

Anxiogenic effects of *Tabernaemontana solanifolia* A. DC. (Apocynaceae) extracts in rats tested in the elevated plus-maze

Determinación mediante la prueba del laberinto elevado en cruz del efecto ansiogénico en ratas de extractos de *Tabernaemontana solanifolia* A. DC. (Apocynaceae)

Dra. Adriana Mary Mestriner Felipe Melo,^I MSc. Luis Fernando Benitez Macorini,^I MSc. André Müller,^{II} Dra. Cláudia Andréia Lima Cardoso,^{II} Lic. Stephanie Gama,^{III} Dra. Sueli Maria Gomes,^{III} Dra. Dâmaris Silveira^{III}

^I University of Grande Dourados. Dourados, MS, Brazil.

^{II} University of Mato Grosso. Sinop, MT, Brazil.

^{III} University of Brasília. Brasília, DF, Brazil.

ABSTRACT

Introduction: *Tabernaemontana solanifolia* A. DC. (Apocynaceae) grows in the Cerrado (savanna), Caatinga (shrublands), and Atlantic Forest of Brazil.

Objective: to investigate the potential anxiolytic activity of the crude extracts and β -amyryn acetate obtained from the leaves of *Tabernaemontana solanifolia* in the elevated plus-maze (EPM) test for anxiety in rats.

Methods: the crude extracts (aqueous, ethanol, and hexane), as well as β -amyryn acetate, obtained from *Tabernaemontana solanifolia* leaves were evaluated for possible anxiolytic effects in rats tested in the elevated plus-maze. Acute toxicity in rats was determined by OECD 423 guidelines. The leaves of *T. solanifolia* specimens collected in Brasília, Brazil, were air dried at 40 °C and macerated with hexane or ethanol. After filtration, the solvents were removed under reduced pressure, yielding the crude hexane and ethanolic extracts (5.96 and 18.62 % yield, respectively). The crude aqueous extract was obtained by infusion, followed by lyophilization (13.5 % yield). Thirty minutes before the elevated plus-maze

experiments, the animals were treated with the crude ethanol extract (1 000 mg/kg of body weight [bw], p.o.), the crude hexane extract (1 000 mg/kg bw, p.o.), the crude extract (1 000 mg/kg bw, p.o.), or β -amyrin acetate (21 mg/kg bw, p.o.).

Results: the extracts caused no mortality up to 2 000 mg/kg, so half of DL50 doses were selected for the present study. The crude extracts (hexane, ethanolic and aqueous) (1.0 g/kg) as well as the substance β -amyrin acetate (21 mg/kg) were administered once, increasing the number of entries into and the time spent in the closed arms of the elevated plus-maze.

Conclusion: the oral administration of the extracts and β -amyrin acetate obtained from *Tabernaemontana solanifolia* had an anxiogenic effect in rats. Further studies are needed in order to identify and characterize the anxiogenic properties of *Tabernaemontana solanifolia* and to understand the anxiogenic mechanisms of the extracts.

Key words: elevated plus-maze, anxiogenic, *Tabernaemontana solanifolia*, Apocynaceae.

RESUMEN

Introducción: *Tabernaemontana solanifolia* A. DC. (Apocynaceae) crece en el Cerrado (sabana), Caatinga (matorrales) y la Mata Atlántica de Brasil.

Objetivo: investigar la posible actividad ansiolítica de los extractos crudos y el acetato de β -amirina obtenidos de las hojas de *Tabernaemontana solanifolia* en la prueba del laberinto elevado en cruz (EPM) para la ansiedad en ratas.

Métodos: los extractos crudos (etanol acuoso y hexano), así como el acetato de β -amirina, se evaluaron para posibles efectos ansiolíticos en ratas mediante la prueba del laberinto elevado en cruz. La toxicidad aguda en ratas se determinó por las directrices OCDE 423. Las hojas de *Tabernaemontana solanifolia* se secaron al aire a 40 °C y fueron maceradas con hexano y etanol. Después de la filtración, los disolventes se eliminaron bajo presión reducida, produciendo los extractos crudos hexano y etanólico (5,96 y 18,62 % de rendimiento, respectivamente). El extracto acuoso bruto se obtuvo por infusión, seguida por liofilización (13,5 % de rendimiento). A 30 min antes de los experimentos de laberinto elevado en cruz, los animales se trataron con el extracto de etanol crudo (1 000 mg/kg de peso corporal [bw], p.o.), el extracto de hexano crudo (1 000 mg/kg de peso corporal, por vía oral), el extracto acuoso (1 000 mg/kg de peso corporal, por vía oral) o β -amirina acetato (21 mg/kg de peso corporal, por vía oral).

Resultados: los extractos no produjeron mortalidad hasta 2 000 mg/kg, por lo que para el presente estudio se seleccionó la mitad de la dosis DL50. Los extractos crudos (hexano, etanol y acuoso) (1 000 mg/kg), así como la sustancia acetato de β -amirina (21 mg/kg) se administraron una vez, aumentando el número de entradas y el tiempo pasado en los brazos cerrados de la prueba del laberinto elevado en cruz.

Conclusión: la administración oral de los extractos y el acetato de β -amirina obtenidos de *Tabernaemontana solanifolia* tuvo un efecto ansiogénico en ratas. Se necesitan estudios adicionales para identificar y caracterizar las propiedades ansiogénicas de *Tabernaemontana solanifolia* y para entender los mecanismos ansiogénicos de los extractos.

Palabras clave: laberinto elevado en cruz, ansiogénicos, *Tabernaemontana solanifolia*, Apocynaceae.

INTRODUCTION

The genus *Tabernaemontana*, is represented by approximately 100 species worldwide, and produces a wide variety of indole alkaloids that have various biological activities.¹ Most *Tabernaemontana* species have been studied in terms of their chemical composition and biological activity. However, for some species of the genus, there is a lack of knowledge related to one or both of those aspects. One such species is *Tabernaemontana solanifolia* A. DC. [*Peschiera solanifolia* var. *fallax* (Muell. Arg) L. Allorge], which is an endemic species of Brazil, found in the Cerrado (savanna), Caatinga (shrublands), and Atlantic Forest.² This species has been useful as an ornamental,³ and the vernacular name (mata-pasto) has rarely been noted.^{4,5} Leaves of *T. solanifolia* are sessile or with petioles up to 3 mm in length, usually rounded or cordate at the base and rounded to acuminate at the apex.⁵

Various *Tabernaemontana* species have been used as folk or traditional medicines for the treatment of central nervous system disorders. For example, *T. sananho* Ruiz & Pav. has been reported to have possible psychoactive effects, ostensibly "calming the nerves" in rituals carried out by the Krahô Indians of Brazil.⁶ In addition, the alcoholic extract of the flowers of *T. divaricata* (Linn) R. Br., administered orally to mice at a dose of 100, 200, or 400 mg/kg of body weight (bw), has been shown to have anxiolytic effects in the elevated plus-maze (EPM), the open-field test, and the light-dark transition test.⁷ Anticonvulsive properties have also been ascribed to *T. divaricata*.⁷

Symptoms of anxiety are quite common and accompany many disorders. Various animal models of anxiety have been developed in order to determine the effectiveness of new drugs, study their mechanisms of action, or investigate the pathophysiological phenomena involved. The EPM is one of the most widely used animal tests of anxiety and was developed on the basis of the observation that rodents avoid open elevated alleys and on the assumption that this avoidance is generated by fear.⁸ In the EPM model, the use of anxiolytic drugs, such as diazepam, has been found to increase the number of entries by the animals into the open arms of the maze, whereas anxiogenic drugs have been found to reduce the number of such entries. The analysis of other aspects, such as ethological parameters, has also been adopted. The advantages of the EPM are its simplicity of use, as well as the low cost of its assembly and maintenance, together with the fact that it can be used for the assessment of anxiolytic and anxiogenic substances alike.^{9,10} The major disadvantage of the EPM model is its sensitivity to external noise and interruptions.¹¹

In view of reports regarding the central nervous system activity of crude extracts and fractions obtained from other *Tabernaemontana* species, the aim of this study was to investigate the potential anxiolytic activity of the crude extracts and β -amyrin acetate obtained from the leaves of *T. solanifolia* in the EPM test of anxiety in rats.

METHODS

Plant material and preparation of the extract

Leaves of *T. solanifolia* were collected in the city of Brasilia, Brazil, in March of 2007. Specimens were deposited in the Herbarium of the University of Brasilia

(voucher number S.M. Gomes & R. G. Tostes 846). The plant material was air dried at 40 °C and ground and powdered in a knife mill. Part of this material was macerated at room temperature for seven days (repeated weekly over a four-week period), first in hexane and then in ethanol. After filtration, the solvents were removed under reduced pressure, at a temperature below 40 °C, yielding the crude hexane and ethanolic extracts (5.9 and 18.62 % yield, respectively). The crude aqueous extract was obtained by infusion, with 100 g of powdered plant material and 1 L of distilled water at 70 °C. After filtration, the extract was lyophilized (13.5 % yield).

Preparation of extract and substance

Animals

Male Wistar rats (*Rattus norvegicus*), 2-3 months of age and weighing 300-400 g, were obtained from the animal facilities of the Federal University of Mato Grosso do Sul. The animals were randomly housed in groups of 6 per polyethylene cage and maintained under normal 12/12 h light/dark cycle (lights on at 07:00 h) in a temperature -and humidity-controlled environment. The rats were given ad libitum access to food and water until 12 h before the experimental procedures.

Determination of acute toxicity

The animals were fasted for 3 h prior to the acute toxicity experiment, in which we employed the Organization for Economic Cooperation and Development (OECD) Acute Toxic Class Method (OECD guideline no. 423).¹² Animals received a single dose of extract, and mortality was monitored over the following 48 h (determination of short-term toxicity). On the basis of the short-term toxicity profile of each extract, the dose for the next animal was determined as per the OECD guideline. Each extract was administered orally at different dose levels, and, up to a dose of 2000 mg/kg bw, none caused mortality. Therefore, half of that dose was selected for use in the present study.¹³

Ethical considerations

The study was approved by the Research Ethics Committee of the University of Grande Dourados (UNIGRAN) with R. No. 171/09), located in the city of Dourados, Brazil.

Administration

The crude extracts were suspended in Tween 80 (8 %) and saline (0.9 % NaCl), followed by homogenization and sonication for 20 min. The β -amyrin acetate (isolated from the hexane extract) was also suspended in Tween 80 (8 %) and saline (0.9 % NaCl).

Animals were treated, by gavage, with the crude ethanolic extract (1000 mg/kg bw), the crude hexane extract (1 000 mg/kg bw), the crude aqueous extract (1 000 mg/kg bw), or β -amyrin acetate (21 mg/kg bw). For convenience, a fixed dose was chosen, given that there have been no previous studies using this plant species in rodent models of anxiety. As positive and negative controls, respectively, one group of rats were i.p. injected with diazepam (2 mg/kg bw) and those in another group received

only vehicle (an equivalent volume of 0.9 % NaCl + 8 % Tween 80, by gavage). All compounds were administered 30 min before the experiments.

Apparatus

The EPM comprised two open arms (50 × 10 × 1 cm) and two closed arms (50 × 10 × 40 cm) extending from a common central platform (10 × 10 cm). The apparatus was constructed of wood and elevated to 50 cm above floor level. Experiments were carried out in a sound-attenuated, temperature-controlled room, illuminated by two 60-w fluorescent lights.

Procedure

Thirty minutes after the administration of the extracts, vehicle, or diazepam, each animal was placed, individually, at the center of the EPM and observed for 5 min (300 s). During the test, the following spatiotemporal parameters were recorded: length of time (in seconds) that each animal spent in the open and closed arms; number of open and closed arm entries (arm entry= all four paws within the arm); percentage of open-arm entries [(open total/300) × 100]; and percentage of open-arm time [(time in the open/300) × 100]. The percentage of open-arm entries and percentage of open-arm time were used as anxiety indices, and the frequency of closed-arm entries was used as an index of locomotor activity.^{9,11} The ethological parameters head dipping, rearing, and fecal boli were also analyzed.^{10,14}

Statistical analysis

Results are expressed as mean ± SEM. The data were evaluated by one-way analysis of variance followed by Tukey's test. Data from experimental groups were compared with those obtained for the vehicle-only and diazepam groups, and values of $p < 0.05$ were considered statistically significant. The analyses were performed with the InStat program, version 3.0 (GraphPad Software, San Diego, CA), and the graph was constructed with GraphPad Prism, version 5.0 (GraphPad Software).

RESULTS

Acute toxicity studies: Acute toxicity of extracts of *T. solanifolia* was determined in rats, by OECD guidelines. Each extract was administered orally at different dose levels and extracts caused no mortality up to 2 000 mg/kg. Therefore DL50 doses were halved for the present study.

Elevated Plus Maze Model (EPM): diazepam has long been known to have anxiolytic effects in rats tested in the EPM. As can be seen in table 1, we also found the administration of diazepam to have a significant anxiolytic effect, in comparison with that of vehicle only, as evidenced by an increased number of entries into open arms, a decreased number of entries into closed arms, a longer time spent on the open, central platform, and a shorter time spent in the closed arms ($p < 0.05$ for all). When the crude ethanolic extract, the crude hexane extract and the crude aqueous extract (1.0 g/kg) as well as the substance β -amyryn acetate (21 mg/kg) were administered once, no significant effects were observed on the number of

entries and time spent in the open arm, central platform and decreased the number of entries and time spent in the closed arm as compared to the control group (table 1).

The application of the crude aqueous extract and the crude ethanolic extract significantly increased the rearing (table 2).

DISCUSSION

Stress has become a part of the modern world and lifestyles. Evidence suggests that anxiety is caused by dysfunction of one or more neurotransmitters and their receptors. The study of anxiety has emerged as a very important area of research.

Behavioral scoring of performance in the EPM has been extended to include a variety of specific acts and postures which, incorporated into more comprehensive factor analyses, have revealed additional dimensions of the EPM behavior patterns, such as vertical activity, directed exploration, decision making, and risk assessment.¹⁵ However, the frequency of entries, which is related to locomotion, is more often evaluated than are ethological measures, which might be more sensitive to anxiety modulating compounds.¹⁰ Other ethological parameters that can be observed in rodents tested in the EPM are the numbers of rears, head dips, fecal boli, freezing episodes, and stretch-attend postures.¹⁶

The EPM is one of the most important animal tests used in the evaluation of the anxiolytic effects of drugs,^{8,9,17} in mice¹⁸ as well as in rats.¹⁹ When used appropriately, the EPM can be a very valuable tool in drug screening and in the study of the neurobiology of anxiety and defense.²⁰ In addition to the classic parameters (frequency of entries into and time spent in open arms), other anxiety-related rodent behaviors, such as freezing/immobility and defecation, are more frequent in the open arms than in the closed arms.⁸ These parameters have been used to investigate the anxiolytic-like effect.²¹

Plants known to possess enormous anxiolytic potential have been used to cure ailments from time immemorial.²¹ Other botanical extracts have been tested in rodent models of anxiety. The anxiolytic effects of *T. divaricata* (Linn) R. Br. were documented by Basavaraj et al.,⁷ who administered the alcoholic extract of the flowers of *T. divaricata* (100, 200, or 400 mg/kg bw, p.o.) for 7 days to mice subsequently tested in the EPM, the open-field test, and the light-dark transition test.⁸ Those authors also found *T. divaricata* to have anticonvulsant properties and effects on spontaneous motor activity. In addition, a mixture of α - and β -amyrin pentacyclic triterpenes, isolated from the stem bark resin of *Protium heptaphyllum* (Aubl.) March., has been found to have anxiolytic and antidepressant effects in mice receiving various doses of the mixture (ranging from 2.5 to 5.0 mg/kg bw, p.o. or i.p.) and tested in the EPM, the open-field test, the rota rod test, the forced swimming test, and the pentobarbital-induced sleeping time test.¹⁹ Furthermore, administration of the hydroalcoholic extract of the stem bark of *Erythrina velutina* (100 mg/kg bw, p.o.) in mice tested in the EPM has been shown to increase the percentage of open arm entries when the administration was chronic but not when it was acute.²¹ In the present study, acute oral administration of an extract prepared from the leaves of *T. solanifolia* had an anxiogenic effect in rats tested in the EPM, increasing the number of entries into and the time spent in closed arms.

We can conclude that the crude ethanol, hexane, and aqueous extracts, as well as the β -amyrin acetate, isolated from *T. solanifolia* have an anxiogenic effect in rats, increasing the number of entries into and the time spent in closed arms of the EPM. However, further studies are required to identify and characterize the anxiogenic properties of *T. solanifolia* and to understand their mechanisms of anxiogenic action of the extracts.

ACKNOWLEDGMENTS

The authors are grateful for the financial support received from the Brazilian Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, National Council for Scientific and Technological Development, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Office for the Advancement of Higher Education), Fundação de Apoio à Pesquisa do Distrito Federal (FAPDF, Foundation for the Support of Research in the Federal District of Brasília), Mato Grosso do Sul State Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia (FUNDECT, Foundation for the Advancement of Education, Science, and Technology), and the Centro Universitário da Grande Dourados (UNIGRAN, University of Grande Dourados).

REFERENCES

1. Van Beek TA, Verpoorte R, Baerheim Svendsen A, Leeuwenberg AJM, Bisset NG. *Tabernaemontana* L. (Apocynaceae). A review of its taxonomy, phytochemistry, ethnobotany and pharmacology. *J Ethnopharmacol.* 1984;10(1):1-156.
2. Rapini A, Koch I, Kinoshita, LS, Simões AO, Spina AP. Apocynaceae in Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. Available at: <http://floradobrasil.jbrj.gov.br/2010/FB004866>
3. Gower AE, Pereira BDS, Marsaioli AJ. Indole alkaloids from *Peschiera campestris*. *Phytochemistry.* 1986;25(12):2908-10.
4. Leeuwenberg AJM: A revision of *Tabernaemontana* 1. The old World species. Kew, United Kingdom; 1991. p. 223.
5. Leeuwenberg AJM: A revision of *Tabernaemontana*. The new World Species and *Stemmadenia*. Vol. 2. Kew, United Kingdom: Royal Botanical Gardens Press; 1994. p. 450.
6. Rodrigues E, Carlini EL, Araujo L. Plants with possible psychoactive actions used by the Krahô Indian, Brazil. *Rev Bras Psiquiatr.* 2006;28(4):277-82.
7. Basavaraj P, Shivakumar B, Shivakumar H. Anxiolytic activity of *Tabernaemontana divaricata* (Linn) R. Br. Flowers extract in mice. *J Pharma Bio Sciences.* 2011;2(3):65-72.
8. Montgomery KC. The relation between fear induced by novel stimulation and exploratory behavior. *J Comp Physiol Psychol.* 1955;48(4):254-60.
9. Pellow S, Chopin P, File SE, Briley M. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods.* 1985;14(3):149-67.

10. Carobrez AP, Bertoglio LJ. Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on. *Neurosci Biobehav Rev.* 2005;29(8):1193-205.
11. Hogg S. A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. *Pharmacol Biochem Behav.* 1996;54(1):21-30.
12. Organization of Economic Co-operation and Development (OECD). OECD guidelines on acute oral toxicity, environmental health and safety monograph series on testing and adjustment: 423 acute oral toxicity. Paris: OECD; 1996.
13. Melo AAMF, Gama SM, Macorini LFB, Nascimento C, Honorato CA, Vasconcelos PC, et al. Acute oral toxicity of extracts of *Tabernaemontana solanifolia* A. DC. (Apocynaceae). *Lat Am J Pharm.* 2012;31(8):1149-54.
14. Graeff FG, Ferreira Neto C, Zangrossi JH. The elevated T-maze as an experimental model of anxiety. *Neurosci Behav Rev.* 1998;23(2):237-46.
15. Cruz AP, Frei F, Graeff FG. Ethnopharmacological analysis of rat behavior on the elevated plus-maze. *Pharmacol Biochem Behav.* 1994; 49(1):171-6.
16. Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc.* 2007;2(2):322-8.
17. Handley SL, MacBlane JW. 5-HT drugs in animal models of anxiety. *Psychopharmacol.* 1993;112(1):13-20.
18. Lister RG. The use of a plus-maze to measure anxiety in the mouse. *J Psychopharmacol.* 1987;92(2):180-5.
19. Aragão GF, Carneiro LMV, Junior APF, Vieira LC, Lemos TLG, Viana GS de B. A possible mechanism for anxiolytic and antidepressant effects of alpha- and beta-amyrin from *Protium heptaphyllum* (Aubl.). *Pharmacol Biochem Behav.* 2006;85(4):827-34.
20. Rodgers RJ, Johnson NJT. Behaviorally selective effects of neuroactive steroids on plus-maze anxiety in mice. *Pharmacol Biochem Behav.* 1998;59(1):221-32.
21. Rauppa IM, Serenikia A, Virtuoso S, Ghislandi C, Cavalcanti Silva EL, Trebien HA, et al. Anxiolytic-like effect of chronic treatment with *Erythrina velutina* extract in the elevated plus-maze test. *J Ethopharmacol.* 2008;118(2):295-9.

Recibido 15 de junio de 2012.

Aprobado: 30 de diciembre de 2012.

Adriana Mary Mestriner Felipe Melo. Department of Pharmacology, University of Grande Dourados (UNIGRAN), 79824-900, Dourados, MS, Brazil. Telephone: +55 67 3411 4121, Fax: +55 67 3411 4167. E-mail: mestriner@unigran.br
