolytic effect as compared to the control group. This result is also comparable to the most active natural molecules (**1** and **2**). The

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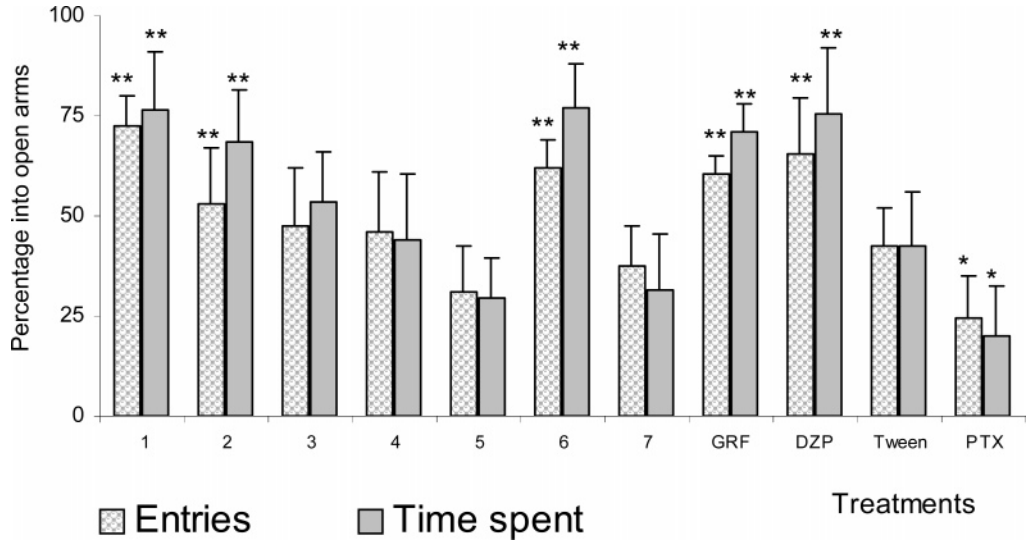


Figure 1. Effect produced by different triterpenes (**1**−**7**) and GRF on the percentage of time spent and entries of mice into the open arms of the EPM test. Except for diazepam (DZP, 1.0 mg/kg), the vehicle (Tween 20 at 5%), and the picrotoxin (PTX, 2.0 mg/kg), all the treatments were administered at a dose of 15 mg/kg.

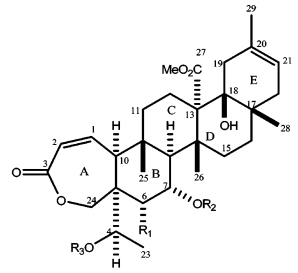
variability between **5**, **6**, and **7** regarding their anxiolytic activity could be due to a conformational modification of the molecule due to the absence of the C_1-C_2 double bond in **5** and to the presence of the tetrahydrofuran ring in **7**, which, therefore, implies a modification of the ligand−receptor interaction.

The anxiolytic activity shown by compounds **1**, **2**, and **6** was not significantly different, and it was also similar to that of GRF. This activity can be attributed to a synergistic effect between **1** and **2**, whose concentration in the fraction is very similar, but considerably lower than **3**, which did not display activity.

A previous SAR study[5] of the spasmolytic activity of natural products **1**−**3** and derivatives **4**−**7** permitted description of the relationship between the structure and the spasmolytic effect, galphimines B (**1**) and A (**2**) being the most active compounds.

When evaluating these compounds in mice exposed to the EPM,[7] in order to determine their possible anxiolytic effect, it was observed that also in this biological model the structures **1** and **2** turned out to be the most active. Apparently, there is a biological correlation between the anxiolytic effects shown in this work and the spasmolytic actions previously described.[5] In the intestine, innervated by an enteric nervous system that can function independently from CNS control,[8] at least 25 chemical messengers of different classes (observed also in the brain) are recognized.[9,10] It is possible that the isolated guinea-pig ileum test (that does not represent a psychiatric disorder model) permits prediction of the interaction of the bioactive molecules with the receptors or neurotransmitters present in both tissues, helping to track and identify useful products with anxiolytic effects.

In accordance with these results, we can argue that the preparation of an extract rich in the active natural galphimines could favor the anxiolytic effect produced by the plant in its traditional preparation, which makes it very attractive for the development and clinical evaluation of a useful medication for the treatment of anxiety disorders.



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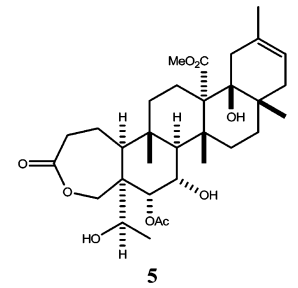
2	H	OH	H
3	H	OAc	H
4	Ac	OAc	Ac



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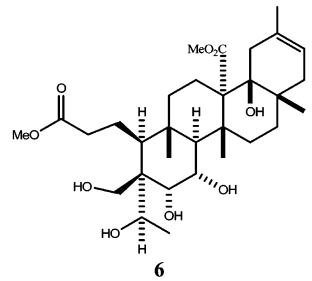

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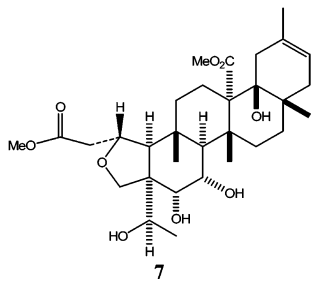

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Experimental Section

Plant Material. The aerial parts of *G. glauca* were collected in an experimental parcel of land in Xochitepec, Morelos, Mexico. Specimens were identified by M. Sc. Abigail Aguilar Contreras, Director of IMSSM Herbarium, where sample vouchers were deposited.

Isolation of the Galphimines-Rich Fraction (GRF). Plant material was dried under dark conditions at room temperature for 10 days. The dry material was milled to obtain 2−5 mm particles and then extracted by percolation with *n*-hexane. Afterward, the plant material was dried again under the same conditions and extracted exhaustively with MeOH.

The MeOH extract (12.2% yield) was dried with a rotary evaporator. The dry extract was then subjected to partition between H_2O and CH_2Cl_2. The resulting organic portion, once dried under reduced pressure, was subjected to a discoloration procedure with a gravitational column packed with activated charcoal and eluted with MeOH; this fraction had a yield of 0.26% with regard to the dried plant.

Quantification of Galphimines. Natural galphimines B (**1**), A (**2**), and E (**3**) were isolated from the GRF by reversed-phase HPLC as described.[5] Quantification of galphimines B (**1**), A (**2**), and E (**3**) in the active fraction was performed using a Waters Delta Prep 4000 modular HPLC system, consisting of a U 6K injector, a 600E pump system controller (Millenium 3.2 software), and a photodiode array UV detector at 210 nm. The analysis was carried out with an XTerra C18 (124 × 4 mm, 5 μm) column; the mobile phase was 1:1 acetonitrile−water at a flow rate of 2.0 mL min^{-1}.

Calibration curves were constructed separately for **1**−**3**, using solutions at the following dilutions: 37.5, 75, 150, 300 μg/mL in MeOH. The identities of each peak were confirmed by co-injection of purified samples of **1**, **2**, and **3**. The calibration curves were based on the peak areas of the HPLC chromatograms. All calibration curves showed good linear regression ($r^2 > 0.9860$) within test ranges. GRF (5 mg/mL) on MeOH was injected at a volume of 15 μL. The experiments were performed in three replicates, and values were expressed in terms of μg of each component on 1 mg of the active fraction as follows: galphimine B (**1**), t_R = 6.23 min, 23.33 μg/mg; galphimine A (**2**), t_R = 4.98 min, 21.73 μg/mg; galphimine E (**3**), t_R = 7.30 min, 93.06 μg/mg.

Compound 7. Galphimine E (**3**) (57 mg, 0.099 mmol) was dissolved in MeOH (5 mL) and treated with K_2CO_3 (125 mg, 0.9 mmol). The reaction was carried out at room temperature under stirring and quenched after 2 h with H_2O. The crude residue was purified by silica gel column chromatography using CH_2Cl_2−acetone (95:5, 140 mL) as isocratic eluent to give 48.1 mg (85.76%) of **7**, whose spectroscopic data were in perfect agreement with literature values.[5]

Galphimine A (2). Compound **3** (54 mg, 0.094 mmol) was dissolved in MeOH (5 mL) and treated with K_2CO_3 (125 mg, 0.9 mmol). The mixture was stirred for 10 min at 0 °C and quenched with H_2O, and the crude residue obtained by the workup was purified by preparative silica gel TLC, using CH_2Cl_2−MeOH (7:3 v/v) as eluent, to give 40 mg (80%) of compound **2**, identical in all aspects with the natural product.

1,2-Dihydrogalphimine E (5). Compound **3** (175 mg, 0.3 mmol) dissolved in MeOH (10 mL) was hydrogenated using 10% PtO_2 as catalyst (17.5 mg, 0.07 mmol). After 10 min at room temperature and under 10 lb/in. of pressure with continuous stirring, the reaction was quenched by filtration, and the residue obtained by evaporation was purified on a Celite column eluting with CH_2Cl_2. After air-dried evaporation, 95 mg (54.10%) of compound **5** (mp 247−253 °C) was obtained.[5]

Compound 6. Hydrogenation was done using 40 mg (0.075 mmol) of compound **2** and 4 mg (0.022 mmol) of PtO_2 in MeOH (10 mL). After 10 min at room temperature and under 30 lb/in. of pressure with continuous stirring, the reaction was quenched by filtration. The reaction product was purified as described for compound **5** to give compound **6** as a white powder (34.8 mg, 88.6%).[5]

4,6,7-Triacetylgalphimine A (4). Compound **3** (67.3 mg, 0.12 mmol) was dissolved in pyridine (1 mL) and treated with Ac_2O (1 mL) overnight at room temperature. The reaction was quenched with ice (5 g), obtaining a precipitate, which was filtered and washed with water to give a white solid (58.2 mg, 75.4%), identical with the natural product derivative.[5]

Animals and Drug Administration. Male ICR mice with a 32 to 38 g average weight were used. All animals were purchased in Harlan, México, and maintained for 3 weeks in our animal house with a cycle of 12 h of light and 12 h of darkness and free access to H_2O and food. Three days before the test, the animals were conditioned to the laboratory environment and to the researcher. All experiments were carried out between 8 and 13 h and conducted in accordance with international standards of animal welfare recommended by the Society of Neuroscience (USA). The experimental protocols were approved by the Institutional Research Committee. The minimum number of animals and duration of observation required to obtain consistent data were employed.

Groups of 8 mice per treatment were formed; each group was i.p. administered (1 h before the test, in a constant volume of 300 μL) with 15 mg/kg of one of the triterpenes (**1**−**7**) or GRF. Products administered were dissolved in 5% Tween 20. A control group, which received only the vehicle in the same volume and through the same route, a positive control group that was treated (i.p.) with 1.0 mg/kg of diazepam (DZP, Sigma), and an anxiogenic control group that was administered 2.0 mg/kg of picrotoxin (PTX, Sigma) were used.

Elevated Plus-Maze (EPM) Test. After the administration of treatments, mice were submitted to the EPM test, which is a widely used model to determine the anxiolytic activity of different substances in mice.[7,11] The device is made of Plexiglas and consists of two open arms (30 × 5 cm) and two closed arms (30 × 5 cm) with clear 25 cm walls. The arms extend from a central square (5 × 5 cm), and the whole device is 38.5 cm high from the floor. Animals were placed in the center of the plus-maze, and during the registration (5 min) the number of entrances and the time the animals spend in the open and closed arms, as well as the total exploring activity (number of entrances), were measured. After each session, the device was thoroughly cleaned with a clean paper towel and a solution of 10% EtOH. Each experimental session was videorecorded.

Statistical Analysis. The statistical analysis of the results was performed with the SPSS 11.0 program and based on an analysis of variance (ANOVA) followed by the Dunnet test, in which a significant difference was established among groups when the p value was lower than 0.05.

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