Anxiolytic-Like Effects of (O-Methyl)-N-2,6-dihydroxybenzoyl-tyramine (Riparin III) from Aniba riparia (NEES) MEZ (Lauraceae) in Mice

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This work presents behavioral effects of (O-methyl)-N-2,6-dihydroxybenzoyl-tyramine (riparin III) isolated from the unripe fruit of Aniba riparia (NEES) MEZ (Lauraceae) in animal models of open field, rota rotod, elevated plus maze and hole board tests in mice. Riparin III (rip III) was administered orally, in male mice, at single doses of 25 and 50 mg/kg. The results showed that rip III, at both doses, had no effects on the spontaneous motor activity in the rota rotod test or the number of squares crossed in the open field test. However, rip III decreased the number of grooming and rearing. In the plus maze test, rip III, at both doses increased the following parameters: percentage of entries in the open arms (PEOA), time of permanence in the open arms (TPOA) and percentage of time of permanence in the open arms (PTOA) and at the dose of 50 mg/kg, increased the number of entries in the open arms (NEOA). Similarly, rip III, at both doses, showed an increase in the number of head dips into the holes of the hole board test. These results show that riparin III presents anxiolytic effects in the plus maze and hole board tests which are not influenced by the locomotor activity in the open field test.

Key words Aniba riparia; riparin III; anxiolytic effect

Aniba is a genus that comprises 41 species of shrubs and trees and is primarily a lowland group with its centre of diversity in Central Amazonia and Guiana. However, this genus extends into the Andes, the mountains of northern Venezuela, the Lesser Antilles and eastern and southern Brazil. One of its species, Aniba riparia (NEES) MEZ, from the Lauraceae family, is popularly known as “louro” in Brazil and occurs in the Humaitá region of the Amazonas state of Brazil.1

From the unripe fruit of Aniba riparia, collected from the Amazonas state of Brazil, were isolated three substances with broad spectrum antimicrobial activity: methyl ethers of N-(benzoyl) tyramine (riparin I), N-(2-hydroxybenzoyl) tyramine (riparin II) and N-(2,6-dihydroxybenzoyl) tyramine (riparin III)2,3 (Fig. 1) which were later synthesized.2,3

It was reported that one of the above compounds, (O-methyl)-N-(2,6-dihydroxybenzoyl) tyramine (riparin III), has potent smooth muscle relaxant activity.4,5 Thus, in concentrations from 8 to 30 μM, riparin III antagonized acetylcholine- and histamine-induced contractions of guinea-pig ileum, and oxytocin- and bradykinin-induced contractions of the rat uterus. Further, in the guinea-pig trachea, riparin III inhibited the spontaneous tone (IC50 7.7 μM) and carbachol-induced contractions (IC50 10 μM). The spasmolytic effect of riparin III, was investigated6 concerning the involvement of the compound in relation to Ca2+ metabolism. It was demonstrated that riparin III produces an inhibition of Ca2+ influx and release of intracellular Ca2+. These results lead to the reduction of intracellular Ca2+ concentration and possibly contribute to the drug spasmolytic effect.

Recently, our group has made a central nervous system pharmacological screening of riparin III, which presented anxiolytic and antidepressant effects in mice, when administered intraperitoneally.6 Thus, the general purpose of the present study was to analyze whether the orally administration of riparin III promotes anxiolytic effects in male mice.

MATERIALS AND METHODS

Animals Male Swiss mice (25–30 g) provided by the Animal House of the Federal University of Ceará (Brazil) were used in each experiment. The animals were housed, groups of 30, in plastic cages with sawdust as bedding and kept in a room with controlled temperature (23±1 °C) and a 12 h light/dark cycle, with food and water ad libitum, except during the experiments. Animals were treated in accordance to the current law and the NIH Guide for Care and Use of Laboratory Animals.

Drugs Riparin III was emulsified with 2% Tween 80 (Sigma-U.S.A.). Animals were treated with the substance at doses of 25 and 50 mg/kg, orally, one hour before the experiments. Controls received 2% Tween 80 (Sigma-U.S.A.) dissolved in distilled water at the same volume as the treated groups (10 ml/kg). Diazepam (DZP) 1 and 2 mg/kg, intraperitoneally, (União Química Brazil) was used as standard.

Experimental Protocol The animals were tested during the light period and were observed in a closed room at constant temperature (23±1 °C) and poorly illuminated with a
15-V red light. One hour after the treatment, the open field and rota rod tests were performed with the same animals in the manner described below: Firstly, the animal was placed in the open field area for 5 min. Immediately after the open field test, the animal was removed to the rota rod where it was evaluated for 1 min. All the other tests were performed in different days with other groups of animals.

**Open Field Test** The open field area was made of acrylic (transparent walls and black floor, 30×30×15 cm) divided into nine squares of equal area. The open field was used to evaluate the exploratory activity of the animal.9) The observed parameters were: number of squares crossed (with the four paws) and numbers of grooming which consists of licking the paws, washing movements over the head, fur licking and tail/genital cleaning10–12) and rearing which is the number of times an animal stood erect on its hind legs with forelegs in the air of against the wall. The animals were divided into four groups with 7—20 mice per group.

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**Rota Rod Test** In this test13) the animals divided into four groups with 7—20 mice per group were placed with the four paws on a 2.5 cm diameter bar, 25 cm above the floor, which was turning at 12 rpm. For each animal, the number of falls (up to three falls) and time of permanence on the bar for 1 min were registered.

**Elevated Plus Maze Test (EPM)** The elevated plus maze for mice14) consisted of two opposed open arms (30×5 cm) and two closed arms (30×5×25 cm) also in opposed position. The open and closed arms were connected by a central platform (5×5 cm). The lateral walls of the closed arms were made of transparent acrylic and the floor was made of black acrylic. The maze was 45 cm above the floor. One hour after the treatment, the animal was placed at the center of the plus maze facing one of the enclosed arms, and observed for 5 min, according to the following parameters: number of entries into the open and closed arms, and time of permanence in each of them. The ratios “number of entries into open arms/number of entries into all (i.e., open and closed) arms” and “time spent in the open arms/time spent in all arms” were calculated and multiplied by 100 to yield the percentages of entries into open arms (PEOA) and the percentage of time of permanence in the open arms (PTOA). Anxiolytic compounds reduce the animal’s aversion to the open arms and promotes the exploration thereof. On the other hand, the forced or voluntary passages of the animal into the open arms of the elevated plus maze are associated with hormonal and behavioral changes indicative of increased anxiety. The animals were divided into four groups with 8—17 per group.

**Hole Board Test** The hole-board test for exploratory behavior in mice was used as described previously by Clark et al. (1971).15) The apparatus used was an Ugo Basile of 60 cm×30 cm with 16 evenly spaced holes with in built infra red sensors. In brief, adult male mice were randomly divided into four groups with 8—20 mice per group. Two groups received graded doses of riparin III (25, 50 mg/kg, p.o.). One group received diazepam (1 mg/kg, i.p.) and the remaining group received vehicle orally to serve as control. One hour later, the number of head dips into the holes was counted for each animal for 5 min. An increase in the head dipping response reveals a positive anxiolytic-like effect.16)

**Statistical Analysis** Data were analyzed by Graphpad Prism version 4.0 software and presented as mean±S.E.M. values. The statistical tests used were one way analysis of variance (ANOVA) followed by Tukey as the post hoc test, except in the parameter of number of falls of rota rod test, in which was used a nonparametric test (Kruskal–Wallis) followed by Dunns as post hoc. Results were considered significant at p<0.05.

### RESULTS

#### Open Field Test

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of squares crosseda</th>
<th>Rearingb</th>
<th>Groomingc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>50.6±4.66 (19)</td>
<td>10.1±0.46 (15)</td>
<td>3.5±0.32 (16)</td>
</tr>
<tr>
<td>RipIII-25</td>
<td>50.6±2.43 (19)</td>
<td>6.1±2.55 (15)**</td>
<td>2.1±0.34 (16)**</td>
</tr>
<tr>
<td>RipIII-50</td>
<td>57.4±2.63 (19)</td>
<td>6.7±2.70 (15)*</td>
<td>1.7±0.21 (16)**</td>
</tr>
<tr>
<td>DZP-2</td>
<td>24.3±7.6 (7)**</td>
<td>0** (9)</td>
<td>1.1±0.29 (8)*</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E.M. of the number of animals in parenthesis. *p<0.05, **p<0.01, ***p<0.001 as compared with the respective control group. a–c) One way ANOVA followed by Tukey as the post hoc.

#### Rota Rod Test

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of fallsd</th>
<th>Time of permanencee (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.45±0.21 (20)</td>
<td>51.75±2.43 (20)</td>
</tr>
<tr>
<td>RipIII-25</td>
<td>1.90±0.22 (20)</td>
<td>46.05±3.18 (20)</td>
</tr>
<tr>
<td>RipIII-50</td>
<td>1.70±0.26 (20)</td>
<td>49.05±3.18 (20)</td>
</tr>
<tr>
<td>DZP-2</td>
<td>2.71±0.18 (7)*</td>
<td>35.25±2.68 (8)*</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E.M. of the number of animals in parenthesis. *p<0.05 as compared with the respective control group. a) Number of falls: nonparametric test of Kruskal–Wallis followed by Dunns as post hoc. b) Time of permanence: one way ANOVA followed by Tukey as post hoc.
studied in animal models of anxiety, such as open field, rota rod, EPM, and hole board tests. These tests are classical models for screening central nervous system actions providing information about psychomotor performance, myorelaxant activity and anxiety.

The assessment of anxiety-related behavior in animal models is based on the assurance that anxiety in animals is comparable to anxiety in humans. Although, as a matter of fact, it cannot be proven that an animal experiences anxiety in the same way as a human being, it is undisputed that distinct behavior patterns in rodents indicate anxiety, i.e. behavioral and peripheral changes presumed to accompany high sympathetic nervous activity.17) Thus, an analogy, if not a homology, between anxiety in humans and rodents may be assumed.

Taking into consideration the various ways to keep the animal under stress, it is well known from both field studies and laboratory observations that rodents tend to avoid the unprotected area of a novel environment when first entering it.18,19) In an experimental setup, usually represented by a defined area, as the open field, rodents will typically start to explore the environment along the walls while avoiding the open, i.e. unprotected area. In order to guarantee that the animal would explore the unprotected area, we placed the animal in the centre of the open field instead of the lateral. Another point of stressful event is that the aversive character of an area can be modulated by illumination levels, with a brightly lit area being more aversive for rodents, thus producing a more pronounced avoidance behavior than a dark area. Likewise, we placed the animals in a closed room poorly illuminated with a 15-V red light. Another possibility to increase a rodent’s aversion against an unprotected area is by elevating it and by enabling the animal to see the edge, as we can see in the EPM and hole board tests respectively.

The EPM is based on the observation that rodents tend to avoid elevated areas.20) Following the concept of avoidance behavior in rodents, avoidance of the open arms is interpreted as anxiety.21—23) Moreover, the EPM test is the most popular test to search for new benzodiazepine-like anxiolytic agents.21,22) Our results showed that riparin III decreased the avoidance to open arms, thereby increasing the number of entries into open arms (NEOA) with the higher dose and also increased, at both doses, the time of permanence in the open arms (TPOA), the percentage of entries into open arms (PEOA) and the percentage of time of permanence in the open arms (PTOA), similar to the effects observed after administration of the reference anxiolytic drug diazepam (1 mg/kg).

The hole board test is a measure of exploratory behavior.24) Exploration is gradually inhibited by anxiety, thereby representing an indirect measurement of anxiety.21—23) The inhibition of exploration behavior can be reversed by anxiolytic compounds25—28) indicated, in the case of hole board test, by the increase of number of head dips.29) The animals treated with riparin III at both doses showed an increase in the number of head dips as the same as diazepam (1 mg/kg), indicating an anxiolytic activity.

Data in literature demonstrated that drugs that alter general motor activity may give false-positive/negative results in the plus maze. In this way, we decided to study the effects of riparin III in the open field test, a classical animal model that
Our findings show that riparin III (25, 50 mg/kg) did not alter the locomotor activity different from diazepam 2 mg/kg which decreased this parameter showing a sedative effect. Likewise, it is unlikely that these effects of riparin III observed in the plus maze and hole board tests are based on the stimulation of general motor activity. Further, in the rota rod test, riparin III, different from diazepam (2 mg/kg), had no effect on the motor co-ordination suggesting that the anxiolytic-like effect of the substance might not be exerted through peripheral neuromuscular blockade, but rather, elicited centrally.\(^{31,32}\)

It is well known that benzodiazepines act as anxiolytics (at low doses), anticonvulsants, and also produce sedation and a myorelaxant effect at higher doses,\(^{33—36}\) thereby our group has used diazepam at 1 mg/kg in EPM and hole board tests and 2 mg/kg in open field and rota rod tests.

In this work, we could observe that riparin III seems to be more active after oral administration as compared to the intraperitoneal administration as detected in previous work\(^8\) in the plus maze test. However, the apparent discrepancy could be due to the different number of animals used in each condition. For instance, in the first condition (after oral administration) number of animals used were higher. It is largely accepted that behavioral tests require a great number of animals due to tests variability. In this way, in the second condition (intraperitoneal administration), although not detected statistically, a clear tendency was observed. Furthermore, recent data from our laboratory using riparin I (methyl ethers of \(N\)-benzoyl tyramine) also isolated from unripe fruit of \(Aniba riparia\) demonstrated anxiolytic activity in both routes of administration.\(^{37}\)

The anxiolytic effect of riparin III detected in the plus maze test was also observed in the hole board test with both routes of administration. Similarly, in the open field test, it could be seen that riparin III had the same effects comparing i.p.\(^8\) and p.o. (present study) administrations.

In conclusion, we showed that acute oral administration of riparin III presented anxiolytic-like effects in the elevated plus maze and hole board tests and was devoid of sedative effect as assessed by the open field test.

REFERENCES