Evaluation of Effects of *N*-(2-Hydroxybenzoyl) Tyramine (Riparin II) from *Aniba riparia* (NEES) MEZ (Lauracea) in Anxiety Models in Mice

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In order to evaluate the effects produced by *N*-(2-hydroxybenzoyl) tyramine (riparin II) isolated from the unripe fruit of *Aniba riparia* (NEES) MEZ (Lauraceae) on the central nervous system, different behavioral tests were performed. Riparin II (rip II) was administered orally (*p.o.*) and intraperitoneally (*i.p.*) in male mice, at doses of 25, 50 and 75 mg/kg, and tested on elevated plus maze (EPM), open field, rota rod and hole board tests. The results revealed that rip II caused considered increase of the number of head dips in hole board test and increased the number of entries and the time of permanence in the open arms in plus maze test in both routes. No significant effect was evidenced on rota rod and open field test, except an increase observed in the number of rearing. These results showed that riparin II presents anxiolytic-like effects in the plus maze and hole board tests which are not influenced by the locomotor activity as detected in the open field test.

Key words *Aniba riparia*; riparin II; antianxiety

Aniba riparia (NEES) MEZ, from the Lauraceae family, is popularly named “louro” in Brazil. It belongs to a genus mainly found in Central Amazonia and Guiana comprising approximately 40 species of lowland shrubs and trees.^{1,2)}

From the green 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)¹⁾ which were later synthesized.³⁾

It was previously reported that (*O*-methyl)-*N*-(2,6-dihydroxybenzoyl) tyramine (riparin III) has potent smooth muscle relaxant activity^{4,5)} and this spasmolytic effect was possibly related to a reduction of intracellular Ca²⁺ concentration.⁶⁾

Recently, it was reported by us that riparin I⁷⁾ and III^{8,9)} presented antianxiety effects in mice treated with the doses of 25 and 50 mg/kg. As far as we know, there are no studies in the literature on the central actions of riparin II. However, this substance is an *N*-(2-hydroxybenzoyl) tyramine, and previous data showed that the spectrum of tyramine actions are similar to those of norepinephrine. In fact, some reports related that tyramine-rich foods when ingested by individuals taking antidepressant drugs, such as monoamino oxidase inhibitors, might result in a prolonged increase in blood pressure.¹⁰⁾

Hereby, these findings led us to investigate the behavioral effects of riparin II on animals models of locomotion, anxiolytic and myorelaxant activities.

MATERIALS AND METHODS

Animals Male Swiss mice weighing 25–30 g were used in these experiments. The animals were housed in plastic cages, 30 to cage, under a 12 h light/dark cycle (light on at 7:00 a.m.) at constant temperature of 23 ± 1 °C with free access to food and water, 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 II was emulsified with 3% Tween 80 (Sigma-U.S.A.) and dissolved in distilled water. Animals were treated with the compound in doses of 25, 50 and 75 mg/kg, once, 30 or 60 min before the experiments when administered by intraperitoneal or oral routes, respectively. Controls received vehicle at the same volume (10 ml/kg) and the same route as the treated groups. Diazepam (DZP), União Química Brazil, (1, 2 mg/kg) 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.

Elevated Plus Maze Test (EPM) The EPM for mice¹¹⁾ 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

Riparin I (R1=R2=H)
Riparin II (R1=OH, R2=H)
Riparin III (R1=R2=OH)

Fig. 1. Structure of Riparin (I, II, III)

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transparent acrylic and the floor was made of black acrylic. The maze was 45 cm above the floor. Thirty minutes after intraperitoneal treatment or 60 min after oral 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 and the percentage of time of permanence in the open arms. Anxiolytic compounds reduce the animal's aversion to the open arms and promote the exploration thereof. The animals were divided into four groups with 8–17 per group.

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.¹²⁾ The observed parameters were: number of squares crossed with the four paws (locomotor activity) and numbers of grooming (number of times the animal makes washing movements over the head) and rearing (number of times the animal stood completely erect on its hind legs).

Rota Rod For the rota rod test, the animal was placed with the four paws on a 2.5 cm diameter bar, 25 cm above the floor, which was rotating at 12 rpm. For each animal, the number of falls (up to three falls) and the time of permanence on the bar for 1 min were registered.¹³⁾

Hole Board Test The hole-board test for exploratory behavior of mice was used as described previously by Clark *et al.* (1971).¹⁴⁾ The apparatus used was an Ugo Basile of 60 cm×30 cm with 16 evenly spaced holes with built infra red sensors. For each animal, the number of head dips into the holes was counted for each animal for 5 min.

Statistical Analysis All results are presented as mean±S.E.M. ANOVA followed by Newman–Keuls as the *post hoc* test was used. Results were considered significant at $p<0.05$.

RESULTS

Figures 2 and 3 show the effects of riparin II from *Aniba riparia* in EPM test at both routes. The results showed that the oral treatment of riparin II (Fig. 2) increased the parameters number of entries into the open arms, percentages of entries into open arms and time of permanence in open arms only at the dose of 75 mg/kg [number of entries into the open arms: 9.33 ± 0.84 (9) $p<0.01$; percentages of entries into open arms: 53.0 ± 2.75 (9) $p<0.05$; time of permanence in open arms: 116.1 ± 10.57 (10) $p<0.05$] compared to respective controls [number of entries into the open arms: 5.77 ± 0.48 (13); percentages of entries into open arms: 41.24 ± 2.05 (12); time of permanence in open arms: 76.08 ± 7.69 (12)]. Figure 3 shows that when riparin II was administered intraperitoneally, it presented a statistical increase in all parameters analyzed at the dose of 25 mg/kg [number of entries into the open arms: 7.07 ± 0.69 (15) $p<0.05$; percentages of entries into open arms: 47.81 ± 1.81 (13) $p<0.05$; time of permanence in open arms: 133.7 ± 8.65 (10) $p<0.05$; per-

Fig. 2. EPM Test of Groups of Mice Which Received Vehicle, Riparin II (25, 50, 75 mg/kg), *p.o.*, and DZP (1 mg/kg)

(A) number of entries in the open arms; (B) percentage of entries in the open arms; (C) time of permanence in the open arms. (D) percentage of time of permanence in the open arms. The results are presented as mean±S.E.M. Significant difference compared with control (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). ANOVA and Newman–Keuls as the *post hoc* test.

centage of time of permanence in the open arms: 53.06 ± 2.43 (8) $p<0.05$] as compared to controls [number of entries into the open arms: 4.62 ± 0.4 (13); percentages of entries into open arms: 38.75 ± 2.58 (15); time of permanence in open arms: 91.92 ± 7.79 (13); percentage of time of permanence in the open arms: 36.16 ± 3.10 (13)]. Similarly, diazepam (1 mg/kg, *i.p.*), used as standard, increased all parameters observed [number of entries into the open arms: 9.5 ± 0.70 (14) $p<0.01$; percentages of entries into open arms: 54.18 ± 3.03 (14) $p<0.05$; time of permanence in open arms: 146.5 ± 10.76 (16) $p<0.001$; percentage of time of permanence in the open arms: 59.28 ± 3.80 (16) $p<0.001$] comparing to *i.p.* and *per os* controls.

In the open field test (Table 1), the groups treated orally

25 mg/kg and intraperitoneally 75 mg/kg with riparin II decreased the number of rearing as compared to controls. No alteration was observed in the number of crossing and

grooming with riparin II at the doses and routes used in the present work. Diazepam (2 mg/kg, i.p.) decreased all the parameters as compared to controls.

In the rota rod test (Table 2), there were no difference after the treatment with riparin II, at the doses and routes used, unlikely diazepam (2 mg/kg, i.p.) when compared with respective controls.

Figure 4 shows the results of the *per os* administration of rip II in hole board test. In this test, rip II increased, with both doses, the number of head dips [ripII-25: 48.71 ± 3.82 (7) $p < 0.01$; ripII-50: 52.00 ± 4.0 (8) $p < 0.001$] as compared to control [31.62 ± 3.08 (8)]. Riparin II also increased this parameter after i.p. treatment (Fig. 5) at both doses [ripII-25: 31.4 ± 0.9 (12) $p < 0.001$; ripII-50: 31.5 ± 1.4 (13) $p < 0.001$] comparing to control [24.3 ± 1.0 (13)]. As the same as riparin II, diazepam (1 mg/kg; i.p.) increased the number of head dips [47.16 ± 1.35 (6) $p < 0.05$].

Fig. 3. EPM Test of Groups of Mice Which Received Vehicle, Riparin II (25, 50, 75 mg/kg), i.p., and DZP (1 mg/kg)

(A) number of entries in the open arms; (B) percentage of entries in the open arms; (C) time of permanence in the open arms. (D) percentage of time of permanence in the open arms. The results are presented as mean $\pm$ S.E.M. Significant difference compared with control (* $p < 0.05$; *** $p < 0.001$). ANOVA and Newman-Keuls as the *post hoc* test.

Table 1. Effects of Riparin II and Diazepam in the Open Field Test

Groups	Number of squares crossed ^{a)}	Rearing ^{b)}	Grooming ^{c)}
Control <i>per os</i>	61.17 $\pm$ 5.06 (12)	10.42 $\pm$ 1.0 (12)	4.16 $\pm$ 0.61 (12)
RipII-25 mg/kg <i>per os</i>	58.0 $\pm$ 5.16 (12)	6.17 $\pm$ 1.06 (12)*	4.67 $\pm$ 0.51 (12)
RipII-50 mg/kg <i>per os</i>	57.25 $\pm$ 3.31 (12)	6.92 $\pm$ 1.18 (12)	4.08 $\pm$ 0.70 (12)
RipII-75 mg/kg <i>per os</i>	74.0 $\pm$ 4.42 (9)	9.33 $\pm$ 1.14 (9)	5.33 $\pm$ 1.07 (9)
Control i.p.	45.83 $\pm$ 1.98 (12)	8.5 $\pm$ 0.97 (12)	3.08 $\pm$ 0.45 (12)
RipII-25 mg/kg i.p.	49.67 $\pm$ 2.41 (12)	6.83 $\pm$ 0.80 (12)	2.17 $\pm$ 0.36 (12)
RipII-50 mg/kg i.p.	47.83 $\pm$ 3.30 (12)	6.27 $\pm$ 0.79 (11)	1.92 $\pm$ 0.36 (12)
RipII-75 mg/kg i.p.	43.43 $\pm$ 3.94 (7)	2.0 $\pm$ 0.52 (6)***	2.57 $\pm$ 0.65 (7)
DZP-2 mg/kg i.p.	24.28 $\pm$ 7.61 (7)##	0 (8)###	1.12 $\pm$ 0.29 (8)#

Each value represents the mean $\pm$ S.E.M. of the number of animals in parenthesis. ** $p < 0.01$, *** $p < 0.001$ as compared to respective controls. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ as compared to both control groups. a—c) One way ANOVA followed by Newman-Keuls as the *post hoc* test.

Table 2. Effects of Riparin II and Diazepam in the Rota Rod Test

Groups	Number of falls ^{a)}	Time of permanence ^{b)} (s)
Control <i>per os</i>	0.76 $\pm$ 0.20 (13)	59.0 $\pm$ 0.28 (13)
RipII-25 mg/kg <i>per os</i>	0.77 $\pm$ 0.32 (13)	55.31 $\pm$ 2.98 (13)
RipII-50 mg/kg <i>per os</i>	1.08 $\pm$ 0.31 (13)	57.31 $\pm$ 1.22 (13)
RipII-75 mg/kg <i>per os</i>	0.62 $\pm$ 0.32 (8)	58.75 $\pm$ 0.65 (8)
Control i.p.	0.58 $\pm$ 0.19 (12)	59.25 $\pm$ 0.25 (12)
RipII-25 mg/kg i.p.	0.67 $\pm$ 0.26 (12)	57.42 $\pm$ 1.96 (12)
RipII-50 mg/kg i.p.	0.92 $\pm$ 0.19 (12)	58.67 $\pm$ 0.33 (12)
RipII-75 mg/kg i.p.	1.0 $\pm$ 0.38 (8)	57.50 $\pm$ 1.18 (8)
DZP-2 mg/kg i.p.	2.5 $\pm$ 0.27 (8)**	35.25 $\pm$ 6.69 (8)***

Each value represents the mean $\pm$ S.E.M. of the number of animals in parenthesis. ** $p < 0.01$, *** $p < 0.001$ as compared to both control groups. a, b) One way ANOVA followed by Newman-Keuls as the *post hoc* test.

Fig. 4. Hole Board Test of Groups of Mice Which Received, Riparin II (25, 50 mg/kg), *p.o.*, and DZP (1 mg/kg)

The figure shows the number of head dips. The results are presented as mean $\pm$ S.E.M. Significant difference compared with control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). ANOVA and Newman-Keuls as the *post hoc* test.

Fig. 5. Hole Board Test of Groups of Mice Which Received, Riparin II (25, 50 mg/kg), i.p., and DZP (1 mg/kg)

The figure shows the number of head dips. The results are presented as mean $\pm$ S.E.M. Significant difference compared with control (*** p < 0.001). ANOVA and Newman-Keuls as the *post hoc* test.

DISCUSSION

In this work, it was studied the effects in anxiety of riparin II from *A. riparia* in several behavioral animal models 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.

Behavior in rodents is determined by the conflict between the drive to explore the unknown area/object and the motivation to avoid potential danger. Exploration behavior in rodents summarizes a broad spectrum of behavioral patterns such as risk assessment behaviors, walking, rearing, climbing, sniffing, and manipulating objects.¹⁵⁾ Exploration is gradually inhibited by anxiety, thereby representing an indirect measurement of anxiety.^{16,17)}

For the EPM test, it has been demonstrated that the preference shown for the closed arms reflects an aversion toward the open arms, caused by fear or anxiety induced by the open space.¹⁸⁾ The EPM test is the most popular test to search for new anxiolytic agents.^{17,19)} Thus, the present results show that the riparin II is active in this test of fear or anxiety since riparin II at 25 mg/kg (i.p.) and 75 mg/kg, (*p.o.*) had anti-anxiety effects, as indicated by increase of the time spent and the number of entries in the open arms. However, even if the doses of 50 mg/kg and 75 mg/kg, i.p., have not presented statistically significant anxiolytic-effect, there was a tendency to have. This fact may not have been detected probably due to the number of animals used, such as the number of animals used in the group of 25 mg/kg was higher than the number used in other groups.

Besides, the anxiolytic-like effect of riparin II was confirmed in the hole board test which measures exploratory behavior.²⁰⁾ Anxiolytics have been shown to increase the number of head dips.²¹⁾ The animals treated with riparin II at both doses (25 and 50 mg/kg, *p.o.* and i.p.) showed an increase in the number of head dips as the same as diazepam (1 mg/kg), indicating an anti-anxiety activity.

The anxiolytic-like effect of riparin II shown in the plus maze and hole board tests seems to be similar to the riparin III anxiolytic effects. This can be seen comparing riparin II and riparin III. For example, the last substance, when administered intraperitoneally, only presented anxiolytic effect with the higher dose on the plus maze test, but presented anxiolytic effect on the hole board test with both routes and doses used (Sousa *et al.*, 2004), similarly to riparin II. Besides, the potencies of riparin I, II and III as anxiolytic agents seem to

be similar once there are no statistical differences among them. Moreover, none of the three substances seem to present dose-dependency effect, as can be seen in previous works with riparin I (Sousa *et al.*, 2005) and with riparin III (Sousa *et al.*, 2004; Melo *et al.*, 2006). Similarly, riparin II did not show dose-dependency effect among the treated groups, suggesting that these three substances present similar effects. This can be explained by the similarities between their chemical structures (Fig. 1).

As riparin II acted similar to DZP in the EPM and hole board tests, it is possible to speculate that its anxiolytic-like effects observed in these tests could be probably related to the potentiation of GABA's inhibitory actions. However, we can not discard that other mechanisms can be involved and further studies need to be performed.

The open-field test is based on rodents' natural tendency to stay near the perimeters of a novel environment (*i.e.*, thigmotaxis), which may serve to protect the animal from avian predators.²²⁾ Anxiolytic effects are indicated by increased entries and time spent in the open-field's central sector, in the absence of concomitant changes in general locomotor activity.²³⁾ Previous works showed that drugs which alter locomotor activity may give false-positive/negative results in the plus maze. Therefore, in order to verify the relation between rip II anxiolytic effects with locomotor activity alteration, we used in our protocol the open field test, which also can be useful in detecting genetic or pharmacological effects on anxiety.²³⁾ The open field test, a classical animal model, is used to evaluate autonomic effects of drugs and general activity of animals.²⁴⁾ Our findings show that the animals treated with riparin II (25, 50, 75 mg/kg) did not alter the locomotor activity, different from diazepam (2 mg/kg) which decreased this parameter showing a sedative effect. In addition, the anxiolytic-like effect of riparin II seems not to be associated with any motor effects since it did not alter significantly the ambulatory behavior in the open field test. This indicates that increased motor activity were not involved in the action seen in both plus maze and hole board tests, and confirms the assumption that the anxiolytic-like effect of riparin II may be specific.

In the rota rod test, different from diazepam (2 mg/kg), riparin II 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.^{25,26)}

In summary, the present data show that riparin II presents anxiolytic-like effects in the EPM and hole board tests, similarly to the other compounds isolated from *Aniba riparia*, riparin I⁷⁾ and III.^{8,9)} Moreover, this effect of riparin II was devoid of sedative or myorelaxant effect as assessed by the open field and rota rod tests respectively.

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