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Evaluation of neurotransmitters involved in the anxiolytic and panicolytic effect of the aqueous fraction of *Paullinia cupana* (guaraná) in elevated T maze

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Abstract: This study investigated the effects of repeatedly administration of an aqueous fraction of *Paullinia cupana* Kunth, Sapindaceae (guaraná) seeds (8 mg/kg) on rats submitted to the elevated T-maze, model of generalized anxiety and panic disorders. The selective serotonin reuptake inhibitor paroxetine (3 mg/kg), was used as a positive control. To evaluate possible neurotransmissions involvement, ineffective doses of metergoline (3 mg/kg - non-selective serotonin receptor antagonist), sulpiride (20 mg/kg - non-selective dopaminergic receptor antagonist) or ketamine (0.125 mg/kg - non-selective glutamate receptor antagonist) were acutely administered in association with the aqueous fraction of *P. cupana*. Both aqueous fraction and paroxetine decrease the inhibitory avoidance latencies of the elevated T-maze, indicating anxiolytic effect and increased one-way escape latencies from the open arm of the elevated T-maze, indicating a panicolytic effect. The pre-treatment with metergoline, sulpiride and ketamine blocked the anxiolytic effect of aqueous fraction. The panicolytic effect of aqueous fraction was blocked by both metergoline and sulpiride. These results show that the serotonergic, dopaminergic and glutamatergic neurotransmission systems are involved in anxiolytic effect promoted by aqueous fraction, whereas only the serotonergic and the dopaminergic neurotransmission systems are involved in the panicolytic effect promoted by aqueous fraction of *P. cupana*. The effects produced by paroxetine, were blocked only by metergoline, validating this experimental procedure.

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Introduction

The anxiety disorder comprises distinct pathological conditions such as generalized anxiety disorder (GAD), panic disorder (PD), social phobias (SP) and posttraumatic stress disorder (PSD) (Kessler et al., 2005). GAD is the most common of all psychiatric disorders leading to great suffering, being defined as a generalized vague and unpleasant feeling (Andrade & Gorestein, 1998). PD is defined by the presence of recurrent panic attacks that consist of a feeling of fear or intense discomfort, accompanied by physical symptoms that abruptly start (Craske & Barlow, 1993). Selective serotonin reuptake inhibitors (SSRI) are considered treatment of first choice due to its high efficacy and tolerability (Yacubian & Minutentag, 2001), however, they have limitations as a delayed onset of effects, an initial worsening of anxiety symptoms (Blier & Montigny, 1999) and resistance occurs in approximately 30% of patients (Thase & Rush, 1995). Those limitations of therapeutically show that the search for new therapeutic strategies is necessary.

It is estimated that Brazil has the greatest plant biodiversity in the world (Guerra & Nodari, 2001), and *Paullinia cupana* Kunth (guaraná) it belongs to the *Sapindaceae* family and is popularly known as guaraná (Henman, 1982), its pharmacological actions have been a target of interest for pharmaceutical laboratories. Its seeds containing high concentrations of xanthenes and traces of theophylline and theobromine, high concentrations of polyphenols and saponins which contain catechins and other condensed tannins (Benowitz, 1990). Popularly, several effects, as antidiarrheal (Basile et al., 2005), antioxidant (Mattei et al., 1998) and stimulant of central nervous system (CNS) (Carlini, 2003), are attributed to the extract of the dried guaraná seeds by the high content of methylxanthines, especially caffeine (Antonelli-Ushiroba et al., 2004). Confirming this CNS stimulant effect popularly attributed, a semi-purified fraction

obtained from guaraná seed fraction, called PEA showed improvement in performance and memory speed (Otobone et al., 2005), produced antidepressant-like effects in rats (Otobone et al., 2007) and panicolytic effect similar to the one produced by the antidepressant paroxetine, in animals subjected to elevated T-maze (ETM), this effect was evident participation of the neurotransmitters serotonin and dopamine (Roncon et al., 2010).

Although the neurotransmitters as noradrenaline (NA), serotonin (5-HT) and dopamine (DA) have been the focus of research in developing treatments related to anxiety disorders for a long time (Cansado et al., 2012), new therapeutic approaches require the search for new treatments on different transmissions. Thus, the glutamate system is emerging as a component of a new generation of antidepressants (Berman et al., 2000). Ketamine (KET), a glutamatergic *N*-methyl-D-aspartate antagonist receptor, showed an antidepressant effect in rodents in the forced swimming test (Mathew et al., 2005). Also, sub-therapeutic dose of KET provides a rapid antidepressant response in patients resistant to treatment (Murrough, 2012). This study evaluated if the FAQ has anxiolytic and panicolytic effects in rats submitted to ETM test, and if the serotoninergic, dopaminergic and glutamatergic neurotransmissions are involved in these effects.

Materials and Methods

Plant material

The *Paullinia cupana* Kunth, Sapindaceae (guaraná) seeds were collected in city of Alta Floresta, State of Mato Grosso, Brazil. They were then dried, identified and pulverized in a hammer mill (Tigre ASN-5). Identification was carried out by Dr. Cássia Sakuragui. A voucher plant specimen (#HUEM9065) was deposited in the herbarium of the State University of Maringá.

Extraction

The extract was prepared from the guaraná seeds (1000 g) by turbolysis using an acetone:water (7:3; v/v) extractor solution in proportion of 10% (w/v). After removal of the organic solvent, the remaining solid material was lyophilized (EBPC-crude lyophilized extract, patent pending PI0006638-9). Then, 158 g of the EBPC was partitioned with ethyl acetate (10×0.5 L), resulting in an aqueous fraction (FAQ: 114 g) and ethyl acetate fraction (PEA: 44 g).

Drugs

FAQ, Paroxetine (PAR, SSRI; IPCA Laborat; 99.1%), Metergoline (MET, Sigma; 5-HT receptor antagonist; ≥98%), Sulpiride (SUL, Sigma; non-selective dopaminergic receptor antagonist; ≥98%) were solubilized in saline solution (0.9% NaCl) containing 2% Tween 80, and Ketamine (KET, non-selective glutamate receptor antagonist, Virbac®) injectable solution, was used from the ampule. The control group was treated with only the vehicle (VEH; 0.9% NaCl plus 2% Tween 80).

Animals

Male Wistar rats (230-250 g) at constant room temperature (22-23 °C) under a 12 h light-dark cycle with free access to food and water were used in the experiments. The experiments were performed between 13 and 18 h. The procedures adopted were approved by the State University of Maringá Ethics Committee (107/2011), and followed the recommended guidelines for Biomedical Research Involving Animals (CIMS), Geneva, 1985.

Apparatus

The ETM was constructed of wood and had three arms of equal dimensions (50 × 12 cm). One arm, enclosed by 40 cm high walls, was perpendicular to two opposed open arms. The entire apparatus was positioned 50 cm above the floor. Locomotion was measured in a circular wooden arena, 70 cm in diameter, with 30 cm high walls. Luminosity at the level of the maze arms and at the center of the circular arena was 60 lux.

Behavioral tests

One day before the test, each animal was pre-exposed to one of the open arms of the ETM for 30 min. A wooden barrier mounted between the central area of the maze and the proximal end of the arm isolated it from the rest of the ETM. It has been shown that such pre-exposure to the open arm makes the escape task a more sensitive measure of the effects of panicolytic drugs.

After 24 h, the ETM test was initiated by the inhibitory avoidance task. For this, each animal was placed at the distal end of the enclosed arm of the ETM facing the intersection. The time taken by the rat to leave this arm with all four paws was recorded (baseline latency). This measurement was repeated in two subsequent trials (avoidance 1 and 2) at 30 s intervals. Thirty seconds after the avoidance trials, the rats were placed at the end of the open arm they had been previously exposed to, and the latency to leave this arm with all four paws was recorded in three consecutive trials (one-way escape 1, 2, and 3) at 30 s intervals. A cut-off time of 300 s was established for the avoidance and escape latencies. In acute treatment, manipulation of the animal is made 5 min in the lap of a researcher and 5 min of habituation in the box after three days of their arrival at the laboratory. On day 4, animals

were handled the same way again and confined to one of the open arms of ETM for 30 min. Thirty seconds after being tested in the ETM, each animal was placed in the circular arena for 5 min to evaluate their locomotion. The total distance traveled was analyzed by a video tracking system (Ethovision; Noldus, Holland).

Treatment

Treatment 1 - Determination the FAQ effect

The animals were treated by gavage (*i.g.*) for 21 days with PAR (3 mg/kg), FAQ (8 mg/kg) or VEH and submitted to the behavioral tests 30 min after this last treatment.

Treatment 2 - Dose response curve of ketamine

The animals were treated with a single dose of KET (0.0625, 0.125 and 0.25 mg/kg) or VEH by intraperitoneal route (*i.p.*) and submitted to the behavioral tests 30 min after this treatment.

Treatment 3 - Associates treatment

The animals were treated for 21 days with PAR (3 mg/kg), FAQ (8 mg/kg) or VEH (*i.g.*) and only in the 21th day of treatment, the animals were acutely pretreated with the VEH or antagonists (*i.p.*), and then treated with PAR (3 mg/kg), FAQ (8 mg/kg) or VEH (*i.g.*) 5 min later. They were submitted to the behavioral tests 30 min after the last treatment.

Statistical analysis

Repeated-measure analyses of variance (RMANOVA) were used to analyze both avoidance and escape data. The treatments were considered as the independent factors and the trials (baseline, avoidance 1 and 2 and escape 1-3) as the repeated measures. When appropriate, one-way ANOVA followed by the *post hoc* Duncan's multiple comparison tests, were used. Locomotion data were analyzed by one-way ANOVAs followed by the *post hoc* Duncan's multiple comparison tests. Differences between groups were considered significant if $p<0.05$.

Results and Discussion

Antidepressant drugs of different classes have been successfully used for the treatment of anxiety disorder subtypes, including GAD (Kim et al., 2006) and PD (Pollack & Doyle, 2003). However, SSRI show delay in the onset of therapeutic effect and initial worse of anxiety symptoms, specially observed in PD patients (Zanoveli et al., 2007). Therefore, the development of new therapeutic strategies with a faster onset of action and fewer adverse effects has been the focus of research developed by industry and academic. Guaraná is popularly used as anorectic, nootropic and aphrodisiac (Henman, 1982; O'Dea, 2003; Oliveira et al., 2005). Animal studies using a semi-purified fraction obtained from an extract of guaraná seeds (PEA), already demonstrated improved performance and memory speed, antidepressant-like effects (Otobone et al., 2005; 2007) and antipanic-like effects in rats (Roncon et al., 2010).

This study evaluated the effects of FAQ on rats in the ETM test, an animal model developed to assess defensive behaviors related to specific subtypes of anxiety disorders, as GAD and PD, and PAR was chosen as positive control in the study, because as the first SSRI licensed by the Food and Drug Administration and has proven effective in treating PD.

Figure 1 show the effects of repeated treatment with control group (VEH), PAR (3 mg/kg), or FAQ (8 mg/kg) in the ETM, and (Figure 1A) showed significant effect on the avoidance to FAQ at baseline (*p=0.02), avoid 1

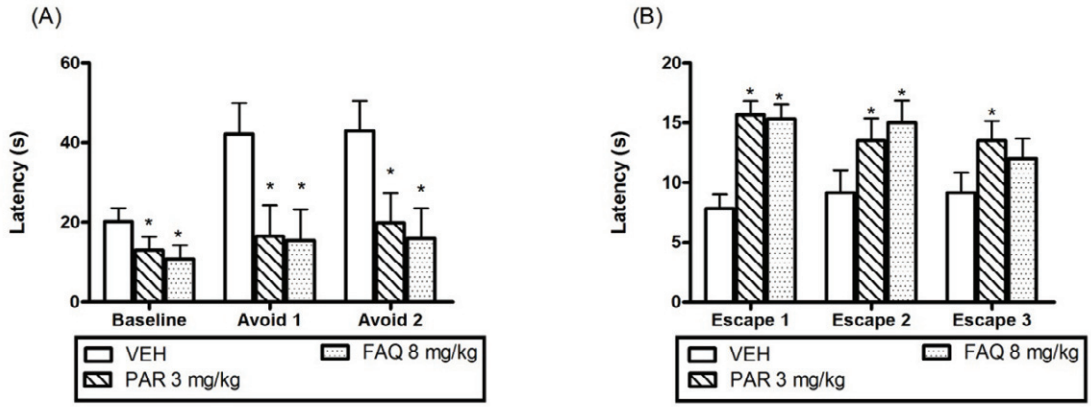

Figure 1. Means±SEM of administration (21 days, *i.g.*) of control group (VEH), PAR (3 mg/kg) or FAQ (8 mg/kg) on inhibitory avoidance (panel A) and one-way escape latencies (panel B) in the ETM test (n=9-10). *p<0.05, compared to the VEH.

(*p=0.007) and 2 (*p=0.001) latencies, and PAR showed significant effect on the avoidance at baseline (*p=0.02), avoid 1 (*p=0.01) and 2 (*p=0.002) latencies, compared to control group (VEH), indicating the anxiolytic-like effect for FAQ and PAR. For the escape trial (Figure 1B), showed that FAQ increased escape 1 (*p=0.001) and 2 (*p=0.003) latencies, and that PAR significantly increased escape 1 (*p=0.001), 2 and 3 (*p=0.01) latencies compared to the control group, indicating the panicolytic-like effect. One-way ANOVA did not show significant differences in distances traveled under the different treatments compared to control group (Table 1).

Table 1. Distance traveled in meters (m) in the circular arena by rats repeatedly treated with FAQ or PAR.

Drug (mg/kg)	Distance traveled (m)
VEH	19.12±1.54
PAR (3)	17.44±1.46
FAQ (8)	20.28±1.54

Data presented as means±SEM. n=9-10. p>0.05 compared to control group (VEH) after repeated administration (*i.g.*) with FAQ and PAR.

In the combined treatment to evaluation of the neurotransmitters involved in the effect of FAQ, Figure 2 shows the results observed in the ETM test for the pretreatment of the VEH or MET (3 mg/kg), to evaluate the involvement of serotonergic neurotransmission in the effect of FAQ. For the inhibitory avoidance trial (Figure 2A), RMANOVA showed that FAQ significantly reduce the latencies of avoid 1 (*p<0.05), and 2 (*p=0.002), and PAR significantly reduce avoid 1 (*p<0.05), and 2 (*p=0.006) latencies compared to control group. For the escape trial (Figure 2B), showed that FAQ increased escape 1 and 2 (*p=0.001) latencies. PAR significantly increased escape 1 (*p=0.001), 2 and 3 (*p<0.05) latencies compared to the control group. These results confirm the anxiolytic and the panicolytic effect of FAQ observed in treatment 1.

MET blocked the anxiolytic effect produced by FAQ, as can be seen by the significant differences in avoid 2 ($^{\$}p$=0.001) latencies between the MET+FAQ and the VEH+FAQ groups. Furthermore, MET blocked the anxiolytic effect produced by PAR, as can be seen by the significant differences in avoid 2 ($^{\#}p$=0.001) latencies between the MET+PAR and the VEH+PAR groups (Figure

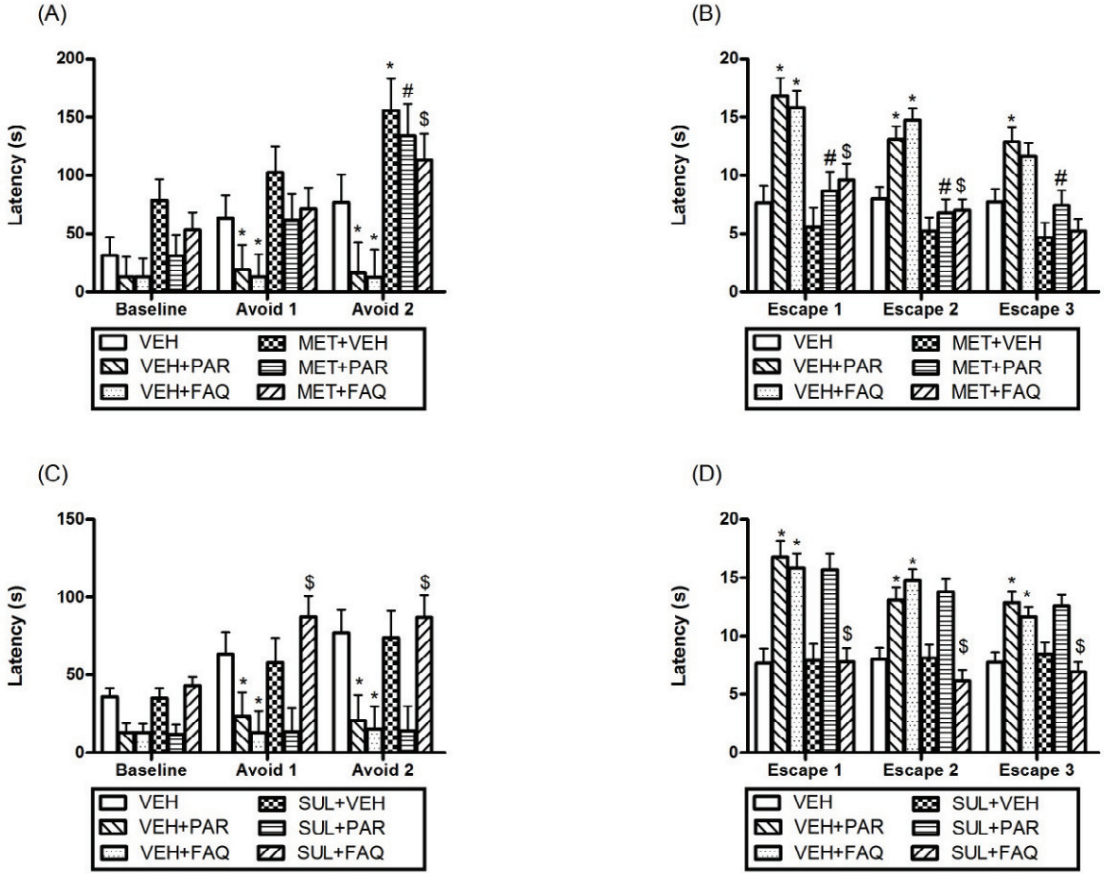

Figure 2. Means±SEM of acute administration (*i.p.*) of MET or SUL in rats treated (21 days, *i.g.*) with VEH, PAR (3 mg/kg) or FAQ (8 mg/kg) on inhibitory avoidance (A and C panel) and one-way escape (B and D panel) latencies in the ETM test (n=9-13). *p<0.05, compared to the control group (VEH+VEH), $^{\#}p$<0.05, compared to the VEH+PAR, $^{\$}p$<0.05, compared to the VEH+FAQ groups.

2A). MET blocked the panicolytic effect produced by FAQ, as can be seen by the significant differences in escape 1 and 2 ($^{\$}p$=0.001) latencies between the MET+FAQ and the VEH+FAQ groups. Furthermore, MET blocked the panicolytic effect produced by PAR, as can be seen by the significant differences in escape 1($^{\#}p$=0.001), 2 ($^{\#}p$=0.002) and 3 ($^{\#}p$=0.008) latencies between the MET+PAR and the VEH+PAR groups (Figure 2B). For the positive control, PAR, the blockage of anxiolytic and panicolitc by MET was expected, and validate the experimental model, according to the literature, this effect was due to the blockade of postsynaptic receptors by sensitization chronic exerted by compound (Humphrey et al., 1986; Ellis et al., 1991). The blockage of anxiolytic and panicolitic effect of FAQ by MET suggests that possible involvement of serotonergic neurotransmission in the anxiolytic and panicolytic effect of the FAQ.

Although SSRI are considered the first-choice treatment for PD, other neurotransmitters, are also important in the etiology and treatment of GAD and depression (Goodnick et al., 1998). DA is a neurotransmitter between these, and drugs capable of blocking the reuptake of DA demonstrate efficacy in the treatment of PD (Gebhart et al., 2008) and improve symptoms of GAD (Reis et al., 2004).

Figure 2 shows the results observed in the ETM for the pretreatment of the VEH or SUL (20 mg/kg), to evaluate the involvement of dopaminergic neurotransmission in the effect of FAQ. For the inhibitory avoidance trial (Figure 2C), FAQ significantly reduce avoid 1 (*p=0.003) and 2 (*p=0.001) latencies, and PAR significantly reduce avoid 1 (*p=0.02) and 2 (*p=0.002) latencies, compared to control group, indicating an anxiolytic effect. SUL blocked the anxiolytic effect produced by FAQ can be seen by the significant differences in avoid 1 and 2 ($^{\$}p$=0.001) latencies between the SUL+FAQ and the VEH+FAQ groups. For the escape trial (Figure 2D) showed that FAQ increased escape 1, 2 (*p=0.001) and 3 (*p=0.009), and PAR significantly increased escape 1, 2 and 3 (*p=0.001) latencies compared to control group, indicating a panicolytic effect. SUL also blocked the panicolytic effect produced by FAQ, as can be seen by the significant differences in escape 1, 2 and 3 ($^{\$}p$=0.001) latencies between the SUL+FAQ and the VEH+FAQ groups, but did not blocked the effect of PAR both the avoidance and escape (p>0.05) latencies, suggesting the possible involvement of dopaminergic neurotransmission in the anxiolytic and panicolytic effect of the FAQ.

In the analysis of glutamatergic neurotransmission, it was necessary to make the dose response curve of KET, showed in Figure 3. For the inhibitory avoidance trial (Figure 3A), the anxiogenic effect appeared in the ETM test in animals treated with a single dose of KET (0.25 mg/kg), on the avoidance at baseline (*p=0.001), avoid 1 (*p<0.05) and 2 (*p=0.004) latencies compared to control group. Anxiogenic effect also has been reported in the study with animals pretreated with a single dose of KET and submitted to the elevated plus maze (Silvestre et al., 1997). For the escape trial (Figure 3B), KET there was no significant effect (p>0.05). A one way ANOVA did not show significant differences in locomotor activity (Table 2), being so established a sub effective dose of KET (0.125 mg/kg) for evaluation the involvement of glutamate in the FAQ effect.

Figure 4 shows the results observed in the ETM test for the pretreatment of the VEH or KET (0.125 mg/kg), to evaluate the involvement of glutamatergic neurotransmission in the effect of FAQ. For the inhibitory avoidance trial (Figure 4A), showed that FAQ significantly reduce avoid 1 (*p<0.05), 2 (*p=0.001) latencies, and PAR significantly reduce avoid 1 (*p<0.05), 2 (*p=0.001) latencies, compared to control group, indicating an anxiolytic effect. KET blocked the anxiolytic effect produced by FAQ, as can be seen by the significant differences in avoid 1

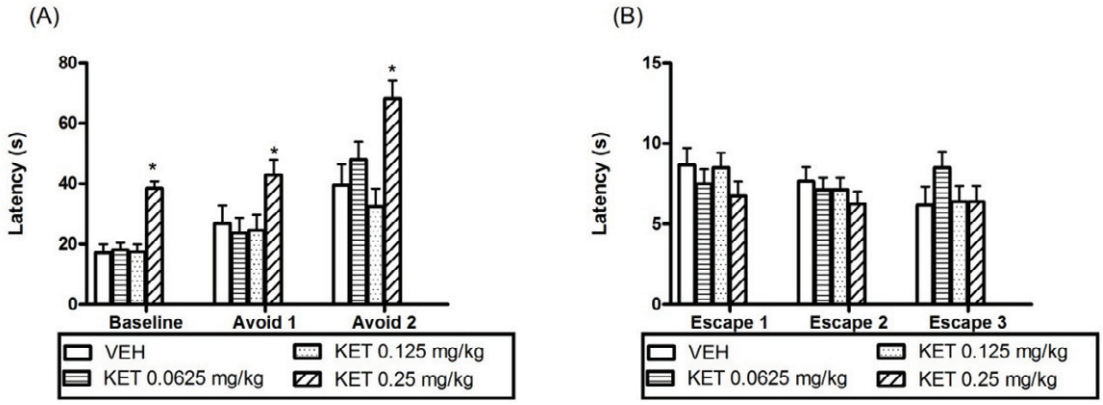

Figure 3. Means±SEM of acute administration (*i.p.*) of VEH or KET (0.0625, 0.125 or 0.25 mg/kg) on inhibitory avoidance (panel A) and one-way escape latencies (panel B) in the ETM test (n=6-8). *p<0.05, compared to the VEH.

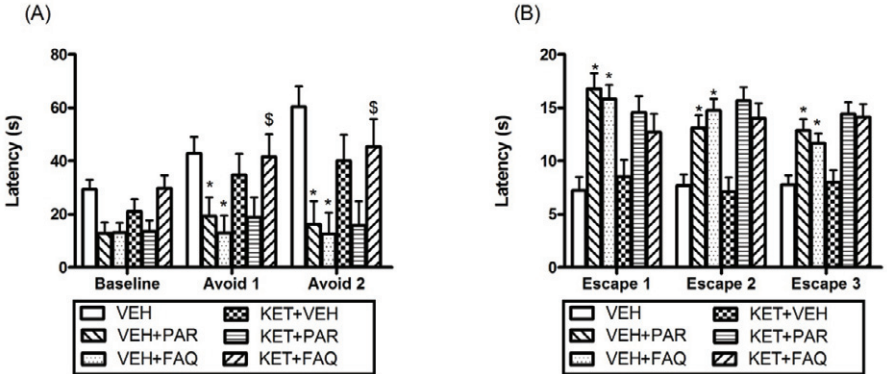

Figure 4. Means±SEM of acute administration (*i.p.*) of KET in rats treated (21 days, *i.g.*) with VEH, PAR (3 mg/kg) or FAQ (8 mg/kg) on inhibitory avoidance (panel A) and one-way escape (panel B) latencies in the ETM test (n=9-13). **p*<0.05, compared to the control group (VEH+VEH), $^{\$}p$<0.05, compared to the VEH+FAQ groups.

($^{\$}p$<0.05) and avoid 2 ($^{\$}p$=0.007) latencies between the KET+FAQ and the VEH+FAQ groups, but not blocked the effect of PAR on avoid (p>0.05) latencies. For the escape trial (Figure 4B), showed that both FAQ as PAR increased escape 1, 2 (**p*=0.001) and 3 (**p*<0.05) latencies compared to the control group indicating a panicolytic effect. KET not blocked the panicolytic effect of PAR and FAQ on the escape (p>0.05) latencies, showing that this effect promoted by FAQ in ETM test does not mediated by glutamatergic receptors. In agreement with our results, the literature does not report effective use of glutamatergic drugs in PD (Harvey & Shahid, 2011).

Table 2. Distance traveled in meters (m) in the circular arena by rats treated acutely with KET.

Drug (mg/kg)	Distance traveled (m)
VEH	12.42±1.58
KET (0.0625)	11.17±1.37
KET (0.125)	14.83±1.37
KET (0.25)	13.61±1.37

Data presented as means±SEM. n=6-8. p>0.05 compared to control group (VEH) after acute treatment (*i.p.*) with KET.

The glutamatergic neurotransmission is related to GAD, because glutamate is the major excitatory neurotransmitter in the CNS, and its blockade can cause symptoms of schizophrenia (Parsons et al., 2007), this involvement of the glutamate neurotransmitter was evident in the results, where KET blocked the anxiolytic effect shown by the FAQ. One way ANOVA did not show significant differences in distance traveled under these different treatments compared to the control group (Table 3).

Our results with PAR and FAQ confirm the need for a latency period until the onset of the therapeutic, antidepressant compounds as well as, data from the literature indicate that period of about 21 days due to the need for changes in the adaptive receivers serotonin receptors, located in the raphe nucleus as well as the raising of different post-synaptic receptors (Pineyro & Blier, 1999).

Table 3. Distance in meters (m) traveled by rats in the circular arena following combined drug administration.

Drug (mg/kg)	Distance traveled (m)
VEH + VEH	18.86±1.01
VEH + PAR (3)	19.96±1.10
VEH + FAQ (8)	17.25±1.01
MET (3) + VEH	16.96±1.16
MET (3) + PAR (3)	18.58±1.16
MET (3) + FAQ (8)	16.54±0.97
SUL (20) + VEH	18.36±1.14
SUL (20) + PAR (3)	17.06±1.08
SUL (20) + FAQ (8)	17.48±0.95
KET (0.125) + VEH	17.51±0.96
KET (0.125) + PAR (3)	16.02±0.96
KET (0.125) + FAQ (8)	16.08±0.96

Data presented as means±SEM. n=7-13. p>0.05 compared to control group (VEH). Acutely administered VEH, MET, SUL or KET (*i.p.*) followed by repeated administration VEH, FAQ or PAR (*i.g.*).

In conclusion, the present study demonstrated that FAQ is active orally, that it produces anxiolytic and panicolytic effect on rats in the ETM test, and that the serotonergic, dopaminergic and glutamatergic neurotransmissions are involved on the anxyolitic effect and that the serotonergic and dopaminergic neurotransmission are involved in the panicolytic effect. These results suggest that FAQ could be a useful drug in the treatment of mood disorders such as GAD and PD.

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Authors' contributions

JCPM contributed on extraction and fractionation of the plant and provided material for pharmacological tests. EAA supervised the laboratory work and critical reading of the manuscript. MPR (graduate student) contributed to running to pharmacological testing, statistical analysis and manuscript production. All the authors have read the final manuscript and approved the submission.

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