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# Flavones-bound in benzodiazepine site on $GABA_A$ receptor: Concomitant anxiolytic-like and cognitive-enhancing effects produced by Isovitexin and 6-C-glycoside-Diosmetin

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ABSTRACT

Increasing evidence suggests that flavones can modulate memory and anxiety-like behaviour. However, these therapeutic effects are inconsistent and induce of adverse effects, which have been associated with interactions at the Benzodiazepine (BZ)-binding site. To improve our understanding of flavone effects on memory and anxiety, we employed a plus-maze discriminative avoidance task. Furthermore, we evaluated the potential of the compounds in modulating $GABA_A$ receptors via BZ-binding site using molecular modelling studies. Adult male Wistar rats were treated 30 min before training session with Vicenin-2 (0.1 and 0.25 mg/kg), Vitexin (0.1 and 0.25 mg/kg), Isovitexin (0.1 and 0.25 mg/kg) and 0.1 mg/kg 6-C-glycoside-Diosmetin, vehicle and a $GABA_A$ receptor agonist. The analysis of the time spent in the non-aversive vs aversive enclosed arms during the test session and percentage of time in the open arms within the training session revealed that treatment with Isovitexin and 6-C-glycoside-Diosmetin had memory-enhancing and anxiolytic-like effects ($P < 0.001$). In contrast, treatment with a higher dose of Diazepam impaired short-and long-term memory when it alleviated anxiety level. Docking studies revealed that flavones docked in a very similar way to that observed to the Diazepam, except by a lack of interaction in residue α1His101 in the BZ-binding site on $GABA_A$ receptors, which may be related to memory-enhancing effect. The occurrence of the $\alpha_1$His101 interaction could justify the memory-impairing observed following Diazepam treatment. These findings provide the first evidence that Isovitexin and 6-C-glycoside-Diosmetin could exert their memory-enhancing and anxiolytic-like effects via $GABA_A$ receptor modulation, which likely occurs via their benzodiazepine-binding site.

## 1. Introduction

Anxiety disorders are the most common psychiatry disorders worldwide. Approximately one-third of the world population will suffer from at least one anxiety episode in their lifetime (WHO International Consortium in Psychiatric Epidemiology, 2000). Anxious individuals report impairments in executive functioning and episodic memory, as well as difficulty concentrating (Balderston et al., 2017; Vytal et al., 2012). Thus, understanding the interplay between anxiety and memory is important to establish appropriate therapeutic strategies for anxiety disorders.

Benzodiazepines (BZs) are the most common class of psychoactive drugs used to treat human anxiety (Griffin et al., 2013; Rudolph and Knoflach, 2011). BZs exert their actions via a modulatory binding site (the benzodiazepine binding site; BZ-binding site) that is present in different subtypes of $GABA_A$ receptors ($GABA_A$Rs). Diazepam, a non-selective agonist, increases the number or the opening frequency of ion channels in the presence of GABA, thus exerting anxiolytic, sedative,

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anticonvulsant, myorelaxant and cognitive-impairing effects (de Oliveira et al., 2014, 2015; Griffin et al., 2013; Rich et al., 2006; Verwey et al., 2005).

$GABA_A$ receptors belong to the superfamily of nicotinic-acetylcholine receptors (nAChRs) and are chloride-ion channels composed of one γ-, two β- and two α-subunits that may belong to different subunit classes. These subunits include $\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_{1-3}$, δ, ε, π, θ, and $\rho_{1-3}$, and the most abundant in the brain is the $\alpha_1\beta_2\gamma_2$ subtype $GABA_AR$ (Sieghart and Sperk, 2002; Stephenson, 1995). The BZ site is situated at the interface of α1, α2, α3, or α5 subunits and a γ subunit (Bergmann et al., 2013; Richter et al., 2012). Studies of functions involving punctual mutations of specific α subunits have provided invaluable information regarding the functions of different $GABA_A$ receptor subtypes. In these studies, the "missing" Diazepam actions in mice carrying the mutated α subunit are ascribed to the mutant α subunit. For example, in Diazepam α1 (H101R)-treated mice, the amnestic and sedative actions of diazepam were absent; however, its anxiolytic-like action was preserved (Rudolph et al., 1999; Rudolph and Mohler, 2004). There is substantial interest regarding the identification of new compounds with fewer side effects than classical benzodiazepines (for example, non-amnestic effects). In this search, an emerging area of interest is the activation of $GABA_A$ receptors by flavonoids (Goutman et al., 2003; Hanrahan et al., 2011).

Previous studies have identified a subclass of plant-derived compounds known as flavones, which exert actions on mammalian cognition (de Oliveira et al., 2014; Spencer, 2008, 2009; Wang et al., 2007, 2011) and anxiety disorders (Wang et al., 2007; Zhang et al., 2012). Vicenin-2 (apigenin-6,8-di-C-glycopyranoside), Vitexin (apigenin-8C-glucoside), Isovitexin (apigenin-6-C-glucoside) and 6-C-glycoside-Diosmetin are flavonoid derivatives isolated from the stem bark of *Erythrina falcata* L., which has been used as an herbal medicine. The memory effects of Vicenin-2, Vitexin, Isovitexin and 6-C-glycoside-Diosmetin on the central nervous system have been investigated by our group. It has been reported that acute Vitexin, Isovitexin and 6-C-glycoside-Diosmetin-treated rats enhance the acquisition of fear memory; however, this was not the case for Vicenin-2 (de Oliveira et al., 2014). These memory-enhancing effects may be related to the ability of the flavone to modulate GABAergic neurotransmission in the brain. Moreover, previous studies from different laboratories have shown that flavones, such as 6,20-Dihydroxyflavone, apigenin, baicalein and baicalin, exhibited contradictory effects on memory and anxiety, which impaired/improved or had no significant effects on memory in healthy or B-amyloid induced-impairment models; moreover, the anxiolytic and anxiogenic-like effects may be mediated by activation of the benzodiazepine binding (BZ-binding) site of $GABA_A$ receptors. These differential effects on memory and anxiety appear to be related to subunits that contain $GABA_A$ receptors and the differential affinities of these drugs. However, the basis of flavone interactions in the BZ-binding site remains unknown.

Accordingly, as inconsistent effects on memory and anxiety have been attributed to flavones and a potential interaction in the BZ-binding site on $GABA_A$ receptors, this study was designed to (i) concomitantly assess the effects of Vicenin-2, Vitexin, Isovitexin and 6-C-glycoside-Diosmetin treatment on memory, as well as anxiety-like and locomotor activity effects using the plus-maze discriminative avoidance task (PM-DAT), and (ii) evaluate the modulatory action of these flavones on the benzodiazepine binding site of $GABA_A$ receptors using molecular docking studies.

## 2. Materials and methods

### 2.1. Animals

A total of 80 male *Wistar* rats from 10 to 12 weeks old were obtained from the Center for the Development of Experimental Medicine and Biology (CEDEME, Federal University of Sao Paulo, SP, Brazil). All animals were housed 5 animals/cage, under controlled lighting with a 12 h light/dark cycle, temperature (21 °C ± 2 °C) and relative humidity (53% ± 2%). The experiments were performed in the light phase of the cycle. Animals were allowed access to food and water ad libitum; all procedures were conducted in accordance with the Brazilian law for the use of animals in scientific research (No. 11.794), as recommended by the guidelines set by the National Institutes of Health Guide for the Care and Use of Nonhuman Animals in Research. The protocol was approved by the local Committee Governing the Ethics on the use of Animal Experimentation of the Federal University of Sao Paulo (CEUA, approval no. 840560/2014).

### 2.2. Compounds

Valium® (Diazepam) was purchased from Roche (São Paulo, Brazil), and Tween® 80 was obtained from Synth (Diadema, Brazil). Vicenin-2, Vitexin, Isovitexin and 6-C-glycoside-Diosmetin were obtained from the Institute of Chemistry, Nuclei of Bioassay, Biosynthesis and Ecophysiology of Natural Products (NuBBE), Sao Paulo State University, UNESP, Araraquara, SP, Brazil (de Oliveira et al., 2014).

### 2.3. Experimental protocol and systemic administration

Rats were randomly assigned to 10 groups (n = 8/group) as follows: 12% Tween® 80 (negative control), Diazepam (4 mg/kg and 0.10 mg/kg, positive control), Vitexin (0.25 mg/kg and 0.10 mg/kg), Isovitexin (0.25 mg/kg and 0.10 mg/kg), Vicenin-2 (0.25 mg/kg and 0.10 mg/kg) and 6-C-glycoside-Diosmetin (0.10 mg/kg). The Diazepam and flavones were re-suspended in water that contained 12% Tween 80®. The dose ranges of all substances were selected according to previous studies conducted in our laboratory (de Oliveira et al., 2014). Flavones, Diazepam and 12% Tween® 80 were administered orally via an intragastric tube (IG) at 30 min before the training session (Tr).

### 2.4. Plus-maze discriminative avoidance task (PM-DAT)

#### 2.4.1. Behavioural apparatus

The plus-maze discriminative avoidance task apparatus was composed of wood, which contained two enclosed arms (aversive enclosed arm and non-aversive enclosed arm (50 × 12 × 40 cm)) opposite to two open arms (50 × 12 cm). A lamp (100 W) and one speaker (that emitted 85 dB) were placed over one of the aversive enclosed arm (Fig. 1) (Frussa-Filho et al., 2016; Silva and Frussa-Filho, 2000).

#### 2.4.2. Behavioural procedure

All rats were individually transferred and maintained in the adjacent room with a controlled intensity of light at 30 min before the Training session (Tr) and Test session (T). Behavioural sessions were recorded using a Panasonic® camera model SDR-T51 (Panasonic®, Sao Paulo, Brazil) fixed on the ceiling above the apparatus. In both sessions, the apparatus was cleaned with 10% ethanol solution prior to the introduction of a rat.

*2.4.2.1. Training session (Tr).* Rats were individually placed in the centre of the apparatus (facing the space between both open arms) and over a period of 10 min. When the rats entered with the four paws in the aversive enclosed arm (AEA), they received the aversive stimuli (a light; 100-Watt lamp and 85 dB sound, produced by a speaker) (Fig. 1A). The aversive stimuli were administered until the rats left the aversive enclosed arm. The percentage of time spent in the aversive enclosed arm was used as a measure of the acquisition of conditioned fear and short-memory. We simultaneously analysed the anxiety-like behaviour in rats by the percentage of time spent in the open arm (OA) and a time risk assessment. Therefore, short-term memory was calculated as a percentage of time spent in the aversive enclosed arm (% AEA) according the following equation: % AEA = [(AEA/(AEA +