Molecular Docking and Panicolytic Effect of 8-Prenylnaringenin in the Elevated T-Maze

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The purpose of this study was to investigate the effects of the chronic administration of a racemic mixture of 8-prenylnaringenin (8-PN) on rats submitted to the elevated T-maze (ETM) model of generalized anxiety and panic disorders. The selective serotonin (SERT) reuptake inhibitor fluoxetine was used as a positive control. Rat locomotion was assessed in a circular arena following each drug treatment. The administration of racemic 8-PN for 21 d in rats increased one-way escape latencies from the ETM open arm, indicating a panicolytic effect. To evaluate the interactions of 8-PN with monoamine transporters, a docking study was performed for both the R and S configurations of 8-PN towards SERT, norepinephrine (NET) and dopamine transporters (DAT). The application of the docking protocol showed that (R)-8-PN provides greater affinity to all transporters than does the S enantiomer. This result suggests that enantiomer (R)-8-PN is the active form in the in vivo test of the racemic mixture.

Key words animal model; anxiety; panic; molecular docking; monoamine transporter’s inhibitor

Anxiety disorders, such as generalized anxiety disorder, panic disorder, obsessive compulsive disorder and phobias, affect one eighth of the world population and have become an important area of interest in psychopharmacology research.1,2 Genetic and neurobiological similarities between anxiety and depressive disorders have been investigated.

Antidepressant compounds are considered the first choice treatment of anxiety and depression disorders. Blockage of serotonin (SERT), norepinephrine (NET) and dopamine transporters (DAT), which cover the majority of antidepressant drug targets, increases the level of these neurotransmitters in the synaptic cleft. Thus, the biogenic amine concentration in the central nervous system (CNS) is maintained by these transporters.3

Selective serotonin reuptake inhibitors (SSRIs) and 5-HT/NE reuptake inhibitor (SNRI)4 are well tolerated and have a better side effect profile than the traditional tricyclic antidepressant (TCA) class,5 but all of them have limitations, such as delayed therapeutic improvement. For this reason, continual efforts have been made to develop new efficacious drugs with earlier therapeutic effect onset and better side effect profiles.6

8-Prenylnaringenin (Fig. 1), together with xanthohumol and isoxanthohumol, is a member of a large group of prenylated chalcones and flavanones isolated from common hop (Humulus lupulus). Their major source in human diet is beer, as hop female flowers are used as a flavouring agent and as a beverage preservative. 8-Prenylnaringenin (8-PN) has been identified as the most potent plant phytoestrogen (more active than coumestrol, genistein and daidzein)6,7 being able to bind to both α- and β-oestrogen receptors.8 Additionally, 8-PN is also recognized for its action as an inflammation inhibitor,9 in angiogenesis10 and against cancer cell proliferation.11,12

Some studies have examined the effects of phytoestrogens on brain and behavior.13–17 Lephart et al. investigated the influence dietary phytoestrogens on anxiety-related behaviors. Animal fed with a phytoestrogen-rich diet (genistein–daidzein) showed a significant reduction of anxiety-like indicators in the elevated plus-maze when compared to control animal.

Besides that the flavanoid 8-prenylnaringenin present a structuratural similarity with 1,3,7-trihydroxy-2-(3-methylbut-2-enyl)-xanthone (Fig. 1), a xanthone extract of Kielmeyera coriacea steams, popularly known as “Pau Santo.” This xanthone proved to be a prototype drug useful in mood disorders such as anxiety, panic and depression, or may indeed to be a beneficial adjunctive treatment, improving the efficacy of antidepressant drugs and/or accelerating the effects of these drugs in patients with mood disorders.18

In light of the above, the aim of the present work was to synthesize and investigate the anxiolytic and panicolytic effects of 8-PN on rats subjected to the elevated T-maze.

![Fig. 1. Structure of 8-Prenylnaringenin (1) and 1,3,7-Trihydroxy-2-(3-methylbut-2-enyl)-xanthone (2)](image)

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(ETM) test. Moreover, to evaluate the interactions of 8-PN with monoamine transporters, we performed a docking study for both R and S configurations of 8-PN towards SERT, NET and DAT.

Results and Discussion

Chemistry Flavanoid 8-PN (I) was synthesized in racemic form using a convenient four-step sequence from commercially available (±)-narigenin (3) following the procedure described by Humpel et al.\textsuperscript{19} (Chart 1). This method is based in method described by Gester et al.\textsuperscript{20} but includes several improves, as no chromatographic steps are needed and the amount of catalyst is drastically reduced. First, the chemoselective acetylation of C(7) and C(4′/uni2032) phenolic hydroxyl groups of 3 was carried out following installation of the requisite prenyl ether moiety at C(5). Mitsunobu reaction of 4 with prenyl alcohol smoothly delivered the desired substrate 5 for the key sigmatropic event. Next, europium(III)-catalysed Claisen–Cope rearrangement of the prenyl group from C(5) to C(8) position occurred. The obtained product 6 is directly used for step 4. Finally, the diacetate was subjected to potassium carbonate-catalysed hydrolysis for deprotection of the C(7) and C(4′) phenolic hydroxyl groups.

Pharmacological Evaluation The results illustrated in Fig. 2A show that the ETM inhibitory avoidance latency was not affected by any of the drug treatments. RM-ANOVA showed a significant effect of trial \([F_{(2,40)}=47.82, p=0.001]\), but no significant effect of treatment \([F_{(2,20)}=1.048, p=0.36]\) or a significant treatment X trial interaction \([F_{(2,40)}=0.80, p=0.52]\). Figure 2B shows that 8-PN (10 mg/kg) and fluoxetine (15 mg/kg) significantly increased the ETM one-way escape latency. RM-ANOVA showed a significant main effect of treatment \([F_{(2,20)}=4.91, p=0.018]\) but no significant effect of trial \([F_{(2,40)}=0.19, p=0.82]\) and no treatment X trial interaction \([F_{(4,40)}=1.58, p=0.198]\). Post-hoc comparisons showed that 8-PN (10 mg/kg) significantly increased escape 2 latency (\(p=0.03\)) and fluoxetine increased escape 3 latency (\(p=0.02\)) when compared to the control group, which was interpreted as a panicolytic-like effect.

In Fig. 3, the ANOVA shows that none of the drug treatments used significantly affected the distance travelled by the rats in the circular arena \([F_{(2,20)}=1.43, p=0.26]\).

The ETM test is the only test that involves two tasks that are performed by the same rat: inhibitory avoidance and one-way escape. For the first one, the rat is placed at the distal end of the enclosed arm and the time to withdraw from this arm with the four paws is measured in three successive trials. For the escape task, the rat is placed at the end of one of the open

![Chart 1. Synthetic Route of 8-PN](image)

Fig. 2. Effects (Mean±S.E.M.) of Repeated Administration with Vehicle (Control Group), Fluoxetine or (±) 8-PN on Inhibitory Avoidance in Rats (Panel A) and One-Way Escape Latencies (Panel B) in ETM Test (\(n=7–9\))

* \(p<0.05\) compared to the control group.
arms and the withdrawal latency is measured three times. Pharmacological validation has shown that the drug profile of inhibitory avoidance is similar to that of generalized or anticipatory anxiety, while the drug response of escape is akin to that of panic disorder. As a consequence, the ETM is considered a sound animal model of both anxiety and panic.22,23 Regarding panic, performance of the one-way escape in the ETM has been shown to be impaired by several antidepressant drugs following chronic, but not acute, administration, paralleling the therapeutic response.21 The administration of racemic 8-PN for 21 d to rats submitted to the ETM consistently impaired the escape indication of panicolytic effect. The acquisition of inhibitory avoidance was not affected by the drugs, showing no anxiolytic effect. These effects are probably not due to unspecific motor effects, since the same drug treatments failed to significantly affect locomotion, measured in the circular arena. SSRI fluoxetine, used as positive control in our study, confirmed the panicolytic effect in the ETM observed in another study. Fluoxetine has been proven to be effective in treating panic disorder and shown to increase extracellular 5-HT in dorsal periaqueductal grey (dPAG).24

Acute treatment with the same doses of 8-PN (10 mg/kg) produced no effects in rats submitted to ETM (results not shown). Our results confirm existence of a latency period until the onset of the therapeutic action. This effect is also observed for other compounds with antidepressant profile, a period of about 21 d is necessary for adaptive changes in serotonin receptors located in the raphe nucleus, as well as raising different post-synaptic receptors.25

**Molecular Docking** In order to rationalize the tendency of activity of the compounds under study, molecular docking of (R)-fluoxetine, (S)-bupropion, (S,S)-reboxetine (Fig. 4) and of both 8-PN enantiomers (Fig. 1) was performed with homology models of SERT, NET and DAT constructed by Ravna et al.26 It is important to point out that pharmacological tests were conducted with the racemic mixture of the enantiomers. The molecular docking assays were performed for each enantiomer to verify the influence of the 8-PN configuration in the binding mode with the transporters.

The compounds were docked in the central binding pocket of SERT, NET and DAT, which corresponds to the substrate binding pocket of leucine in the LeuT crystal structure. The principal ligand-receptor interactions were analysed and the best orientation for each model and both 8-PN enantiomers were compared (Fig. 5).

Figures 5A and B show the docking orientation of (R)-8-PN, (S)-8-PN and (R)-fluoxetine (model) in SERT. The trifluorophenyl moiety of fluoxetine is stabilized through van der Waals contact with van der Waals contact with Thr175, Tyr176 and Phe335. The aromatic ring that contains this group has stacking interactions with Tyr175 and Ile179. The hydrogen of the protonated amino group forms hydrogen bonds with Glu493.

For enantiomer (R)-8-PN (Fig. 5A), one can see that the prenyl group is stabilized by van der Waals contact with Tyr176, Tyr175 and Phe335, the same residues that stabilize the trifluoromethyl group in the standard. Additionally, three hydrogen bonds can be observed, one between the carbonyl and Lys399, another between hydroxyl 7 of ring A and Tyr175, and the other between hydroxyl 4 of ring B and Asp400. For enantiomer (S)-8-PN (Fig. 5B), the prenyl group is in a region defined by Ile179, Lys399 and Asp400, in contrast to what occurs for enantiomer (R)-8-PN and the trifluoromethyl group in fluoxetine. Additionally, hydrogen bonds were observed between hydroxyl 7 and 4 of rings A and B with Lys399 and Tyr176, respectively.

Figures 5C and D show the active site of transporter noradrenaline (NET) complexed with standard (S,S)-reboxetine and enantiomers (R)-8-PN and (S)-8-PN, respectively. The aromatic rings of the standard are stabilized by van der Waals contact with Trp80, Ala77, Arg81 and Asp473. The amine group makes a hydrogen bond with Thr381. The prenyl group of enantiomer (R)-8-PN (Fig. 5C) is stabilized by van der Waals contact with Ala77, Arg81 and Asp473, which are the same residues that stabilize the aromatic rings of the standard, while the prenyl group of (S)-8-PN (Fig. 5D) is stabilized by van der Waals contact with Thr470, Thr381 and Glu382.

Figure 5E shows the active site of the dopamine transporter (DAT) complexed with standard (S)-bupropion and enantiomer (R)-8-PN. The aromatic ring of the standard is stabilized by van der Waals contact with Ala81, Ile159 and Phe155, and the tert-butyl group with Lys384 and Tyr88. In (R)-8-PN, the prenyl group is stabilized by van der Waals contact with Phe472, Thr473 and Lys384; furthermore, there is a hydrogen bond between Arg85 and hydroxyl 4 of ring B. The molecular docking of enantiomer (S)-8-PN showed that it does not overlap in the active site of this transporter effectively.

Table 1 compares the calculated (Autodock) and experimental (Binding Database) values of inhibition constants (Ki) of each of the ligands with their respective targets. The Ki values reported in the Binding Database (literature)
show that \((R)\)-fluoxetine has greater affinity to SERT, followed by DAT, which is confirmed by the lower theoretical \(K_i\) values given by Autodock. However, this ligand can also bind to NET with smaller affinity (greater \(K_i\)).

The prediction of the drug targets based on the ligand structures by SEA predictions indicated that the SERT protein is the most probable target of \((R)\)-fluoxetine. The \(e\)-values of this analysis indicate the probability of a false positive result, therefore, the lower this value, the greater the reliability of the results. Summing up, these results show that the theoretical docking data agree with the experimental results.

There are no data in the literature that indicate that \((S,S)\)-
reboxetine can bind to SERT or DAT; however, NET is known to be its main target. Again, the theoretical $K_i$ values from Autodock confirmed this observation. The small theoretical $K_i$ calculated for the (S)-bupropion showed that this ligand has greater affinity to DAT, again in agreement with the literature.

This validation shows that the reproducibility of the in silico experimental data based on classical ligands of receptors SERT, NET and DAT provides reliable theoretical results and thus allows the use of the docking protocol presented here in the evaluation of the interaction of other ligands with these protein targets.

The data in Table 1 show that (R)-8-PN presents greater affinity (lower inhibition constant) for all transporters (SERT, NET and DAT) than enantiomer (S)-8-PN, indicating that (R)-8-PN interacts more effectively with the residues of the active sites of the transporters.

These results suggest that the panicolytic effect obtained with the racemic form of 8-PN in the ETM test can be attributed mainly to the R enantiomer employed. It is known that the activity of a drug is often related to only one of its enantiomeric forms, such as is the case of the antidepressant escitalopram. Clinical and preclinical studies have shown that escitalopram interacts more actively with SERT and that it is more efficacious than racemic citalopram. \(^{27,28}\) (R)-Citalopram counteracts the actions of escitalopram, and it has been hypothesized that this antagonistic effect is mediated via interaction with the allosteric binding site on SERT.\(^{29}\)

Reboxetine, a potent and selective NET inhibitor ligand, is marketed as a racemic mixture of (R,R)- and (S,S)-reboxetine. It is a non tricyclic antidepressant drug, the (S,S) enantiomer being two times more potent in receptor binding and in \textit{in vivo} models of norepinephrine re-uptake inhibition than the (R,R) enantiomer.\(^{30,31}\)

Therefore, further pharmacological studies could be performed only with enantiomer (R)-8-PN to increase the panicolytic effect observed for the racemic mixture of 8-PN.

**Conclusion**

This study showed that (±)8-PN promoted a specific panicolytic effect on ETM test. Our results too confirm existence of a latency period until the onset of the therapeutic action. This effect is also observed for other compounds with antidepressant profile.

The docking simulations performed in this study were guided by the crystallographic pose of ligand (R)-fluoxetine, a compound with experimentally demonstrated binding to the three transporters evaluated in this study. Furthermore, the excellent correlation between the experimental and theoretical data obtained for the docking of ligands (S,S)-reboxetine and (S)-bupropion leads to the conclusion that (R)-8-PN has greater affinity to the evaluated transporters than enantiomer (S)-8-PN and, therefore, the former must be the active form in the racemic mixture tested \textit{in vivo}.

**Experimental**

**Chemistry** Flavanoid 8-prenylnaringenin (8-PN) was synthesized in racemic form using a convenient four-step sequence from commercially available (±)-naringenin (Sigma-Aldrich, U.S.A.) following the procedure described by Humpel \textit{et al.}\(^{39}\) by distillation. The product is dried \textit{in vacuo} at 40°C overnight.

**Pharmacology: Animals** Male Wistar rats (State University of Maringá) weighing 230–300 g were housed in groups of five in Plexiglas-walled cages at a room temperature of 22±1°C, with alternating 12 h:12 h light/dark cycles (lights on from 07:00 to 19:00h) and free access to food and water, except during testing. The experimental procedures adopted were approved by the Committee of Ethical Conduct in the Use of Animals in Experiments of the State University of Maringá (072/2010-CEAE) and the recommendations for Biomedical Research Involving Animals (CIMS) (Geneva, 1985) were observed.

**Drugs** The following drugs were used: 8-PN, fluoxetine (Sigma, U.S.A.) or vehicle. All drugs were dissolved in sterile saline with 2% Tween 80. The SSRI, fluoxetine, was used as a positive control.

**Apparatus** The ETM was made of wood and has three arms of equal dimensions (50×12 cm). One arm, enclosed by 40-cm high walls, was perpendicular to two opposed open arms. To avoid falls, the open arms were rimmed with 1-cm high Plexiglas. The whole apparatus was elevated 50 cm above the floor. Locomotion was measured inside a wooden circular...
arena (70 cm diameter) with 30-cm high walls. Brightness at the level of the maze arms and open-field centre was 60 lx.

Procedure The ETM is an anxiety model that evokes two defensive responses in the same rat, namely, inhibitory avoidance latency from the closed arm (baseline, avoidance 2 and 3) and one-way escape latency from the open arm (escape 1, 2 and 3), which have been related to generalized anxiety disorder and panic disorder, respectively. Locomotion was assessed in the open field following each drug treatment as a control for nonspecific motor effects.

After 21 d of treatment with 8-PN (10 mg/kg), fluoxetine (15 mg/kg) or the vehicle were administered by gavage and behavioural tests were performed. The doses of fluoxetine were chosen based on their effects in previous studies performed in the ETM.32) The doses of 8-PN were chosen based on in vivo study using the adult rat model33) and in a previous study performed in the ETM with a similar compound.34) On the 20th day of treatment, the animals were gently handled for 5 min and pre-exposed to one of the open arms of the ETM for 30 min. The open arm exit was closed with a wooden barrier mounted between the central area of the maze and the proximal end of the arm. It has been shown that such pre-exposure makes the escape task more sensitive to antipanic drugs, as it shortens the withdrawal latencies from the open arm during the test.35) The ETM test was performed 24h later.

The ETM test started with the inhibitory avoidance task. Each animal was placed at the distal end of the enclosed ETM arm facing the intersection. The time that the rats took to leave this arm with four paws was recorded (baseline latency). This measurement was repeated in two subsequent trials (avoidance 1 and 2) at 30-s intervals. Thirty second after the last avoidance trial, the rats were placed at the end of the open arm to which they had been previously exposed and the latency to leave this arm with four paws was recorded in three consecutive trials (escape 1, 2 and 3) at 30-s intervals. A cut-off time of 300 s was established for avoidance and the escape latency. Thirty second after the ETM test, the animals were placed inside the circular arena for 5 min to evaluate locomotion. The total distance travelled was recorded with a video tracking system (Ethovision; Noldus, Holland) for analysis.

The results observed in the ETM and circular arena were submitted to one-way ANOVA. When appropriate, the Duncan post hoc test was used. The significance level was set at p<0.05. The Statistica Six Sigma software was used for statistical analysis.

Molecular Docking: Ligand Preparation Standards (R)-fluoxetine, (S,S)-reboxetine, (S)-bupropion (Fig. 4) were selected according to literature data16–39) and confirmed as the actually desired transporters using the SEA search tool.40) The three-dimensional structures of these compounds were obtained from the ZINC database (codes ZINC01530638, ZINC0002284 and ZINC00022284, respectively). It is noteworthy that at pH 7.0, all standards have positively charged nitrogen; the protonated version of the structures were used in the molecular docking calculations.

The enantiomers structures (R)-8-PN and (S)-8-PN were generated with the Gaussian 09 program.41) The stable geometries of the compounds were obtained by calculating the potential energy surface (PES) through the HF/3-21G level of theory. The most stable geometries were optimized by density functional theory (DFT) calculations with the B3LYP/6–31++g(d,p) level.

Preparation of the Receptors The structures of the transporters of serotonin (SERT), dopamine (DAT) and norepinephrine (NET) modelled in Apo form were kindly provided by Ravna et al.26) The active sites were identified through the overlapping of the three-dimensional structure of the LeuT submitted to crystallography with (R)-fluoxetine (PDB: 3GWV)22) in the three modelled transporters. Ligand (R)-fluoxetine was added to the transporter structures by geometric docking. LeuT from Aquifex aeolicus (pdb: 3GWV) was chosen as a geometric docking mold for fluoxetine because this protein belongs to the Sodium Neurotransmitter Symporter Family (SNF), the same as the transporters SERT, DAT and NET (protein family id: PF00209), according to evaluation performed by server Pfam.43) Furthermore, pdb 3GWV is the only structural mold linked to (R)-fluoxetine that was available.

(R)-Fluoxetine-receptor complexes were submitted to 60000 energy minimization steps by conjugated gradient for the removal of any steric hindrance with the NAMD2 program.44) The objective of docking (R)-fluoxetine to the three receptors was to serve as a docking guide for other ligands which had unknown bonding mechanisms.

Ligand Docking The docking protocol was validated by redocking assays of (R)-fluoxetine in the three minimized complexes. The protocol was considered valid when the rmsd calculated from the overlapping of the best pose onto the ligand was smaller than an average of 0.5 Å in four assays of each complex, in order to avoid false positive results.

The docking simulations for the standards (R)-fluoxetine, (S,S)-reboxetine and (S)-bupropion and for enantiomers (R)-8-PN and (S)-8-PN were performed with the AutoDock 4.2.3 program implemented at the interface PyRx 0.9.45) applying the redocking-validated protocol (hybrid Lamarckian Genetic Algorithm).

The energy evaluation grid was chosen according to the crystallographic structure of (R)-fluoxetine for each receptor and was centred on coordinates (X=27.980, Y=20.251 and Z=24.870) in SERT, (X=28.301, Y=21.269 and Z=20.420) in DAT and (X=25.639, Y=20.555 and Z=20.010) in NET, with grid points in the x, y and z axes set to 50×50×50 and separated by 0.375 Å. The initial population size and maximum number of energy evaluations were set to 10. The docked results within an rmsd of 2.0 Å were clustered and the final results of each ligand were selected considering both the embedded empirical binding free energy evaluation and the clustering analysis.

Energy Minimization The lowest energy poses obtained by docking the ligands (S,S)-reboxetine and (S)-bupropion and enantiomers (R)-8-PN and (S)-8-PN were exported, in B3LYP/6–31+g(d,p) level.
Atm.

After energy minimization, the protein–ligand complexes were redocked using the same parameters used in docking, giving rmsd values between 0.4 and 1.5 Å, which made the final results reliable.

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