Anxiolytic-like actions of the hexane extract from leaves of Annona cherimolia in two anxiety paradigms: Possible involvement of the GABA/benzodiazepine receptor complex

ARTICLE *in* LIFE SCIENCES · FEBRUARY 2006

Impact Factor: 2.7 · DOI: 10.1016/j.lfs.2005.05.078 · Source: PubMed

Journal of Ethnopharmacology 139 (2012) 164–170

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

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

Antidepressant-like effects of an alkaloid extract of the aerial parts of *Annona cherimolia* in mice

M. Martínez-Vázquez [a], R. Estrada-Reyes [b,*], A.G. Araujo Escalona [b], I. Ledesma Velázquez [b], L. Martínez-Mota [c], J. Moreno [d], G. Heinze [e]

[a] *Instituto de Química, Universidad Nacional Autónoma de México, Coyoacán, México, D.F. 04510, Mexico*
[b] *Laboratorio de Fitofarmacología, Dirección de Investigaciones en Neurociencias, Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz, Calzada México-Xochimilco 101, Col. San Lorenzo Huipulco, Delegación Tlalpan, Mexico City 14370, Mexico*
[c] *Laboratorio de Farmacología Conductual, Dirección de Investigaciones en Neurociencias, Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz, Calzada México-Xochimilco 101, Col. San Lorenzo Huipulco, Delegación Tlalpan, Mexico City 14370, Mexico*
[d] *Laboratorio de Servicios Clinicos, Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz, Calzada México-Xochimilco 101, Col. San Lorenzo Huipulco, Delegación Tlalpan, Mexico City 14370, Mexico*
[e] *Departamento de Psiquiatría y Salud Mental, Facultad de Medicina, Universidad Nacional Autónoma de México, Edificio F s/n, Circuito Interior y Cerro del Agua, Ciudad Universitaria, Coyoacán, México, D.F. 04510, Mexico*

ARTICLE INFO

Article history:
Received 2 June 2011
Received in revised form 12 October 2011
Accepted 26 October 2011
Available online 9 November 2011

Keywords:
Antidepressant
Alkaloids
Aporphines
Forced swimming test

ABSTRACT

Ethnopharmacological relevance: Several species of *Annona* (Annonaceae) are used in traditional Mexican medicine by their anti-anxiety, anticonvulsant and tranquilizing properties. It has been reported that the alkaloids isolated from some species of the *Annona* have affinity to serotonergic 5-HT_{1A} receptors and modulate dopaminergic transmission, which is involved in depressive disorders.
Aim of the study: To investigate the antidepressant-like effect of an alkaloid extract from the aerial parts of *Annona cherimola* (TA) in mice.
Materials and methods: The antidepressant-like effect was evaluated in the forced swimming test. To elucidate a possible mechanism of action, experiments of synergism with antidepressant drugs, such as imipramine (IMI), clomipramine (CLIMI), and fluoxetine (FLX), were carried out. The neurotransmitter content (DA: dopamine, 5HT: serotonin and its metabolites, HVA: homovanillic acid and 5HIAA: 5-hydroxyindoleacetic) in the whole brain of mice were also determined by HPLC method. TA chemical composition was determined using high performance liquid chromatography–electrospray mass spectrometry.
Results: The results showed that repeated treatment with TA produced antidepressant-like effects in mice. This effect was not related to an increase in locomotor activity. Administration of TA facilitated the antidepressant effect of IMI and CLIMI as well as increased the turnover of DA and 5-HT. The alkaloids: 1,2-dimethoxy-5,6,6a,7-tetrahydro-4*H*-dibenzoquinoline-3,8,9,10-tetraol, anonaine, liriodenine, and nornuciferine were the main constituents of TA.
Conclusions: Results showed that TA produces an antidepressant-like action from a generalized increase in monominergic turnover, supporting the use in tradicional medicine of *Annona cherimolia*, and strongly suggest its therapeutic potency as an antidepressant agent.

1. Introduction

Depression is a common mental disorder and one of the most important causes of disability in the world, with a heavy social burden and a substantial lifetime risk (WHO, 2010). Depression is frequently recurrent and chronic, and it has been associated with suicide risk and psychosocial dysfunction (Emslie et al., 2005). Antidepressant therapy includes drugs with exceptional structural chemical diversity; most of them increase monoaminergic neurotransmission (Elhuwuegi, 2004). Although the majority of the antidepressant drugs ameliorate depressive symptoms, they exert multiple unwanted side effects; moreover, 30% of depressive patients do not react appropriately to the first-line treatment (Fava and Rush, 2006). Thus, the search for more efficacious and well-tolerated drugs is in progress. The need for the discovery and development of new pharmaceuticals for the treatment of depression demands that all approaches to drug discovery be exploited. Among the possible approaches, the use of natural products has made many unique and vital contributions to drug discovery

* Corresponding author. Tel.: +52 41605080l; fax: +52 5556559980.
E-mail addresses: estrarosa@hotmail.com, restrada@imp.edu.mx (R. Estrada-Reyes).

0378-8741/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jep.2011.10.033

(Newman et al., 2003). In this regard, many medicinal plants have been used as a treatment for stress, sadness, anxiety and depression (Zhang, 2004).

Several species of *Annona* (Annonaceae) are used in traditional Mexican medicine due to their anti-anxiety, anticonvulsant, and tranquilizing properties (Tortoriello and Romero, 1992; Gupta, 1995). Experiments with animal models have confirmed the anxiolytic-like and anticonvulsant activities of the polar and hexane extracts of *Annona diversifolia*, *Annona muricata* and *Annona cherimolia* (Gouemo et al., 1996; Hasrat et al., 1997a; González-Trujano et al., 2001). We previously reported the anxiolytic-like effect of a hexane extract of *Annona cherimolia* in mice, on the same way we described the toxicity of methanol extract of this specie (López-Rubalcava et al., 2006).

It is well known that the Annonaceae family is rich in alkaloids (Leboeuf et al., 1982). Previous studies have shown that alkaloids isolated from species of the *Annona* genus possess an affinity for the 5-HT_{1A} receptors *in vitro* and participate in dopamine biosynthesis (Protais et al., 1995; Hasrat et al., 1997a,b; Lee et al., 2008). The 5-HT_{1A} receptors have been implicated in depressive disorders and in the effect of antidepressant drugs (Blier and Montigny, 1994; Martínez-Mota et al., 2002; Blier and Ward, 2003). Furthermore, dopamine mediates the antidepressant effect of tricyclic antidepressants in the forced swimming test (FST) (Cervo and Samanin, 1987). Thus, it has been proposed that alkaloids derived from the *Annona* could induce antidepressant-like effects, however, to our knowledge there have been no *in vivo* behavioral studies to confirm this proposal. The first objective of the present study was to evaluate the antidepressant-like effect of an alkaloid extract from aerial parts of *Annona cherimolia* (TA) in mice using the forced swimming test (FST). On the other hand is very well-known that mechanism of some classic antidepressants, such as imipramine (IMI), clomipramine (CLIMI) and fluoxetine (FLX), involves their interaction with neurotransmitter systems, for example, IMI interacts mainly with the noradrenergic system, while CLIMI and FLX interact with the noradrenergic/serotonergic and serotonergic systems, respectively (Elhuwuegi, 2004). To evaluate if TA produces an effect similar to those of IMI, CLIMI and FLX, the participation of these neurotransmitter systems in the antidepressant-like actions of the TA was analyzed. To this end, two experiments were conducted: (1) sub-effective doses of both TA and antidepressant drugs were combined to determine a possible synergism, and (2) the levels of monoamines and their main metabolites were measured in the whole brain of mice treated with TA. Finally, to identify the exact identity of the potentially active alkaloids as well as their proportion in the antidepressant-like extract, the chemical composition of TA was examined by HPLC–ESI-MS analysis.

2. Methods

2.1. Plant material

Aerial parts of *Annona cherimolia* were collected in Metepec, Puebla, Mexico, and identified by Dr. N. Diego (Facultad de Ciencias, UNAM). A voucher specimen (No. 584162) was deposited at the *Herbario del Instituto de Biología*, UNAM.

2.2. Preparation of alkaloids extract from aerial parts of Annona cherimolia

The air-dried leaves (3600 g) were ground into powder and exhaustively extracted with hexane and methanol, successively, at room temperature for 48 h, and the solvent was evaporated by vacuum; 74.23 g of hexane extract (2.85% dry weight) and 29 g of methanol extract (0.80% dry weight) were obtained. The methanol extract was diluted with H_2O and subsequently basified (pH = 8) with a concentrated solution of NH_4OH followed by extraction with $CHCl_3$ (3× 20 mL). The organic layer afforded 400 mg (0.011%) of total crude alkaloids of *Annona cherimolia* (TA), which gave a positive result when examined by the Dragendorff and Mayer tests for alkaloids (Domínguez, 1973). One aliquot of the extract was separated by preparative TLC and eluted with $CHCl_3$–MeOH (9:1) the alkaloids; anonaine, liriodenine and nornuciferine were identified by comparison of their physical and spectroscopic data with those reported. The alkaloids isolated from the TA were used as standards in the HPLC–ESI-MS analysis.

2.3. Chemical characterization of TA

To identify the constituents of the active alkaloid extract, an aliquot (5 mg) was analyzed using high performance liquid chromatography–electrospray mass spectrometry (HPLC–ESI-MS). The analysis was carried out with an analytical HPLC Waters 600 system equipped with a Supelcosil LC-NH_2, 5 μm, 250.0 mm × 4.6 mm column and the photodiode array detector, Waters 996, coupled to the ion tramp mass spectrometer, Bruker Esquire 6000. The entire analysis was performed at 25 °C. The mobile phase was a mixture of acetonitrile/water (5:5) at flow rate 1.0 mL/min, and the detection wavelength was set at 220 nm. A full-scan mass spectrum, in positive ion mode, was acquired over a range of *m*/*z* 50–1000 amu with a scan cycle time of 2 s. The ESI-IT source conditions were adjusted as follows: for N_2 drying the capillarity temperature was set to 350 °C, the spray voltage was set to 40.0 V, the auxiliary gas pressure was 30 psi, and the dry gas flow rate was 10 L/min. An additional aliquot from this extract was used for biological tests. The compounds of the active extract were identified and quantified by comparison of their retention time (RT), HPLC peak, and M^+. Anonaine [B], liriodenine [D] and nornuciferine [C] were used as standards. The HPLC peak areas were not corrected for response factors and are reported as the relative percentage of area (Estrada-Reyes et al., 2010).

2.4. Animals

Adult male Swiss Webster mice, weighing 25–30 g, were used in all experiments. All animals were housed in plastic cages (44 cm × 21 cm × 21 cm), with eight animals per cage, in a temperature-controlled room (20–21 °C) under a 12:12 h inverted light/dark cycle condition (lights on at 22:00 h). Animals had free access to Purina mice chow and water during the complete experiment. The management of the animals was performed in agreement with the general principles of laboratory animal care (NIH publication # 85-23, revised in 1985) and the "Norma Oficial Mexicana" (NOM-062-ZOO-1999). Additionally, the local ethics committee approved the experimental protocol.

2.5. Drugs

All drugs used in this study were injected intraperitoneally (i.p.) in a total volume of 10.0 mL/kg. The TA fresh extract was dissolved in saline solution (0.9%). Sigma-Ultra IMI hydrochloride (Sigma Chemical Co., St. Louis, MO, USA), FLX hydrochloride (Sigma Chemical Co., St. Louis, MO, USA) and CLIMI hydrochloride (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in saline solution (0.9%). The animals from the control group received the vehicle (NaCl 0.9%) in a volume of 10.0 mL/kg. EDTA disodium salt, L-cysteine hydrochloride anhydrous and 1-octanesulfonic acid sodium salt was purchased from Sigma (St. Louis, MO, USA); PIC Reagent D-4 dibutylamine phosphate was obtained from Millipore, Waters Chromatography Division (Millford, MA, USA). HPLC-grade glacial acetic was purchased from J.T. Baker Inc. (Paris,

Kentucky). Anhydrous sodium acetate from E. Merck (Darmstadt FR, Germany) and dibasic ammonium phosphate from Mallinckrodt AR. Milli Q WATER (Millipore, Bedford, MA, USA) were used. Chromatography-grade acetonitrile was obtained from J.T. Baker Inc. (Paris, Kentucky).

HPLC standards: Dopamine hydrochloride (DA) and its metabolite, homovanillic acid (HVA), and serotonin (5HT, creatinine sulfate complex) and its major metabolite 5-hydroxyindoleacetic acid (5HIAA), were purchased at reagent-grade purity from Sigma Chemical Co (St. Louis, MO, USA).

2.6. Forced swimming test (FST)

The forced swimming is the most widely used and recognized test for assessing antidepressant-like activity in rodents (Porsolt et al., 1977a,b; Martínez-Mota and Fernández-Guasti, 2004; Martínez-Mota et al., 2008). In this model, rodents are forced to swim in conditions from which they cannot escape and rapidly become immobile, floating in an upright position and making only small movements to keep their heads above water. The development of immobility reflects the cessation of persistent escape-directed behavior or learned helplessness, and a decrease in the duration of immobility, i.e., with antidepressant drugs, is interpreted as an antidepressant-like effect (Porsolt et al., 1977b).

Mice were individually placed in glass cylinders (height: 21 cm, diameter: 14.5 cm) containing 15 cm of water at 23 ± 1 °C. All animals were forced to swim for a 15-min period (pre-test), followed by a 3-min session (test) at 24 h later. Drugs and TA were administered before the test depending on the different experimental designs (*vide infra*).

After the swimming sessions, the mice were removed from the cylinder, carefully dried, placed in heated cages for 20 min and then returned to their home cages. All test sessions were videotaped and later registered by an observer that was unaware of the pharmacological treatments.

2.7. Experiment I: Effect of a single administration of TA in the FST

It has been demonstrated that a single administration of antidepressant drugs is able to produce antidepressant-like reactions in the FST (Porsolt et al., 1977b). On this basis, fifteen independent groups of mice ($n = 16$ per group) were used in this experiment. Four groups were injected with TA at 0.0, 5.0, 10.0, and 20.0 mg/kg (i.p. 60 min before the test) while eleven independent groups were used as positive controls receiving IMI at 0.0, 12.5, 25.0 and 32.0 mg/kg (i.p. 30 min before the test), CLIMI at 0.0, 12.5, 25.0 and 50 mg/kg (i.p. 30 min before the test) and FLX at 0.0, 10.0 and 15.0 mg/kg (i.p. 30 min before the test). Doses and latencies for TA were obtained from previous pilot studies conducted in the laboratory; the schedule of antidepressant drugs was selected from literature data (Porsolt et al., 1977b).

2.8. Experiment II: Effect of repeated administration of TA in the FST

Considering that repeated administration of antidepressants drugs is effective in reducing immobility in the FST (Harkin et al., 1999; Castagné et al., 2001; Rogóz et al., 2005), this schedule was also used to evaluate the effects of TA. Four groups of mice were treated with TA (0.0, 5.0, 10.0, and 20.0 mg/kg) at 24, 7 and 1 h before the FST. The control group received vehicle at the same scheme.

Positive control groups ($n = 16$) were administered with IMI (0.0, 12.5, 25.0, and 32 mg/kg), CLIMI (0.0, 12.5, and 25.0 mg/kg) or FLX (0.0, 5.0, 10.0, and 15.0 mg/kg), respectively, at 24, 7 and 0.5 h before the test. The last injection of each drug was given with consideration of the latency for each compound that was established in the acute experiment. The number of animals for each group was 16. All groups of animals were used to determine changes in the levels of monoamines and their main metabolites in the whole brain.

Table 1
Schedule of administration of total alkaloids extract (TA) from *Annona cherimolia* in combination with antidepressant drugs imipramine (IMI), clomipramine (CLIMI) and fluoxetine (FLX).

Time before the FST 3-min session	
−60 min	−30 min
Vehicle	Vehicle
TA 5 mg/kg	IMI 12.5 mg/kg
TA 10 mg/kg	IMI 12.5 mg/kg
TA 5 mg/kg	CLIMI 12.5 mg/kg
TA 10 mg/kg	CLIMI 12.5 mg/kg
TA 5 mg/kg	FLX 10 mg/kg
TA 10 mg/kg	FLX 10 mg/kg
TA 5 mg/kg	FLX 15 mg/kg
TA 10 mg/kg	FLX 15 mg/kg

Schedule of administration for TA and the antidepressant drugs (timing of administration and dosage) was taken from experiment I. All animals used in this study were subjected to the pre-test session 24 h before the 3-min FST session.

2.9. Experiment III: Combination of the TA with IMI, CLIMI or FLX in the FST

To determine whether TA is able to enhance the effect of antidepressant drugs in the FST, an experiment of synergism with sub-threshold doses was designed. Independent groups of mice ($n = 16$ per group) were treated with ineffective doses of TA in combination with sub-threshold doses of each antidepressant drug, according to the schedule presented in Table 1. The doses were chosen based on the results obtained in experiment I.

2.10. Activity test (open field test, OFT)

To discard nonspecific effects of the antidepressant drugs or TA, the spontaneous locomotor activity of all mice was measurement in the open field test just before the beginning the FST. The apparatus consisted of an opaque-Plexiglas box (40 cm × 30 cm × 20 cm) with the floor divided in 12 equal squares. Each animal was placed in the center of the apparatus and its behavior was videotaped during a 5 min session. An observer, blind to the pharmacological treatment, registered the number of times the animal crossed to each square (counts/5 min). A count is considered when an animal crosses a square to the next with 100% of its body. A decreased number of counts are considered as a lower locomotor activity. The test box was carefully cleaned after each recording. To prevent behavioral changes of the animals after the first experience, mice were tested only once.

2.11. Analysis of neurotransmitters and their metabolites by HPLC

It has been described that the antidepressant effects of drugs in the FST are related to changes in monoaminergic neurotransmitters (Miura et al., 1999), therefore we evaluated whether TA is able produce changes in the concentrations of biogenic monoamines and their main metabolites in the whole brain of mice subjected to the FST.

Mice used on the experiment II were sacrificed by decapitation at 20 min after FST and the brains were rapidly removed. The brains were placed in 800 μL of ice-cold 0.1 M perchloric acid. Individual brain samples were homogenized and centrifuged at 20,000 × *g* at

4 °C and stored in the dark freezer at −70 °C until further analysis. The pellets were dissolved in a 10 mM NaOH solution for protein determinations using the Bradford protein assay (Bradford, 1976).

The samples were filtered through a 0.45-μm filter and 10 μL was injected in an HPLC system. The HPLC eluent system consisted of an aqueous component of acetonitrile (95:5, v/v), with the aqueous component of 12.16 mM citric acid, 11.60 mM $(NH_4)_2HPO_4$, 2.34 mM sodium octylsulfonate, 3.32 mM DBAP and 1.11 mM disodium EDTA. The pH of the eluent was adjusted to 3.71 with NaOH 2N after acetonitrile addition and filtered through a 0.45-μm filter; the eluent was used at a flow-rate of 1 mL/min (the pressure was approximately 13.1 MPa).

2.12. Conditions of HPLC analysis

The 250 mm × 4.6 mm I.D., stainless-steel column (Phenomenex, Rancho Palos Verdes, CA, USA) was packed with 5 μm Spherisorb ODS-2 (phase separations, Deeside, UK). The column was used at 30 ± 0.2 °C. The chromatographic system consisted of a Jasco PU-2085 plus pump, with a Jasco AS-2057 plus autosampler, a Guard-Pak guard-column (with μBondapak C_{18} inserts), and a Millennium 32 software system (for system control, data acquisition and reduction, operating via a System Interface Module); the entire system was obtained from Millipore Waters. The detection system included an Antec Leyden model Decade II Electrochemical detector with an Antelec Leyden model Sencell ISAAC flow-cell assembly (range: 1 nA, filter: 0.005 Hz, E_{ox} = 0.60 V, basal ±0.001 V, I_c = 2.72 nA).

2.13. Statistical analysis

The results of behavioral tests are presented as the averages ± SEM. Data were analyzed with a Kruskal–Wallis analysis of variance on ranks ($^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$), followed by the Mann–Whitney rank sum test.

The results of the analysis of neurotransmitters and their metabolites by HPLC are presented as the averages ± SEM of groups of 4–6 animals. One-way ANOVA tests followed by the *post hoc* Tukey's test ($^*p < 0.05$) were used to make comparisons.

The Sigma Stat Program (version 3.5, Jandel Scientific) and Sigma Plot (version 10, Jandel Scientific), respectively were used to carry out al statistical analyses and graphics.

3. Results

3.1. Chemical analysis of TA

The chromatographic separation of TA resulted in the isolation of the oxo-aporphine alkaloid, liriodenine ($C_{17}H_9O_3N$), and two aporphine alkaloids, anonaine ($C_{17}H_{15}O_2N$) and nornuciferine ($C_{18}H_{19}O_2N$). These compounds were identified by their physical and spectroscopic properties and by comparison with published data (Smith and Sood, 1971; Achenbach and Schwinn, 1995; Hu et al., 2010). Fig. 1 shows the HPLC profile and formulae of each alkaloid identified. The TA is a mixture of the oxo-aporphine alkaoid, liriodenine [D] (RT = 5.38 min, 22.38% relative area; M^+ 275), and three aporphine products, 1,2-dimethoxy-5,6,6a,7-tetrahydro-4*H*-dibenzoquinoline-3,8,9,10-tetraol [A] (RT = 3.1 min; 30.59% relative area; M^+ 345), anonaine [B] (RT = 3.6 min; 33.99% relative area; M^+ 265), and nornuciferine [C] (RT = 5.1; 2.28% relative area; M^+ 281).

3.2. Behavioral study

3.2.1. Experiment I

The effect of a single administration of TA on FST is shown in Fig. 2.

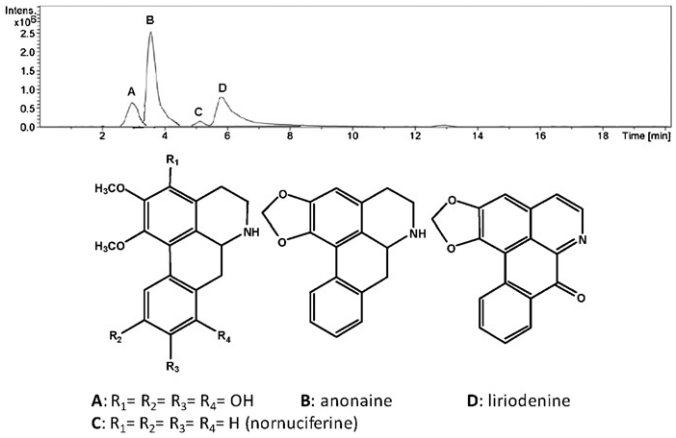

Fig. 1. HPLC profile of TA and formulae of alkaloids identified.

Both TA (H = 6.937, df = 3, p = 0.074) and FXL (H = 0.923, df = 2, p = 0.630) produced a non-significant reduction of immobility time. In contrast, IMI (H = 25.497, df = 3, $p \leq 0.001$) and CLIMI (H = 20.810, df = 3, $p \leq 0.001$) reduced the immobility time significantly at the medium and highest doses. As shown in Table 2, none of the tested doses of the antidepressant drugs or TA affected the general locomotor activity.

3.2.2. Experiment II

The results in Fig. 3 show that a repeated administration of IMI (H = 56.134, df = 3, $p \leq 0.001$), CLIMI (H = 44.314, df = 2, $p \leq 0.001$), FLX (H = 62.156, df = 3, $p \leq 0.001$), and TA (H = 24.650, df = 3, $p \leq 0.001$) produces a significant decrease in the time of immobility. Dose–response curves were obtained with IMI, CLIMI, and FLX; interestingly, TA showed a pharmacological effect similar to the antidepressant drugs. The results shown in Table 2 show that these treatments did not produce significant differences in the number of crossings when compared with the control groups in the open field test.

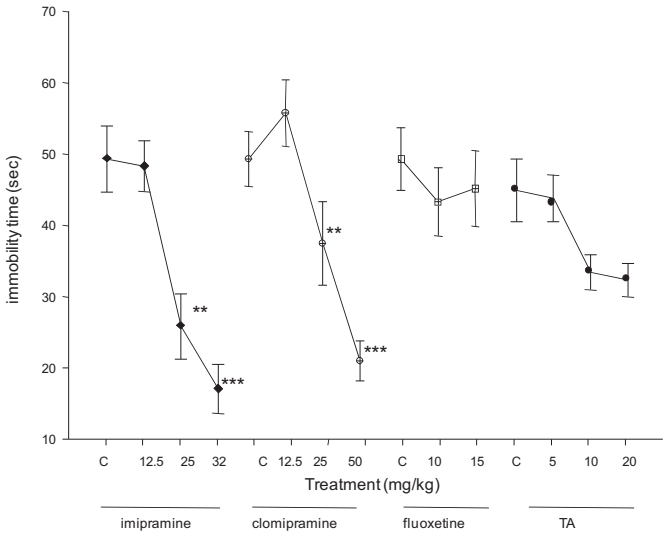

Fig. 2. Effect of a single administration of TA on the FST. Effects of acute administration of TA, imipramine, clomipramine, and fluoxetine on the FST. All results are expressed as the averages ± SEM of 16 animals. Comparisons were made using a Kruskal–Wallis one-way analysis of variance on ranks, followed by the Mann–Whitney *U*-test: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 2
Effect of single and repeated administration of TA and several antidepressant drugs on the ambulatory activity in the open field test.

Treatment (mg/kg)	Activity (number of counts/5 min) average ± SEM Single administration	Activity (number of counts/5 min) average ± SEM Repeated administration
Control	70.37 ± 7.32	68.37 ± 2.82
TA 5	74.53 ± 5.2	68.00 ± 5.62
TA 10	69.53 ± 6.32	63.50 ± 3.67
TA 20	72.37 ± 5.89	60.62 ± 6.23
IMI 12.5	61.29 ± 3.20	75.50 ± 5.86
IMI 25	59.12 ± 6.18	72.00 ± 4.45
IMI 32	73.37 ± 4.03	75.62 ± 3.58
CLIMI 12.5	62.02 ± 3.67	78.12 ± 2.58
CLIMI 25	71.42 ± 3.44	78.50 ± 4.71
CLIMI 50	73.650 ± 5.19	—
FLX 5	—	67.66 ± 7.96
FLX 10	65.90 ± 6.11	76.16 ± 4.19
FLX 15	67.62 ± 5.47	59.66 ± 3.81

Single administration	Repeated administration
[a]IMI data: $H = 0.171$, df = 3, $p = 0.982$	[a]IMI data: $H = 1.021$, df = 3, $p = 0.796$
[b]CLIMI data: $H = 4.630$, df = 3, $p = 0.20$	[b]CLIMI data: $H = 1.466$, df = 2, $p = 0.480$
[c]FLX data: $H = 6.392$, df = 2, $p = 0.151$	[c]FLX data: $H = 5.945$, df = 3, $p = 0.114$
[d]TA data: $H = 3.602$, df = 3, $p = 0.308$	[d]TA data: $H = 3.453$, df = 3, $p = 0.327$

All of the results were expressed as the averages ± SEM of groups of 16 animals each. Comparisons were made by using an Kruskal–Wallis one way analysis of variance on ranks, followed by Mann–Whitney *U*-test.
$^{*}p < 0.05$ versus vehicle treated group.
$^{**}p < 0.01$ versus vehicle treated group.
$^{***}p < 0.001$ versus vehicle treated group.
[a] IMI data versus vehicle treated group.
[b] CLIMI data versus vehicle treated group.
[c] FLX data versus vehicle treated group.
[d] TA data versus vehicle treated group.

3.2.3. Experiment III

Table 3 shows the results of the effect of the combined administration of sub-optimal doses of TA (5 and 10 mg/kg) with sub-threshold doses of IMI (12.5 mg/kg), CLIMI (12.5 mg/kg) and FLX (10 and 15 mg/kg). The combination of TA at 5 and 10 mg/kg facilitated the antidepressant effect of a suboptimal dose of CLIMI (12.5 mg/kg), producing a reduction of immobility time of 29.6% and 46.8% ($H = 15.83$, df = 2, $p \leq 0.001$), respectively, whereas TA at 10 mg/kg in combination with IMI (12.5 mg/kg, $H = 18.26$, df = 2, $p \leq 0.001$) produced a statistically significant reduction (58.73%) in the immobility time. These results suggest that TA enhanced the effects of IMI and CLIMI. In contrast, the combined administration of TA (5 and 10 mg/kg) and FLX (10 and 15 mg/kg) produced an insignificant decrease (23.63%) in the immobility time at the highest doses of both compounds ($H = 5.63$, df = 4, $p = 0.072$). None of the combinations tested in the FST produced significant changes in the locomotor activity test (data not shown).

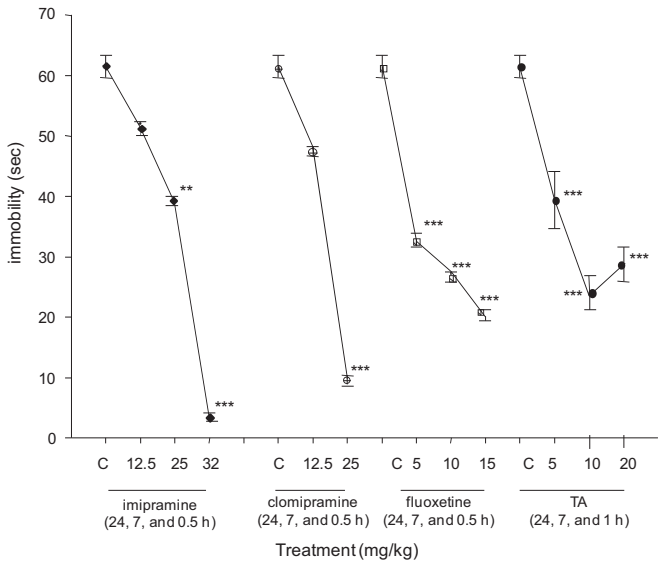

Fig. 3. Effect of a sub-chronic administration of TA on the forced swimming test. Effect of triple administration of imipramine, fluoxetine, clomipramine, and TA on the forced swimming test (immobility time; seconds). All results are expressed as the averages ± SEM of 16 animals. Comparisons were made by using an Kruskal–Wallis one way analysis of variance on ranks, followed by Mann–Whitney *U*-test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 3
Effect of the combined treatment of the total alkaloids extract (TA) from *Annona cherimolia* with the antidepressant drugs imipramine (IMI), clomipramine (CLIMI) and fluoxetine (FLX) on immobility behavior in the FST.

Treatment 1	Treatment 2	% Immobility time
Vehicle	Vehicle	100
TA 5 mg/kg	IMI 12.5 mg/kg	85.69
TA 10 mg/kg	IMI 12.5 mg/kg	41.27***
TA 5 mg/kg	CLIMI 12.5 mg/kg	70.33*
TA 10 mg/kg	CLIMI 12.5 mg/kg	53.23**
TA 5 mg/kg	FLX 10 mg/kg	105
TA 10 mg/kg	FLX 10 mg/kg	127.23
TA 5 mg/kg	FLX 15 mg/kg	98
TA 10 mg/kg	FLX 15 mg/kg	76.37

All doses used in this study were considered ineffective according results of experiment I. Results of Mann–Whitney *U*-test.
$^{*}p < 0.05$ versus vehicle treated group.
$^{**}p < 0.01$ versus vehicle treated group.
$^{***}p < 0.001$ versus vehicle treated group.

3.3. Neurotransmitters and their metabolites by HPLC

An effective dose of TA (10 mg/kg, repeated administration) produced in the whole brain a consistent increase of DA and 5-HT levels, and it also produced an increase in HVA and 5-HIAA levels, main metabolites of DA and 5-HT, respectively. Thus, the extract induced an increase in the turnover rates of these neurotransmitters.

The antidepressant drugs IMI (25 mg/kg) and CLIMI (25 mg/kg) reduced significantly the HVA/DA ratio, which was associated with an insignificant reduction of both the neurotransmitter and its metabolite. IMI increased the 5HIAA/5-HT ratio turnover by a reduction in the 5-HT levels; whereas, CLIMI produced an insignificant decrease in 5-HT and 5HIAA levels without modifying significantly the 5HIAA/5-HT ratio. Finally, FXL (15 mg/kg) induced a reduction in HVA/DA ratio due to a significant increase in the DA levels and a reduction in its metabolite (Table 4).

4. Discussion

The antidepressant drugs used in the clinic today have heterogeneity in the therapeutically response, multiple side effects and high economic cost. Furthermore, treatment of depression with conversional antidepressant drugs provides a complete remission in 70% of the individuals treated (Fava and Rush, 2006). Therefore, the study of the antidepressant-like effects of herbs is an increasing interest (Newman et al., 2003). Medical therapies with plants may be effective alternatives in the treatment of depression, and the research of their effects has progressed significantly since the past decade (Hasrat et al., 1997a,b). In this regard, several *Annona* species have been used in traditional medicine due to their sedative, anxiolytic and tranquilizing properties. Previous

Table 4
Effects of the alkaloid extract of *Annona cherimolia*, imipramine, clomipramine, and fluoxetine on the levels of the monoamine concentration in the mouse whole brain.

Treatment (mg/kg)	Tissue content of monoamines and indolamines (ng/mg of protein)					
	DA	HVA	HVA/DA	5HT	5HIAA	5-HIAA/5-HT
Vehicle	6.637 (0.274)	2.157 (0.262)	0.324 (0.051)	2.863 (0.189)	1.0 (0.0910)	0.354 (0.047)
TA10	7.770* (0.292)	4.944** (0.542)	0.636*** (0.036)	3.050** (0.19)	1.433* (0.041)	0.475*** (0.034)
IMI 12.5	5.656 (0.145)	0.743*** (0.011)	0.131* (0.005)	1.945 (0.0802)	1.073 (0.066)	0.569*** (0.032)
CLIMI 12.5	5.314 (0.155)	0.622*** (0.015)	0.117** (0.003)	1.865 (0.0841)	0.773 (0.033)	0.420 (0.022)
FLX 15	8.452* (0.992)	0.549*** (0.05)	0.072** (0.008)	3.492 (0.279)	1.237 (0.204)	0.279 (0.013)
	$F_{4,21} = 10.52$, $p \leq 0.001$	$F_{4,21} = 35.37$, $p \leq 0.001$	$F_{4,15} = 41.90$, $p \leq 0.001$	$F_{4,13} = 14.57$, $p \leq 0.001$	$F_{4,19} = 5.67$, $p \leq 0.004$	$F_{4,20} = 11.16$, $p \leq 0.001$

DA: dopamine; HVA: homovanillic acid; 5HT: serotonin; 5HIAA: 5-hydroxyindoleacetic acid; IMI: imipramine; CLIMI: clomipramine; FLX: fluoxetine; TA: alkaloids extract *Annona cherimolia*; ND: not determined.
Data represent the averages ± SEM of groups of 14–16 animals. Comparisons were made by using a one-way ANOVA followed by the *post hoc* Tukey's test.
* $p < 0.05$ versus vehicle treated group.
** $p < 0.01$ versus vehicle treated group.
*** $p < 0.001$ versus vehicle treated group.

results obtained by *in vitro* assays implicate the therapeutic potential of aphorphinic and berberinic alkaloids from the Annonaceae species on mood disorders (Gupta, 1995). The present study is the first, to our knowledge, to show an antidepressant-like activity produced by an alkaloid extract from the aerial parts of *Annona cherimolia*, as determined by the forced swimming test, in the most useful animal model that is sensitive to antidepressant drugs. FST induced a state of "hopelessness" and/or "abandonment" in mice that was analogous to those showed by depressed people. It has been demonstrated that antidepressant drugs reduce this behavior of abandonment in mice (Castagné et al., 2001). In addition, several extracts from plants have been evaluated in this model with positive results (Chen et al., 2005; Sánchez-Mateo et al., 2007; Machado et al., 2009).

In the present study, the acute administration of TA (5–20 mg/kg) produced a reduction of immobility time, although this effect was not statistically significant. Notably, the immobility time after TA treatment could be qualitatively positioned between the treatments with FLX, which did not show any effect and those effects of treatment with IMI and CLIMI that both showed convincing antidepressant effects in this schedule. As for other antidepressant drugs (Porsolt et al., 1977b), repeated administration of TA (three injections) produced a more consistent antidepressant-like effect in the FST as evidenced by a major reduction in the immobility time. Treatment with 5 mg/kg doses resulted in a reduction of 17.5% for acute administration and 34.0% for repeated administration, while treatment with 10 mg/kg doses resulted in reductions of 43.3% and 60.4%, respectively. Interestingly, the dosage required for TA to produce its antidepressant-like effect (5–10 mg/kg) was the same as the doses required for FLX in this schedule.

To avoid false positive results in the FST, it is important to rule out the possibility that reductions in the immobility time were not merely a result of the psychostimulant effects of the extract (Martínez-Mota et al., 2008). In our study, the repeated administration of TA did not increase locomotor activity at doses that produced an antidepressant-like effect, indicating that the specific actions of this extract on the behavioral model are predictive of antidepressant activity. In addition, the lack of a significant antidepressant-like effect with a single administration of TA was not influenced by changes in locomotor activity, i.e., by hypoactivity.

The results showed in the neurochemical evaluation indicate that the repeated administration of TA at 10 mg/kg produced a generalized increase in the 5-TH and DA turnover in the whole brain of mice, through both an increase in the concentration of the neurotransmitters and their metabolism. Considering the behavioral actions of this treatment it is possible to suggest that the antidepressant-like actions of TA (at a repeated treatment) are mediated by a mechanism in which participate monoamines. In agreement with the neurochemical data, the effects of TA are similar to those induced by IMI and CLIMI in virtue of producing an increase in the serotonergic turnover, but different on the dopaminergic system, in which both antidepressant drugs induced a reduction in the DA turnover. Previous information of *in vitro* studies suggest that the alkaloids derived from Annonaïs species participate in both DA synthesis and dopaminergic neurotransmission (Protais et al., 1995; Lee et al., 2008), and some of them have been proposed as agonist of serotonergic receptors that participate in depression (Hasrat et al., 1997a,b). Thus this is the first study that relates the antidepressant effects of TA with an enhancement of the DA and 5-HT neurotransmission. Several data of our study may suggest that the serotonergic system have secondary participation in the actions of TA in the FST since: (a) TA did not synergize with the serotonin selective reuptake inhibitor, FLX, to enhance their antidepressant-like actions in mice; (b) TA increases the turnover rate of 5-HT in contrast with FLX, which induces a reduction in the turnover rate of 5-HT in the whole brain of mice. Although both behavioral and neurochemical evaluations point towards a mechanism of action mediated by monoamines, we cannot rule out, the mediation of other neurotransmitter systems in the antidepressant-like effect of TA. Further studies should be conducted to test the specific participation of each monoamine or other neurotransmitter systems in such actions of TA in the FST.

The HPLC analyses of TA revealed the presence of 1,2-dimethoxy-5,6,6a,7-tetrahydro-4*H*-dibenzoquinoline-3,8,9,10-tetrol, anonaine, nornuciferine and liriodenine. The agonistic properties of anonaine and nornuceferine have been reported in functional assays on NIH-3T3 cells stably transfected with 5-HT_{1A} receptors from man (Hasrat et al., 1997a). The activities of these compounds could be relevant for the antidepressant-like effects the TA. Further studies are necessary to determine the individual participation of the alkaloids present in the active extract.

These results are consistent with the reported ethnopharmacological effect of this specie. Notably, the effect produced by the alkaloid extract of *Annona cherimolia* was similar to IMI and CLIMI, classical antidepressant drugs that are widely used in clinical therapy, and this alkaloid extract could help in antidepressant therapy.

5. Conclusions

Repeated administration of TA induces antidepressant-like effects in mice as determined by the FST. TA did not induce changes in the locomotor activity or sedative effect because no sedation is observed in mice receiving doses of up to 40-mg/kg. The results of the present study provided evidence of the antidepressant-like effect produced by an alkaloid extract from the aerial parts of *Annona cherimolia*. This effect seems to result from a generalized

increase in monominergic turnover. The results support the use of *Annona cherimolia* in traditional medicine and strongly suggest its therapeutic potency as an antidepressant agent.

Conflict of interest

The authors state no conflict of interest.

Acknowledgments

The authors would like to thank Rubén Luviano Jaramillo for assisting in the animal care and Erendira García Rios, Carmen Márquez, and Eliuth Juarez Cruz for technical assistance. Partial support came from project No. 190 of Fondo de apoyo para proyectos de Investigación 2009 del Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz.

References

Achenbach, H., Schwinn, A., 1995. Aporphinoid alkaloids and terpenoids from *Piptostigma fugax*. Phytochemistry 38, 1037–1048.

Blier, P., Montigny, C., 1994. Current advances and trends in the treatment of depression. Trends in Pharmacological Sciences 15, 220–226.

Blier, P., Ward, N.M., 2003. Is there a role for 5-HT1A agonists in the treatment of depression? Biological Psychiatry 53, 193–203.

Bradford, M.M., 1976. Rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Castagné, V., Moser, P., Roux, S., Porsolt, R.D., 2001. Rodent models of depression: forced swimming and tail suspension behavioral despair tests in rats and mice. Current Protocols in Neuroscience 8, 10A.

Cervo, L., Samanin, R., 1987. Potential antidepressant properties of 8-hydroxy-2-(di-n-propylamino) tetralin, a selective serotonin 1A receptor agonist. European Journal of Pharmacology 144, 223–229.

Chen, Y., Kong, L-D., Xia, X., Kung, H-F., Zhang, L., 2005. Behavioral and biochemical studies of total furocoumarins from seeds of *Psoralea corylifolia* in the forced swimming test in mice. Journal of Ethnopharmacology 96, 451–459.

Domínguez, X.A., 1973. Métodos de Investigación Fitoquímica. Editorial Limusa, México, 218 pp.

Elhuwuegi, A.S., 2004. Central monoamines and their role in major depression. Progress in Neuropsychopharmacology and Biological Psychiatry 28, 435–451.

Emslie, G.J., Mayers, T.L., Ruberu, M., 2005. Continuation and maintenance therapy of early-onset major depressive disorder. Pediatric Drugs 7, 203–217.

Estrada-Reyes, R., Martínez-Vázquez, M., Gallegos-Solís, A., Heinze, G., Moreno, J., 2010. Depressant effects of *Clinopodium mexicanum* Benth. Govaerts (Lamiaceae) on the central nervous system. Journal of Ethnopharmacology 130, 1–8.

Fava, M., Rush, A.J., 2006. Current status of argumentation and combination treatments for major depressive disorder: a literature review and a proposal for novel approach to improve practice. Psychotherapy and Psychosomatics 75, 139–153.

González-Trujano, M.E., Navarrete, A., Reyes, B., Cedillo-Portugal, E., Hong, E., 2001. Anticonvulsant properties and bio-guided isolation of palmitona from leaves of *Annona diversifolia*. Planta Medica 67, 136–141.

Gouemo, P.N., Koudogbo, B., Tchivounda, P., Akono-Nguema, C., Etoua, M.M., 1996. Effects of etanol extract of *Annona muricata* on pentylentetazol-induced convulsive seizures in mice. Phytotherapy Research 11, 243–245.

Gupta, M.P., 1995. In: Gupta, M.P. (Ed.), 270 Plantas Medicinales Iberoamericanas. Cyted, pp. 26–28.

Harkin, A., Kelly, J.P., McNamara, M., Connor, T.J., Dredge, A.R., Leonard, B.E., 1999. Activity and onset of action of reboxetine and effect of combination with sertaline in a model of depression. European Journal of Pharmacology 364, 123–132.

Hasrat, J.A., De Bruyne, T., De Baker, J.P., Vauquelin, G., Vlietinck, J., 1997a. Isoquinoline derivates isolated from the fruit of *Annona muricata* as 5-HTergic 5-HT_{1A} receptor agonists in rats: unexploited antidepressive (lead) products. Journal of Pharmaceutical and Pharmacology 49, 1145–1149.

Hasrat, J.A., Pieters, L., De Baker, J.P., Vauquelin, G., Vlietinck, J., 1997b. Screening of medicinal plants from Suriname for 5-HT_{1A} ligands: bioactive isoquinoline alkaloids from fruit of *Annona muricata*. Phytomedicine 4, 133–140.

Hu, R., Dai, X., Pan, Y., 2010. Preparative separation of isoquinoline alkaloids from Stephania yunnanensis by pH-zone-refining counter-current chromatography. Journal of Chromatographic B: Analytical Technological and Biomedical Life Science 874, 1881–1884.

Leboeuf, M., Cavé, A., Bhaumik, P.K., Mukherjee, B., Mukherjee, R., 1982. The phytochemistry of the *Annonaceae*. Phytochemistry 21, 2783–2813.

Lee, J.J., Jin, C.M., Kim, Y.K., Ryu, S.Y., Lim, S.C., Lee, M.K., 2008. Effects of anonaine on dopamine biosynthesis and L-DOPA-induced cytotoxicity in PC12 cells. Molecules 13, 475–487.

López-Rubalcava, C., Piña-Medina, B., Estrada-Reyes, R., Heinze, G., Martínez-Vázquez, M., 2006. Anxiolytic actions of the hexane extract from leaves of *Annona cherimolia* in two paradigms: possible involvement of the GABA/benzodiazepine receptor complex. Life Science 78, 730–737.

Machado, D.G., Bettio, L.E.B., Cynha, M.P., Capra, J.C., Dalmarco, J.B., Pizzolatti, M.G., Rodrigues, A.L.S., 2009. Antidepressant-like effect of the extract of *Rosmarinus officinalis* in mice: involvement of the monoaminergic system. Progress in Neuropsychopharmacology and Biological Psychiatry 33, 642–650.

Martínez-Mota, L., Fernández-Guasti, A., 2004. Testosterone-dependent antidepressant-like effect of noradrenergic but not of serotonergic drugs. Pharmacology Biochemistry and Behavior 78, 711–718.

Martínez-Mota, L., Cruz-Martínez, J.J., Márquez-Baltazar, S., Fernández-Guasti, A., 2008. Estrogens participate in the antidepressant-like effect of desimipramine and fluoxetine in male rats. Pharmacology Biochemistry and Behavior 88, 332–340.

Martínez-Mota, L., Estrada-Camarena, E., López-Rubalcava, C., 2002. Indorrenate produces antidepressant-like actions in the rat forced swimming test via 5-HT_{1A} receptors. Psychopharmacology 165, 60–66.

Miura, H., Naoi, M., Nakahara, D., Ohta, T., Nagatsu, T., 1999. Changes in monoamine levels in mouse brain elicited by forced-swimming stress, and the protective effects of a new monoamine oxidase inhibitor, RS-8359. Journal of Neural Transmission General Section 94, 175–187.

Newman, J.D., Cragg, G.M., Snader, K., 2003. Natural products as sources of new drugs over the period 1981–2002. Journal of Natural Products 66, 1022–1037.

Porsolt, R.D., Le Pichon, M., Jalfre, M., 1977a. Depression: a new animal model sensitive to antidepressant treatments. Nature 266, 730–732.

Porsolt, R.D., Bertin, A., Jalfre, M., 1977b. Behavioral despair in mice: a primary screening test for antidepressants. Archives Internationales de Pharmacodynamie et de Therapie 229, 327–336.

Protais, P., Arbaoui, J., de Roue, E.-H., 1995. Effects of various isoquinoline alkaloids on in vitro 3H-dopamine uptake on striatal synaptosomes. Journal of Natural Products 58, 1475–1484.

Rogóz, Z., Budziszewska, B., Kubera, M., Basta-Kaim, A., Jaworska-Feil, L., Skuza, G., Lason, W., 2005. Effects of combined treatment with imipramine and metyrapone on the immobility time, the activity of hypothalamus-pituitary-adrenocortical axis and immunological parameters in the forced swimming test in the rat. Journal of Physiology and Pharmacology 56, 49–61.

Sánchez-Mateo, C.C., Bonkanka, C.X., Prado, B., Rabanal, R.M., 2007. Antidepressant activity of some *Hypericum reflexum* L. fil extracts in the forced swimming test in mice. Journal of Ethnopharmacology 112, 115–121.

Smith, R.V., Sood, S.P., 1971. In vitro metabolism of certain nornuciferine derivatives. Journal of Pharmaceutical Sciences 60, 1654–1658.

Tortoriello, J., Romero, O., 1992. Plants used by Mexican traditional medicine with presumable sedative properties: an ethnobotanical approach. Archives of Medical Research 23, 111–116.

WHO World Health Statistics, 2010. Ten Statistical Highlights in Global Public Health. http://www.who.inturveydata.who.int/topics/depression/ (accessed 29 October 2010), 16 pp.

Zhang, Z., 2004. Therapeutic effects of herbal extracts and constituents in animal models of psychiatric disorders. Life Science 75, 1659–1699.