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Research Paper

Acute effect of *Copaifera reticulata* Ducke copaiba oil in rats tested in the elevated plus-maze: an ethological analysis

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Abstract

Objectives Copaiba oil oleoresin exuded from *Copaifera reticulata* Ducke (CRD) is commonly used in anti-inflammatory, healing and anti-tumoral folk medicines. The purpose of this study was to investigate the putative anxiolytic effect of acute administration of CRD.

Methods CRD was administered (100, 400 and 800 mg/kg, p.o.) to male Wistar rats submitted to the elevated plus-maze model of anxiety using an ethopharmacological analysis.

Key findings In comparison with control rats, CRD increased the percentage of entries in the open arms over the entire dose range tested (vehicle, 33.6 ± 4.5; CRD 100 mg/kg, 44.67 ± 3.68; CRD 400 mg/kg, 47.2 ± 2.3; CRD 800 mg/kg, 50.7 ± 2.2) and the percentage of time spent in the open arms of the elevated plus-maze at the highest dose (800 mg/kg) (vehicle, 26.4 ± 5.7; CRD 800 mg/kg, 52.0 ± 2.7). A standard anxiolytic, diazepam (3 mg/kg, p.o.), was used as a positive control. In a similar way, diazepam increased the percentage of entries and time spent in the open arms when compared with vehicle (% open entries: vehicle, 45.4 ± 1.3; diazepam, 50.7 ± 1.9; % time spent in open arms: vehicle, 28.2 ± 0.9; diazepam, 38.9 ± 1.2). Regarding ethological measures, CRD at the highest dose (800 mg/kg) reduced peeping out (anxiety-related behaviour) (vehicle, 3.1 ± 0.6; CRD, 0.9 ± 0.2) and increased end-arm activity (vehicle, 0.2 ± 0.2; CRD, 2.0 ± 0.4), indicating an enhanced tendency of the rats to explore actively the potentially dangerous areas of the maze. Diazepam decreased peeping out (vehicle, 3.3 ± 0.3; diazepam, 1.0 ± 0.2) and flat-back approach (vehicle, 0.8 ± 0.2; diazepam, 0.2 ± 0.1) and increased end-arm activity (vehicle, 0.3 ± 0.1; diazepam, 2.5 ± 0.3) and head-dipping (vehicle, 8.2 ± 0.4; diazepam, 12.0 ± 0.5).

Conclusions These data showed, for the first time, that acute treatment with CRD copaiba oil produced a dose-dependent anxiolytic-like effect over the dose range tested, on conventional and ethological parameters, without adversely affecting general activity levels.

Keywords anxiolytic; *Copaifera reticulata* Ducke; elevated plus-maze; ethological analysis

Introduction

Anxiety disorders are among the most prevalent psychiatric disorders. Since chlordiazepoxide was introduced for the treatment of anxiety, in 1960, benzodiazepines have been the mainstay of treatment for anxiety disorders. Although benzodiazepines show clear efficacy, considerable concern has been expressed regarding their ability to induce undesirable features such as sedation, muscle relaxation, amnesia, interaction with alcohol/barbiturates and dependency liability.[1]

Copaiba oils are produced by some *Copaifera* L. species as an exudate obtained from the trunk of the trees. The native population of the Amazon forest in the north region of Brazil have known their medicinal properties (anti-inflammatory, healing and tonic) since ancient times.[2,3]

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Several pharmacological actions of these oils have been tested and some popular knowledge has been confirmed, such as the anti-inflammatory,[4–6] antimicrobial[7,8] and anti-cancer activity.[9] Recent studies have described the chemical composition of the oils from some species, including *C. reticulata* Ducke (CRD).[6] However, thus far, no tonic or behavioural activity has been tested using scientifically validated models. For instance, there is no evidence that CRD can affect locomotor activity or avoidance response in any behavioural test.

Animal models of anxiety are used as screening tools in the search for compounds with therapeutic potential and as stimulus for research on mechanisms underlying emotional behaviour.[10] The continued search for new anxiolytic compounds that are without adverse side effects has led to a proliferation of potential anxiolytic agents, which has reinforced the need for efficient pre-clinical screening tests of their effects.[11]

The elevated plus-maze (EPM) is currently the first-choice test for screening anxiolytic drugs. Anxiety, in this test, is routinely assessed by measures of open-arm avoidance, while locomotor activity is most reliably measured by the frequency of closed-arm entries.[12–14] Avoidance of the open arms of the maze has been related to generalized anxiety recognized in clinical practice.[15–17]

A number of authors have argued that test sensitivity and reliability, and ecological validity, may be improved by focusing upon what the animals actually do in the maze, as well as their physical localization.[18] In this context, several research groups have incorporated a variety of behavioural elements derived from ethological analysis in addition to the standard parameters of entries into, and time spent in, the aversive open arms.[10,18,19] The application of ethological techniques to the EPM came from studies on rodent defensive behaviour (e.g. risk assessment) and, in particular, the elegant work of the Blanchards on anti-predator defence in wild rats. The comprehensive behavioural profiles yielded by this technique also provide information invaluable to the question of the behavioural specificity of drug action.[20] Pharmacological studies have shown that the incorporation of such measures in plus-maze scoring not only reduces the likelihood of false positives and negatives, but also enhances the sensitivity of the model to novel anxiolytics.[12]

On the basis of these considerations, the purpose of this study was to verify the therapeutic activity of acute administration of CRD copaiba oleoresin (100, 400 and 800 mg/kg, doses chosen from a pilot study where several copaiba oils were tested and this specific species was selected) using an ethopharmacological analysis of behaviour of rats tested in the EPM. Soy oil was used as vehicle control. Since the anxiolytic potential of CRD was under investigation, we conducted a second experiment including a positive control group, diazepam (3 mg/kg), to compare the effects of a standard anxiolytic with those of vehicle.

Methods

Subjects

Male Wistar rats, 250–300 g (approximately on the day of the test), from the animal house of the University Estácio de Sá, Nova Friburgo, RJ, Brazil, were used. The rats were housed in groups of six per cage under a 12-h light–dark cycle (lights on at 0700 h) at 23 ± 1°C, and given free access to food and water. The rats were taken to the test laboratory at least 1 h before testing. All protocols of this study were approved by the ethics committee of the University Estácio de Sá (06.125.8) and were followed according to the rules for animal experimentation of the SBNeC (Brazilian Society of Neuroscience and Behavior), which are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals.

Compound administration

The copaiba oleoresin from CRD was collected near Belém, State of Pará, in the north region of Brazil, and an exsiccate was deposited at the INPA herbarium (No. INPA61.212). Diazepam (Sigma, St Louis, MO, USA) was dissolved in saline (0.9% NaCl solution) shortly before use. All solutions were prepared on the day of the experiment and administered orally by gavage, in a volume of 1 ml/kg.

Apparatus

The plus-maze consisted of two open arms, 50 × 10 cm (length × width) and two enclosed arms 50 × 10 × 50 cm (length × width × height), arranged such that the two arms of each type were opposite to each other. The whole maze was made of wood and was elevated to a height 50 cm from the floor.

Procedures

All testing was conducted during the mid-portion of the light phase of the light–dark cycle. The maze was located in a dimly lit room and the level of white light illumination was 15 lux on the floor level of the walled arms. The rats were placed individually in the center of the maze facing a closed arm and allowed 5 min of free exploration. The behaviour of the rats was recorded by a video camera positioned 120 cm above the maze, allowing for the discrimination of all behaviours, with the signal relayed to a monitor installed in an adjacent room via a closed-circuit TV camera. The maze was thoroughly cleaned after each test with a solution of 20% ethanol and then dried. Each rat was tested only once.

Two experiments were conducted. In experiment 1 (acute CRD) groups of rats ($n = 10$) were tested following pretreatment with soy oil or CRD copaiba oleoresin (100, 400 and 800 mg/kg). In experiment 2 (acute diazepam), rats were assigned to two treatment conditions ($n = 10$), soy oil or diazepam (3 mg/kg).

In both experiments, soy oil was used as vehicle control, and rats were tested in the EPM 45 min after treatment. Selection of diazepam dose was based on previous study.[21]

Behavioural analysis

Videotapes were subsequently scored, by two highly trained observers (intra- and inter-rater reliability equal to 0.9) blind to treatment condition, using the ethological analysis software (X-Plot-Rat, version 3.3.0, 2002). The behavioural parameters recorded comprised both conventional spatiotemporal and ethological measures.

The behaviour of each rat in the maze was analysed, taking into account the standard measures recorded in each section of the maze (closed and open arms), comprising the frequency of open- and closed-arm entries (arm entry defined as all four paws into an arm), total arm entries and the amount of time spent by the rats in each section of the maze. These data were additionally used to calculate the percentage of open-arm entries and the percentage of time in the open arms.

The ethological items recorded were rearing, head dipping, end-arm activity, peeping out, stretched-attend posture and flat-back approach. These categories were defined following work in rats[19,22] and in mice:[18] (a) rearing (REAR), partial or total rising onto the hind limbs; (b) head dipping (DIPS), exploratory movement of head/shoulders over sides of the maze and down towards the floor; (c) end-arm activity (EAA), number of times the rat reached the end of an open arm; (d) peeping out (PEEP), stretching the head/shoulders from the closed arms to the central platform; (e) stretched-attend posture (SAP), when the rat stretches to its full length and turns back to the anterior position; and (f) flat-back approach (FLAT), locomotion when the rat stretches to its full length and cautiously moves forward.

Statistical analysis

Data are reported as mean ± SEM. Results of experiments were analysed by one-way analyses of variance. Newman–Keuls post-hoc comparisons were carried out if significant overall F-values ($P < 0.05$) were obtained.

Results

Experiment 1: acute CRD copaiba oil

One-way analysis of variance revealed that acute treatment with CRD copaiba oil significantly increased the percentage of open-arm entries F (3,39) = 4.89, $P < 0.05$, (Figure 1a), and the percentage of time spent in the open arms F (3,39) = 5.21, $P < 0.05$, (Figure 1b). Post-hoc analysis revealed that the percentage of open-arm entries increased significantly over the entire dose range tested (100, 400 and 800 mg/kg), whereas only at the highest dose (800 mg/kg), the increase in the percentage of time spent in the open arms reached statistical significance. Moreover, the copaiba oil did not change the number of entries into the enclosed arms F (3,39) = 0.42, $P > 0.05$. Regarding the ethological measures, acute treatment with copaiba oil significantly decreased rearing F (3,39) = 3.31, $P < 0.05$, and peeping out F (3,39) = 4.02, $P < 0.05$, and increased end-arm activity F (3,39) = 4.38, $P < 0.05$. Post-hoc analyses revealed that these effects were due to the 400 mg/kg dose for rearing, the 100 and 800 mg/kg doses for peeping out, and 800 mg/kg dose for end-arm activity. No significant effects were seen in any of the following categories: head-dipping F (3,39) = 0.58, $P > 0.05$; flat-back approach F (3,39) = 0.89, $P > 0.05$ and stretch-attend posture F (3,39) = 1.66, $P > 0.05$ (Table 1).

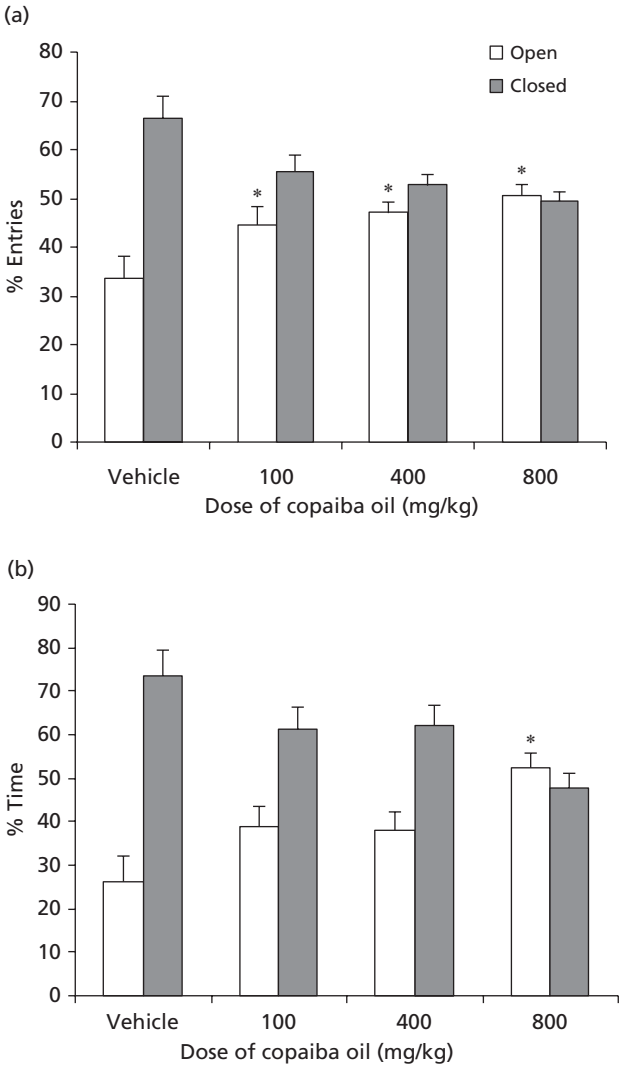

Figure 1 Effect of acute administration of *Copaifera reticulata* Ducke copaiba oil on (a) the percentage of entries made by rats in the elevated plus-maze test and (b) the time spent in open and closed arms of the plus maze. *Copaifera reticulata* Ducke (CRD) copaiba oil (100, 400 and 800 mg/kg, p.o) was administered 45 min before testing. *$P < 0.05$ compared with vehicle (Newman–Keuls test). Data are presented as mean ± SEM, $n = 10$ for each dose.

Experiment 2: acute diazepam

In a very similar way to copaiba oil, diazepam (3 mg/kg) significantly increased the percentage of open-arm entries F (1,19) = 4.91, $P < 0.05$ (Figure 2a) and the percentage of time spent in the open arms F (1,19) = 48.03, $P < 0.05$ (Figure 2b) without affecting the number of entries into the enclosed arms F (1,19) = 0.35, $P > 0.05$. Regarding the ethological measures, acute treatment with diazepam decreased rearing F (1,19) = 16.78, $P < 0.05$, peeping out F (1,19) = 26.3, $P < 0.05$ and flat-back approach F (1,19) = 4.5, $P < 0.05$, and increased end-arm activity F (1,19) = 41.0, $P < 0.05$ and head-dipping F (1,19) = 28.5, $P < 0.05$. The effect on stretch-attend posture F (1,19) = 2.7, $P > 0.05$ was non-significant (Table 2).

Table 1 Effect of *Copaifera reticulata* Ducke copaiba oil on ethological measures of behaviour displayed by rats in the elevated plus-maze test

Behaviour	Vehicle	CRD (100 mg/kg)	CRD (400 mg/kg)	CRD (800 mg/kg)
Rearing	12.6 ± 0.8	10.3 ± 1.0	8.0 ± 0.8*	13.2 ± 1.0
Peeping out	3.1 ± 0.6	1.5 ± 0.3*	1.9 ± 0.4	0.9 ± 0.2*
End-arm activity	0.2 ± 0.2	0.8 ± 0.3	1.2 ± 0.3	2.0 ± 0.4*
Head dipping	7.9 ± 1.3	8.2 ± 0.8	7.8 ± 1.4	9.7 ± 0.8
Flat-back approach	0.6 ± 0.2	0.6 ± 0.2	0.4 ± 0.1	0.2 ± 0.1
Stretched-attend posture	9.6 ± 0.7	7.5 ± 1.2	7.3 ± 0.7	7.5 ± 0.4

The effect of *Copaifera reticulata* Ducke (CRD) copaiba oil (100, 400 and 800 mg/kg, p.o) on ethological measures of behaviour displayed by male Wistar rats in the elevated plus-maze test was examined. Compound was administered 45 min before testing. *$P < 0.05$ compared with vehicle (Newman–Keuls test). Data are presented as mean ± SEM, $n = 10$, for each dose.

Discussion

Copaifera reticulata Ducke (CRD) is a native tree distributed only in the Amazonian region, from Pará to Acre States, in Brazil. Its oil is the most commonly commercialized in the Belém region, especially for exportation to be used in the perfumery and cosmetic industries. Copaiba oil is popularly used as a medicine in several countries of Central and South America. Its use in the treatment of respiratory and urinary tract inflammation has been described since the first Europeans start living in the American tropical forests. Actually, this oil is currently found only in the Amazonian region, where people still use it as a cure for ailments ranging from toothache, rheumatism and syphilis to snake bite and aconite poisoning. Moreover, at different places within this region, people use the copaiba oil (as a tea from the bark of the *Copaifera* L. trees) as a tonic to prevent several diseases.[3]

A recent study describes the chemical composition of CRD and its anti-inflammatory activity, with the oleoresin inhibiting nitric oxide production and pleurisy, but with less intensity.[6] Among the sesquiterpenes, the main compound described was β-caryophyllene 40.9%, followed by α-humulene, α-copaene, α-bergamotene and δ-cadinene. The two main diterpenes were kaurenoic (3.9%) and kolavenic acids (3.4%).[6]

In rats, acute treatment with CRD copaiba oil produced an anxiolytic profile characterized by increased number of entries (over the entire dose range tested) and time spent in the open arms of the maze in a way similar to the reference drug diazepam. These effects are not the result of changes in motor activity, since copaiba oil did not affect the number of entries into the enclosed arms considered as an index of general locomotor activity of the rats in this model.[13,14,23] Diazepam produced a clear anxiolytic effect, reducing avoidance of the open arms without changing the locomotor activity of the rats in the closed arms.

Research employing the EPM to assess anxiety in rodents has incorporated a variety of behavioural elements derived from ethological analysis, collectively referred to as risk assessment.[10,18,19,24–26] Cruz and colleagues,[22] in a study of factor analysis of rat behaviour in the EPM, identified four distinct factors with loadings greater than 0.4: Factor 1 (anxiety); Factor 2 (activity); Factor 3 (decision making); Factor 4 (displacement). In addition, they found that their measure of risk assessment co-loaded in three factors (1, 3 and 4) that measure different aspects of anxiety (avoidance of danger, decision making, approach-avoid conflict).

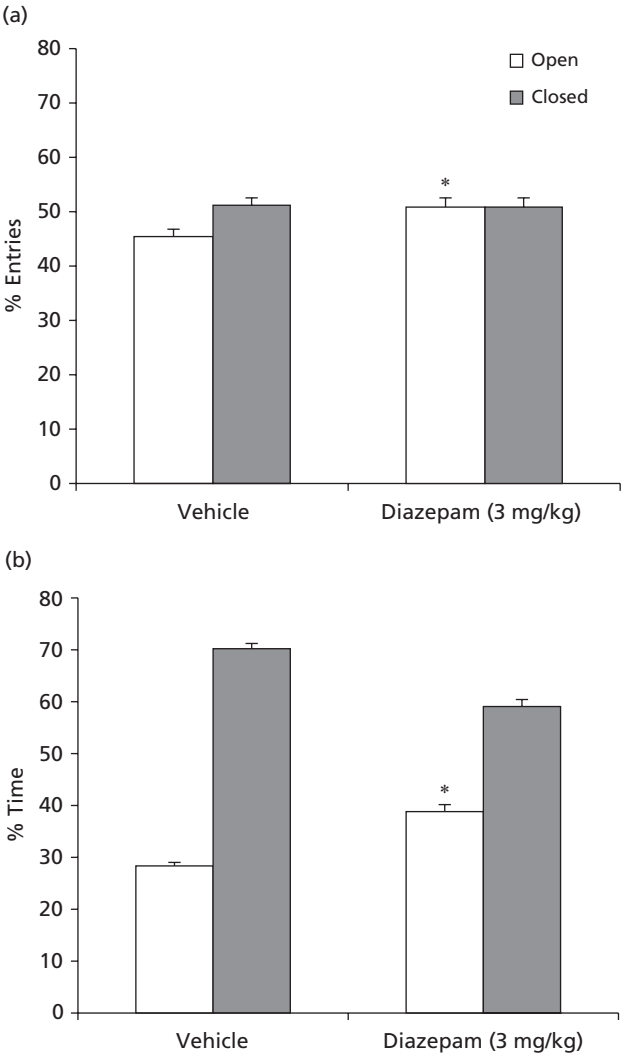

Figure 2 Effect of acute administration of diazepam on the (a) percentage of entries made by rats in the elevated plus-maze test and (b) the percentage time spent in open and closed arms of the plus maze. Diazepam (3 mg/kg, p.o) was administered 45 min before testing. *$P < 0.05$ compared with vehicle (Newman–Keuls test). Data are presented as mean ± SEM, $n = 10$.

Assessment of the ethological measures, in this study, revealed that copaiba oil and diazepam produced an anxiolytic profile of action in some behavioural parameters. Both treatments reduced peeping out (decision making-related behaviour) and increased end-arm activity (exploratory activity-related behaviour), indicating an enhanced tendency to explore actively the potentially dangerous areas of the EPM.[24] These results are consistent with reports showing that

Table 2 Effect of diazepam on ethological measures of behaviour displayed by rats in the elevated plus-maze test

Behavioural itens	Vehicle	Diazepam 3 mg/kg
Rearing	13.7 ± 0.6	9.5 ± 0.7*
Peeping out	3.3 ± 0.3	1.0 ± 0.2*
End-arm activity	0.3 ± 0.1	2.5 ± 0.3*
Head dipping	8.2 ± 0.4	12 ± 0.5*
Flat-back approach	0.8 ± 0.2	0.2 ± 0.1*
Stretched-attend posture	9.8 ± 1.0	7.9 ± 0.5

Diazepam (3 mg/kg, p.o) was administered 45 min before testing. *$P < 0.05$ compared with vehicle (Newman–Keuls test). Data are presented as mean ± SEM, $n = 10$.

anxiolytic effects can be revealed through analysis of risk assessment measures because, in general, they are more sensitive to drug action than are the traditional indices of anxiety in this test.[11,22,24,26] Diazepam also decreased flat-back approach (anxiety-related behaviour) and increased head dipping, an ethological measure expressing exploratory activity.[18] These parameters were not affected by treatment with copaiba oil. However, we can see a clear tendency of the treatment to decrease the first and increase the second behavioural category over the entire dose range.

Rearing was also reduced by the treatment with copaiba oil and diazepam. This showed that rearing may reflect an anxiety factor not linked to risk assessment.[25] It may represent an attempt to avoid threatening situations associated with the height clues in the apparatus. In this context, it is worth mentioning that aversive stimulation of some structures associated with the genesis and elaboration of fear states/generalized anxiety, such as the dorsomedial hypothalamus, produces high scores for rearing.[22,27,28]

In this study, stretched-attend posture was not affected by copaiba oil or diazepam. Other studies on the effects of benzodiazepine receptor ligands in rats also failed to confirm that the SAP is superior to traditional indices of anxiety. In the same direction, another study on the effects of a 5-$HT1_A$ agonist, gepirone, and a selective inhibitor of serotonin reuptake, fluoxetine, also failed to confirm that SAP is superior to traditional indices of anxiety.[10]

The present preclinical studies demonstrated that CRD copaiba oil produced dose-dependent anxiolytic-like effects, over the entire dose ranges tested, on conventional and ethological parameters without adversely affecting general activity.

These results provide further support for the anxiolytic potential of this agent, and point us towards the necessity of extending this study to other animal models of anxiety to facilitate the study of different facets of anxiety (e.g. the traditional learning paradigms – conditioned emotional responding; conditioned active avoidance; Geller conflict test). Considering the idea that emotionality is not unidimensional, but varies along several independent axes that are only accessible through a series of tests involving different stressful stimuli (e.g. novelty, brightness, openness and punishment), increasing the number of tests, the range of stressful stimuli and the behavioural tasks involved would certainly contribute to gaining a broad understanding about the underlying mechanisms of the emotional behaviour of rodents.[29]

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Harada K *et al*. Anxiolytic activity of a novel potent serotonin 5-HT2C receptor antagonist FR260010: a comparison with diazepam and buspirone. *Eur J Pharmacol* 2006; 553: 171–184.
2. Pio-Corrêa M. *Dicionário de Plantas Úteis do Brasil*, Vol. II. Rio de Janeiro: Ministério da Agricultura, 1931.
3. Veiga Junior VF, Pinto AC. O gênero *Copaifera* L. *Quím Nova* 2002; 25: 273–286.
4. Veiga Junior VF *et al*. Phytochemical and anti-oedematogenic studies of commercial copaíba oils available in Brazil. *Phytother Res* 2001; 15: 476–480.
5. Veiga Junior VF *et al*. The inhibition of paw oedema formation caused by the oil of *Copaifera multijuga* Hayne and its fractions. *J Pharm Pharmacol* 2006; 58: 1–6.
6. Veiga Junior VF *et al*. Chemical composition and anti-inflammatory activity of copaiba oils from *Copaifera cearensis* Huber ex Ducke, *Copaifera reticulata* Ducke and *Copaifera multijuga* Hayne: a comparative study. *J Ethnopharmacol* 2007; 112: 248–254.
7. Santos AO *et al*. Antimicrobial activity of Brazilian copaiba oils obtained from different species of the *Copaifera* genus. *Mem Instituto Oswaldo Cruz* 2008; 103: 277–281.
8. Santos AO *et al*. Effect of Brazilian copaíba oils on *Leishmania amazonensis*. *J Ethnopharmacol* 2008; 120: 204–208.
9. Lima SRM *et al*. *In vivo* and *in vitro* studies on the anticancer activity of *Copaifera multijuga* Hayne and its fractions. *Phytother Res* 2003; 17: 1048–1053.
10. Silva RCB, Brandão ML. Acute and chronic effects of gepirone and fluoxetine in rats tested in the elevated plus-maze: an ethological analysis. *Pharmacol Biochem Behav* 2000; 65: 209–216.
11. Treit D *et al*. Anxiogenic stimuli in the elevated plus-maze. *Pharmacol Biochem Behav* 1993; 44: 463–469.
12. Holmes A *et al*. Behavioral profile of wild mice in the elevated plus-maze test for anxiety. *Physiol Behav* 2000; 71: 509–516.
13. Pellow S, File SE. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. *Pharmacol Biochem Behav* 1986; 24: 525–529.
14. Pellow S *et al*. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in rat. *J Neurosci Methods* 1985; 14: 149–167.
15. Ribeiro MD *et al*. Effect of *Erythrina velutina* and *Erythrina mulungu* in rats submitted to animal models of anxiety and depression. *Brazil J Med Biol Res* 2006; 39: 263–270.
16. Handley SL, McBlane JW. 5-HT drugs in animal models of anxiety. *Psychopharmacology* 1993; 112: 13–20.
17. Rodgers RJ, Cole JC. The elevated plus-maze: pharmacology, methodology and ethology. In: Cooper SJ, Hendrie CA, eds. *Ethology and Psychopharmacology*. Chichester: Wiley, 1994: 9–44.

18. Rodgers RJ, Johnson NJT. Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. *Pharmacol Biochem Behav* 1995; 52: 297–303.
19. Blanchard DC *et al.* Risk assessment and animal models of anxiety. In: Olivier B *et al*. eds. *Animal Models in Psychopharmacology*. Bale: Birkhauser, 1991: 117–134.
20. Rodgers RJ *et al*. Ethopharmacological analysis of the effects of putative 'anxiogenic' agents in the mouse elevated plus-maze. *Pharmacol Biochem Behav* 1995; 52: 805–813.
21. Stemmelin J *et al*. Stimulation of the beta3-adrenoceptor as a novel treatment strategy for anxiety and depressive disorders. *Neuropsychopharmacology* 2008; 33: 574–587.
22. Cruz APM *et al*. Ethopharmacological analysis of rat behavior on the elevated plus-maze. *Pharmacol Biochem Behav* 1994; 49: 171–176.
23. File SE. Behavioural detection of anxiolytic action. In: Elliot JM *et al.,* eds. *Experimental Approaches to Anxiety and Depression*. Chichester: John Wiley and Sons, 1992: 25–44.
24. Cole JC, Rodgers RJ. An ethological analysis of the effects of chlordiazepoxide and bretazenil (Ro-6028) in the murine elevated plus-maze. *Behav Pharmacol* 1993; 4: 573–580.
25. Anseloni VZ, Brandão ML. Ethopharmacological analysis of behaviour of rats using variations of the elevated plus-maze. *Behav Pharmacol* 1997; 8: 533–540.
26. Griebel G *et al*. Risk assessment behavior: evaluation of utility in the study of 5-HT-related drugs in the rat elevated plus-maze test. *Pharmacol Biochem Behav* 1997; 57: 817–827.
27. Brandão ML *et al*. Escape behavior produced by the blockade of glutamic acid decarboxylase (GAD) in mesencephalic central gray and medial hypothalamus. *Pharmacol Biochem Behav* 1986; 24: 497–501.
28. Griebel G *et al*. The use of the rat elevated plus-maze to discriminate between non-selective and BZ-1 (w1) selective, benzodiazepine receptor ligands. *Psychopharmacology* 1996; 124: 245–254.
29. Ramos A. Animal models of anxiety: do I need multiple tests? *Trends Pharmacol Sci* 2008; 29: 493–498.