

Full Length Research Paper

## Phytochemical screening and central nervous system effects of ethanolic extract of *Annona vepretorum* (Annonaceae) in mice

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*Annona vepretorum* Mart. (Annonaceae) is a native tree from Caatinga biome (semiarid region of Brazil) popularly known as “araticum” and “pinha da Caatinga”. This study was conducted to investigate the possible anxiolytic, motor and sedative effects of the crude ethanolic extract (Av-EtOH) on the central nervous system (CNS) in mice. The behavioral screening, open-field, elevated plus-maze, hole-board and rota-rod tests were used in this study. Av-EtOH (25, 50 and 100 mg/kg, p.o.) and diazepam (2.5 mg/kg, i.p.) were administered to different groups of mice and appropriate observations were made. Preliminary phytochemical analysis of the extract was also performed. A preliminary analysis of Av-EtOH revealed that it contained phenols, steroids, terpenoids and flavonoids. The initial pharmacological screening revealed behavioral changes in animals at 0.5, 1, 2 and 4 h after Av-EtOH treatment, such as decrease of spontaneous activity, palpebral ptosis and decrease of response to the touch. Av-EtOH at dose of 25 mg/kg reduced ( $p < 0.01$ ) the ambulation and rearing number and also increased the immobility time in open-field test. In elevated plus-maze test, the extract in all doses increased the time spent in closed arms and reduced ( $p < 0.05$ ) the number of head dips in hole-board test at doses of 50 and 100 mg/kg. In addition, Av-EtOH did not impair motor coordination in rota-rod test. Taken together, the findings in this study suggest that *A. vepretorum* has sedative activity but does not affect the motor coordination of animals.

**Key words:** Sedative, central nervous system, *Annona vepretorum*, Annonaceae.

### INTRODUCTION

Central nervous system (CNS) disorders occupy a prominent place in modern therapy. Health problems calling for the use of anxiolytic and sedative drugs

(anxiety, nervous tension, agitation and insomnia), antidepressants and antipsychotics are a common and costly problem worldwide (Ajao and Akindele, 2012).

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Anxiety and stress disorders are currently among the ten most important public health concerns, and in recent years have reached epidemic proportions (Doron et al., 2012).

Approximately 60% of the world's population relies almost entirely on plants for medication and the natural products have long been recognized as an important source of therapeutically effective medicines (Harvey, 2000). The interest in drugs of plant origin is due to several reasons, namely: conventional medicine can be inefficient (for example, side effects and ineffective therapy), abusive and/or incorrect use of synthetic drugs results in other problems, a large percentage of the world's population does not have access to conventional pharmacological treatment and folk medicine suggests that natural products are harmless (Rates, 2001).

Medicinal plants represent a growing and important source of new drugs for the treatment of different conditions. They have been made one of the main sources for the isolation of bioactive organic compounds (Adebiyi et al., 2012). The disadvantages of conventional pharmacological treatment for CNS disorders have prompted the search for alternative treatments. Many interesting compounds have been isolated and identified from Annonaceae fruits, gaining research attention because of their novel structure and wide range of bioactivities (Barreca et al., 2011). The Annonaceae is a large family comprising 135 genera and 2500 species which are distributed mainly in tropical and subtropical regions of the world. The genus *Annona* L. comprises approximately 175 species of trees and shrubs (Dutra et al., 2012). Chemical studies with species of this family have reported the isolation of terpenoids, essential oils whose composition is predominantly of mono and sesquiterpenes, and alkaloids, especially isoquinoline alkaloids (Silva et al., 2009).

Although species of the genus *Annona* are generally consumed as fresh fruits, they are also widely used in folk medicine. Several reports have characterized the pharmacological activity of these plants because of their bioactive compounds found in roots, leaves, bark, seeds and fruit (Carballo et al., 2010). Several extracts obtained from *Annona* species have been shown to be active on the CNS and because of these effects, some species of *Annona* have been used in traditional medicine (López-Rubalcava et al., 2006). Due to their anti-anxiety, anti-convulsant, and tranquilizing properties, several species of *Annona* are used in traditional medicine. Experiments with animal models have confirmed the anxiolytic-like and anti-convulsant activities of the polar extracts of *Annona diversifolia* and *Annona muricata*, as well as hexane extracts of *Annona cherimolia* (González-Trujano et al., 2006; Martínez-Vázquez et al., 2012). There has been the report of the CNS depressant effects produced by a hexane extract of *A. cherimolia* through its interaction

with the GABA<sub>A</sub> receptor complex (López-Rubalcava et al., 2006). Anti-convulsant effect of *A. diversifolia* ethanolic extract was also reported (González-Trujano et al., 2006). Leaves and crude extracts of *A. muricata*, *Annona glabra* and *Annona montana* have been employed in folk medicine due to the fact that they present tranquilizing and sedative properties (López-Rubalcava et al., 2006). *Annona senegalensis* possesses anticonvulsant, central depressant and anxiolytic-like properties (Okoli et al., 2010).

Although *A. vepretorum* is widely used in folk medicine in the semiarid region of Brazil, no report about the neuropharmacological activity of this plant is recorded in the literature. The aim of the present study was to investigate the effects of the ethanolic extract of *A. vepretorum* on the CNS in mice.

## MATERIALS AND METHODS

### Plant

The leaves of *A. vepretorum* Mart. were collected in the city of Jaguarari, State of Bahia, Brazil, in October, 2010. The samples were identified by André Paviotti Fontana, a botanist from Centro de Recuperação de Áreas Degradadas da Caatinga (CRAD). A voucher specimen (#946) was deposited at the Herbarium Vale do São Francisco (HVASF) of the Federal University of San Francisco Valley.

### Extraction

The dried and pulverized leaves (400 g) were macerated with ethanol 95% at room temperature for 72 h. The solution was filtered and concentrated under reduced pressure in a rotatory evaporator oven at 50°C, producing 42 g of crude ethanolic extract (Av-EtOH).

### Preliminary phytochemical screening

Preliminary phytochemical analysis of the extract was performed. The presence of alkaloids was determined with Dragendorff's and Mayer's reagents, flavonoids were detected with HCl and Mg powder, phenols were measured with ferric chloride and both steroids and terpenoids were detected by Liebermann-Burchard reaction (Matos, 1997).

### Animals

Male adult albino Swiss mice (25 to 35 g) were used throughout this study. The animals were randomly housed in appropriate cages at 22 ± 2°C on a 12 h light/dark cycle (lights on at 6:00 a.m.) with free access to food and water. They were used in groups of six animals each. Experimental protocols and procedures were approved by the Federal University of San Francisco Valley Animal Care and Use Committee under the number 0005/261011.

### Behavioral screening

Behavioral screening was performed following parameters

described by Almeida (2006). This test was used in order to check for possible general CNS effects of the Av-EtOH. Groups of six mice were treated with vehicle (saline 0.1 ml/10 g body weight, p.o.) or Av-EtOH at the doses of 25, 50 and 100 mg/kg, p.o. Animals were observed for 4 h to detect general signs like sedation, analgesia, palpebral ptosis, piloerection, grooming and dyspnea. Their behavior was observed at 30, 60, 120, 180 and 240 min after the treatment. At the end of each observation, whether deaths occurred was verified.

### Open-field test

The test apparatus (Insight, Brazil) consisted of a box with dimensions of 50 cm (height) × 60 cm (diameter) and the floor divided in 12 equal squares. A 60 W white bulb illuminated the apparatus. The open-field test was used to evaluate the exploratory activity of the animal (Archer, 1973). The mice were randomly distributed into groups of six animals. Sixty minutes after the treatment with saline (control group), Av-EtOH (25, 50 and 100 mg/kg, p.o.) or diazepam (2.5 mg/kg, i.p.), each mouse was placed in the central square of the open field and observed for 5 min as to the behavioral parameters. The observed parameters were: ambulation, immobility time (s), rearing and grooming number. The floor of the open field was cleaned with ethanol 5% solution after each session.

### Elevated plus-maze test

The elevated plus-maze (Insight, Brazil) consists of two open arms and two closed arms elevated to a height of 40 cm above the floor. This task is based on the natural tendency of mice to avoid open and elevated places. The elevated plus-maze has been widely validated to evaluate anxiety in rodents. The animals were treated with saline (control group, p.o.), Av-EtOH (25, 50 and 100 mg/kg, p.o.) and diazepam (2.5 mg/kg, i.p.). Sixty minutes after the treatments, each mouse was placed in the center of the plus maze with its nose in the direction of one of the closed arms. The cumulative times spent by each mouse in the open and closed arms of the maze and the respective number of entries were recorded for 5 min. Anxiolytic drugs reduce the animal's natural aversion to the open arms and promote the exploration thereof (Santos et al., 2012). After each trial, the apparatus was cleaned with ethanol 5% solution.

### Hole-board test

The hole-board apparatus (Insight, Brazil) consisted of 5 equidistant holes 3 cm in diameters on the floor. Photocells below the surface of the holes measured the number of head-dips. The apparatus was elevated to a height of 40 cm above floor level. This test is based on the observation that the activity of head dipping of animals is inversely proportional to the anxiety state thereof (Almeida, 2006). Groups of six animals randomly distributed were used. Sixty minutes after the administration of saline, Av-EtOH (25, 50 and 100 mg/Kg, p.o.) and diazepam (2.5 mg/kg, p.o.) each mouse was placed in the center of the board facing away from the observer and its behavior was recorded for 5 min. Its total number of head dips, duration of head dips, time of first head dip and number of rearings were recorded (Netto et al., 2009). After each trial, the apparatus was cleaned with ethanol of 5% solution.

### Motor coordination test (rota-rod test)

A rota-rod treadmill device (Insight, Brazil) was used for the evaluation of motor coordination (Melo et al., 2011). Initially, 24 h before the test, mice capable of remaining on the rota-rod apparatus longer than 180 s (7 rpm) were selected. Thirty minutes after the administration of either Av-EtOH (25, 50 and 100 mg/kg, p.o.), vehicle (saline/Tween 80 0.2%; control group) or diazepam (2.5 mg/kg, i.p.), each animal was tested on the rota-rod apparatus at 0.5, 1 and 2 h post-treatment, and the time(s) the mice were able to remain on top of the bar was (were) recorded for up to 180 s.

### Statistical analysis

The data obtained from animal experiments were analyzed using the GraphPad Prism program version 4.0 and expressed as mean ± standard error of mean (SEM). Statistically significant differences between treated and control groups were calculated by means of the application of analysis of variance (ANOVA) followed by Dunnett's test. Values were considered significantly different at  $p < 0.05$ .

## RESULTS

### Preliminary phytochemical screening

Preliminary analysis demonstrated that Av-EtOH was positive for the presence of phenols, steroids, terpenoids and flavonoids. However, the ethanolic extract was negative for the presence of alkaloids.

### Behavioral screening

The initial pharmacological screening revealed behavioral changes in animals at 0.5, 1, 2 and 4 h after treatment: decrease of spontaneous activity, palpebral ptosis and decrease of response to the touch. Behavioral changes were more evident in the first 1 h. These effects do not appear to be dose-dependent. These behavioral changes suggest a possible depressant effect on CNS and are similar to drugs that reduce the CNS activity (Campêlo et al., 2011).

### Open-field test

Av-EtOH at a dose of 25 mg/kg significantly reduced ( $p < 0.01$ ) the ambulation and the rearing number compared to the control group. These effects were significantly higher than those of diazepam. The immobility time also was increased ( $p < 0.01$ ) when compared to the control group. The number of grooming was reduced ( $p < 0.01$ ) at doses of 50 and 100 mg/kg. Diazepam also significantly reduced the ambulation, rearing and grooming number and also increased the immobility time (Table 1).

**Table 1.** Effect of acute treatment with ethanolic extract of *Annona vepretorum* (Av-EtOH) and diazepam in open-field test in mice.

Treatment	Dose (mg/kg)	Ambulation	Immobility time (s)	Rearing (number)	Grooming (number)
Control	---	68.33±5.62	43.83±9.17	24.50±3.99	6.16±1.35
Av-EtOH	25	14.83±4.07**	161.30±14.85**	2.33±2.33**	3.83±0.54
	50	57.50±4.80	1.50±1.02	21.67±4.06	2.16±0.47**
	100	56.67±8.28	16.17±7.65	29.50±3.10	2.83±0.54*
Diazepam	2.5	27.33±3.71**	195.20±11.99**	5.50±2.74**	2.66±0.80*

Values are mean ± SEM; \*p < 0.05; \*\*p < 0.01, significantly different from control; ANOVA followed Dunnett's test (n = 6, per group).

**Table 2.** Effect of ethanolic extract of *Annona vepretorum* (Av-EtOH) and diazepam in elevated plus-maze test in mice.

Treatment	Dose (mg/kg)	Time spent in open arms (s)	Time spent in closed arms (s)	Number of entries into open arms	Number of entries into closed arms
Control	---	8.66±4.20	240.50±25.25	1.00±0.44	3.66±0.98
Av-EtOH	25	4.66±3.22	228.50±24.14	0.33±0.21	3.33±0.91
	50	3.66±2.78	278.20±6.18	0.50±0.34	2.16±0.65
	100	3.83±3.83	255.70±19.18	0.16±0.16	1.83±0.40
Diazepam	2.5	81.33±20.12**	165.70±18.45*	10.00±1.59**	11.83±2.77**

Values are mean ± SEM.; \*p < 0.05, significantly different from control; ANOVA followed Dunnett's test (n = 6, per group).

### Elevated plus-maze test

In this test, no statistical difference was observed in the effect of Av-EtOH in elevated plus-maze in mice. However, Av-EtOH in all doses increased the cumulative time spent in the closed arms and increased the number of entries into the closed arms relative to the control group. The extract did not elicit any significant effect (p > 0.05) on the parameters recorded (Table 2). While diazepam showed an increase in all parameters recorded, in this test these results indicate an anxiolytic effect.

### Hole-board test

Pre-treatment with Av-EtOH at doses of 50 and 100 mg/kg significantly reduced (p < 0.05) the number of head dips relative to the control group. Diazepam significantly reduced (p < 0.05) the number of head dips compared to the control group (Table 3).

### Motor coordination test (rota-rod test)

In this test, Av-EtOH did not impair motor coordination at

0.5, 1 and 2 h post-administration. Diazepam (2.5 mg/kg) caused a significant decrease in the time that the animals remained on the rota-rod apparatus, compared to the control group (Figure 1).

### DISCUSSION

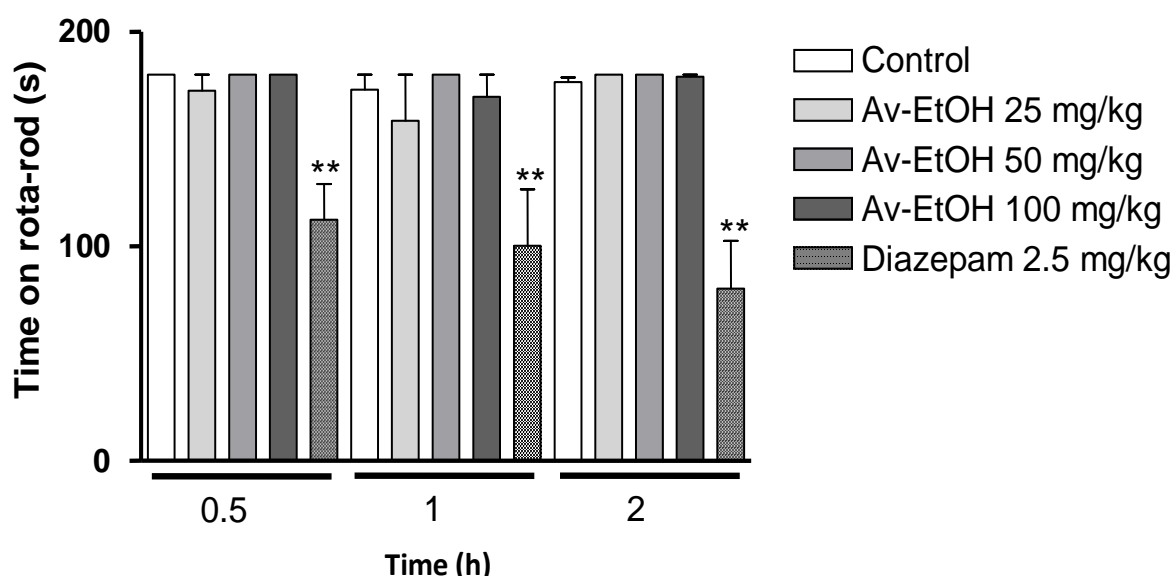
Several *Annona* species have been used in traditional medicine due to their sedative, anxiolytic and tranquilizing properties (Martínez-Vázquez et al., 2012). The present study is the first to demonstrate the effects of *A. vepretorum* on the CNS in mice. Although *A. vepretorum* is widely used in the folk medicine in the semi-arid region of Brazil, no report about its activity on CNS is recorded in the literature. Mice treated with different doses of Av-EtOH (25, 50 and 100 mg/kg) presented behavioral alterations, such as decrease in spontaneous activity, palpebral ptosis, decreased response to touch and sedation. These effects showed possible evidence that the effects on the CNS are similar to those of drugs that reduce central activity (Morais et al., 2004) such as benzodiazepines, for example.

The open-field test is used to measure not only anxiety-like behaviors, but also sedative (Okoli et al., 2010) ones.

**Table 3.** Effects of ethanolic extract of *Annona vepretorum* (Av-EtOH) and diazepam in hole-board test in mice.

Treatment	Dose (mg/kg)	Head dips (number)	Duration of head dips (s)	First head dip (s)	Rearing (number)
Control	---	12.83±2.60	9.75±4.49	79.33±35.17	12.00±3.57
Av-EtOH	25	14.60±1.20	16.50±1.45	35.33±9.67	16.17±3.89
	50	7.40±1.43*	11.83±2.90	29.33±6.23	4.33±1.87
	100	7.00±1.04*	11.50±3.78	16.50±6.71	7.83±3.16
Diazepam	2.5	4.75±1.37*	3.00±1.76	175.00±48.07	8.33±4.59

Values are mean ± SEM; \*p < 0.05, significantly different from control; ANOVA followed Dunnett's test (n = 6, per group).



**Figure 1.** Effect of ethanolic extract of *Annona vepretorum* (Av-EtOH) and diazepam in rota-rod test in mice. Values are mean ± SEM; \*\*p < 0.01, significantly different from control; ANOVA followed Dunnett's test (n = 6, per group).

The extract at a dose of 25 mg/kg significantly reduced the exploratory activity and the rearing number, and increased the immobility time when compared to the control group. The rearing is a function of the excitability level of the CNS (Santos et al., 2012). These effects are indicative of a possible sedative effect and/or muscle relaxant as well as reduced excitability of the CNS or sedation (Okoli et al., 2010). The quantity of grooming was also reduced at doses of 50 and 100 mg/kg. The reduction of the locomotor activity might be due to either an inhibitory effect of the *A. vepretorum* in CNS or by peripheral muscular relaxant activity.

Elevated plus-maze (EPM) is one of the most important animal models used in the evaluation of new anxiolytic drugs. Moreover, it is known that anxiolytic agents increase the frequency of entries and the time spent in

open arms of the EPM (Netto et al., 2009). The administration of Av-EtOH (25, 50 and 100 mg/kg) did not change the anxiety level in the EPM test. Although no effect on anxiety's parameters was detected after the administration of the extract, a trend in increasing the closed arm exploration was observed.

To rule out that Av-EtOH treatment can produce sedative effect without producing anxiolytic profile, we assessed in hole-board test. This test has gained popularity as a model of anxiety, offering "a simple method for measuring the response of an animal to an unfamiliar environment, with advantages that several behaviors can be readily observed and quantified in this test" (Silva and Elisabetsky, 2001). Generally, drugs as benzodiazepines at lower doses significantly increase the number of head-dips in the hole-board test (Netto et al.,

2009). However, we demonstrated that diazepam (2.5 mg/kg) or Av-EtOH (50 and 100 mg/kg, p.o.) treatments decreased the head-dip counts. Drugs with activity at the GABA<sub>A</sub> receptor, such as ethanol, barbiturates and benzodiazepines have well-established effects in hole-board apparatus that are thought of as tests of anxiety-like behaviors in rodents (Kliethermes and Crabbe, 2006). However, drugs with diverse pharmacological properties alter head dipping, suggesting that many neurotransmitter systems are involved in the expression of exploratory behavior. This variability in responses in hole-board remains unclear, but our results are consistent to suggest that Av-EtOH has a sedative effect in this method without apparently producing anxiety-like behaviors.

The rota-rod test designed to assess motor coordination, balance and equilibrium is used to evaluate the pharmacological actions of psychotropic agents on the central or peripheral nervous system (Dunhan and Miya, 1957). Impairment of the rota-rod performance has been thought to reflect, at least in part, a behaviorally depressive state. However, it is well known that the riding time on the rota-rod is also decreased by a relaxation or weakness of the muscles or motor dysfunction. Thus, in addition to central inhibition, the ability of the extract to affect motor coordination may also indicate peripheral blockade of the neuromuscular system (Amos et al., 2001). It is well known that benzodiazepines act as anxiolytics (at low doses), anticonvulsants and also produce sedation and myorelaxant effect at higher doses (Okoli et al., 2010). The results revealed that the extract did not produce changes in motor coordination of treated animals. This is a beneficial effect, indicating that the extract may have no motor coordination deficits as adverse effect like some central nervous system depressants used in the treatment of anxiety (Ya'u et al., 2011).

Taken together, the results of our study suggest for the first time that the ethanolic extract from the leaves of *A. vepretorum* possesses a sedative effect, whose mode of action remains to be elucidated. Continuing pharmacological and chemical studies are necessary in order to characterize the mechanism responsible for this effect, but our study is consistent with the use of *A. vepretorum* as sedative in folk medicine.

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