Chrysin, but not the flavone backbone, decreases anxiety-like behavior in animal tests

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Abstract

Chrysin (5,7-dihydroxyflavone), a flavonoid present in diverse plants, has a backbone structure shared with the basic structure of the flavones, with additional hydroxyl groups that confers its antioxidant properties. Moreover, chrysin is also an aromatase inhibitor, as well as a ligand at the benzodiazepine site; these properties suggest an anxiolytic potential, which has been shown in rodent biobehavioral assays. However, whether these effects are due to the hydroxyl groups is unknown. Here we report the effects of chrysin (1 mg/kg) and the flavone basic structure (1 mg/kg) on rat behavior in the elevated plus-maze and a locomotor activity test (arena), as well as in the zebrafish light/dark test. Chrysin, but not flavone, increased entries and time in the open arms of the elevated plus-maze, as well as time on white in the zebrafish light/dark test. These effects were comparable to diazepam, and were devoid of motor effects in both tests, as well as in the rat locomotor activity test. On the other hand, the flavone backbone increased risk assessment in the zebrafish light/dark test but decreased rearing in the rat arena, suggesting effects threat information gathering; important species differences suggest new avenues of research. It is suggested that the specific effects of chrysin in

relation to the flavone backbone are due to its free radical scavenging abilities and/or its higher affinity for the $GABA_A$-benzodiazepine receptor complex.

Introduction

Flavonoids are polyphenolic compounds present in diverse plants (Ghosh and Scheepens, 2009; Krafczyk et al., 2009). The majority of these compounds have been linked with protective effects against oxidative damage (Hwang and Yen, 2008; Wolfe and Liu, 2008), in addition to its pharmacological action on the Central Nervous System (Germán-Ponciano et al., 2018). Particularly, the flavonoid chrysin (5,7-dihydroxyflavone) has been one of the most studied by its antioxidant properties (Siddiqui et al., 2018), but currently also due to its neuropharmacological actions produced on specific brain structures implicated in neuropsychiatric disorders (Filho et al., 2016; Bortolotto et al., 2018).

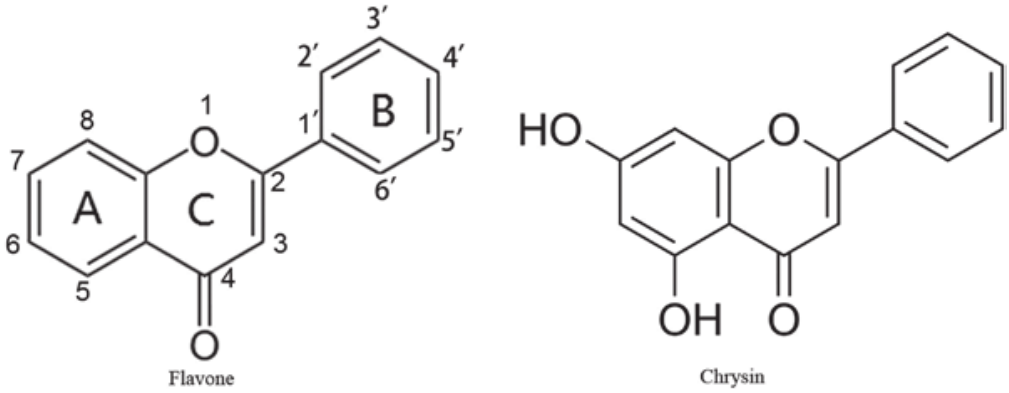

Figure 1. Chemical structures of flavone and chrysin.

Chrysin has a backbone structure that consists in two aromatic rings (A and C), and a phenyl B ring, which is attached to the second position of ring C and shares the basic structure of the flavones, with an additional hydroxyl group in the 5 and 7 positions of the A ring (Mani et al., 2017; Figure 1). The potential of chrysin to act as free radical scavenger has been attributed to the presence of these hydroxyl groups (Sathiavelu et al., 2009; Souza et al., 2015), and it has been suggested that this functional group represents the main action site of this flavonoid to produce a great variety of potential therapeutic effects to ameliorate diverse physiological and psychiatric disorders (Sheela and Angusti, 1995). However, no studies have directly compared the effects of identical doses of chrysin and the basic structure of flavones on the potential therapeutic effects reported at preclinical research.

Chrysin exerts anxiolytic-like effects through action on the $GABA_A$ receptors without the associated side effects (i.e. sedation, amnesia, myrelaxation) that have been attributed to other $GABA_A$ modulators (benzodiazepines, ethanol, etc.); these effects have been evaluated in behavioral models using rodents (Wolfman et al., 1994; Salgueiro et al., 1997; Zanoli et al., 2000; Rodríguez-Landa et al., 2019). Although it has been suggested that presence of the hydroxyl groups in the chrysin structure is the responsible for this pharmacological action, this effect has not been compared with the effects produced by basic structure of the flavones in experimental tests of anxiety-like behavior. The basic flavone moiety has an hydrogen bond donor site, lipophilic pockets, and an electron rich site (Marder and Paladini, 2002); this last is thought to be important for the binding of both flavonoids and 1,4-benzodiazepines to the central benzodiazepine (BZD) site at $GABA_A$ receptors (Marder and Paladini, 2002). Since the

chrysin hydroxyl groups are located in one region of the flavone basic ring that is important for the steric interactions associated with binding, this flavonoid is expected to bind BZD sites with a higher affinity than flavone. As a result, while chrysin is expected to produce a more potent anxiolytic-like effect due to both its higher affinity to the BZD site and its free radical scavenging ability, the electron rich site of flavone is expected to produce at least some level of anxiolysis due to the moderate affinity to the BZD site.

In addition to mammalian model organisms used to study the anxiolytic-like effects of diverse substances, non-mammalian models has been developed that allow researchers to explore the potential therapeutic effects of drugs in a short time (Norton and Bally-Cuif, 2010). The use of zebrafish in preclinical research in the psychopharmacology field has increased recently. Biobehavioral assays of anxiety, such as the rodent light/dark test (LDT), have been adapted to zebrafish, considering that behavioral phenotypes are similar between rodents and zebrafish (Maximino et al., 2010a). Behavioral screens using zebrafish have evaluated the effects of clinically effective anxiolytic drugs, including benzodiazepines (Maximino et al., 2011) and ligands of both the central and peripheral BZD sites (Lima-Maximino et al., 2018), suggesting good pharmacological isomorphism and the ability of such screens to identify potential anxiolytic drugs that can progress in the drug development pipeline. In addition to increasing the toolbox of behavioral screens for psychoactive drugs, zebrafish screens and biobehavioral assays also increase our understanding of the "core" mechanisms of behavioral functions, such as fear and anxiety, that are altered in human patients and shared between humans and rodents (Gerlai, 2014).

The aim of the present study was to compare the effects of chrysin and the flavone backbone (the basic structure of the flavone without the hydroxyl radical groups), using the light/dark test (LDT) in zebrafish, as well as the rat elevated plus-maze (EPM) and locomotor activity test. This could contribute to identify if anxiolytic-like effects produced by chrysin are due to the presence of hydroxyl radicals in its basic structure, which could impact in the specific design of novel molecules destined to pharmacotherapy of anxiety. This manuscript is a complete report of all the studies performed to test the effect of chyrsin, flavone, or diazepam on anxiety-like behavior in rats and zebrafish. We report all data exclusions (if any), all manipulations, and all measures in the study.

Materials and Methods

Animals, housing, and baseline conditions.

Rats. 32 adult male Wistar rats (2.5 months old), weighing 200-250 g, were used. Animals were housed in Plexiglas cages (4-5 rats per cage) in the Laboratorio de Neurofarmacología (Universidad Veracruzana, Xalapa, Mexico) under a 12 h/12 h light/dark cycle (lights on at 7:00 AM) at 25°C ± 1°C with *ad libitu*m access to food and water. The experimental procedures were performed in accordance with national (*Especificaciones Técnicas para la Producción, Cuidado y Uso de Animales de Laboratorio* NOM-062-ZOO-1999) and international (National Institutes of Health Guide for the Care and Use of Laboratory Animals) ethical recommendations. All efforts were made to minimize animal discomfort during the study.

Zebrafish. 48 adult zebrafish of the longfin phenotype were used in the present experiments. Animals were acquired in a local aquarium shop (at Belém, Brazil) and kept in collective tanks at the Laboratório de Neurofarmacologia e Biofísica (UEPA, Marabá, Brazil) for at least 2 weeks before experiments started. Conditions in the maintenance tank were kept stable, as described by Lawrence (2007) and recorded in *protocols.io* (Maximino et al., 2019). Recommendations in the Brazilian legislation (Conselho Nacional de Controle de Experimentação Animal - CONCEA, 2017) were followed to ensure ethical principles in animal care and throughout experiments.

Drugs and treatments.

Chrysin (CAS no. 480-40-0; Sigma-Aldrich, C80105) and flavone (CAS no. 525-82-6; Sigma-Aldrich, F2003) were dissolved in 5% dimethylsulfoxide (DMSO) and injected *i.p.* For all drugs, the dose was 1 mg/kg, a dose of chrysin shown to be anxiolytic in rat elevated plus-maze (Wolfman et al., 1994). Thirty-two rats were assigned to four independent groups (n = 8/group). The vehicle group received 5% dimethylsulfoxide (DMSO) solution (Golden Bell Reactivos, México City, México); a second group (CHR) received 1 mg/kg chrysin; a third group (FLAV) received 1 mg/kg flavone; and a fourth group (DZP) received diazepam (CAS no. 439-14-5; Tocris, 2805) as a positive control. Sixty minutes after the injection, the rats were evaluated in the elevated plus maze and then in the locomotor activity test. Zebrafish were randomly drawn from the holding tank immediately before injection and assigned to four independent groups (n = 12/group). Groups were identical to those used for rats. The injection volume was 1 μL/0.1 g b.w. (Kinkel et al., 2010). The order with which groups were tested was randomized via generation of random numbers using the randomization tool in `http://www.randomization.com/`. Experimenters were blinded to treatment by using coded vials for drugs. The data analyst was blinded to phenotype by using coding to reflect treatments in the resulting datasets; after analysis, data was unblinded.

Apparatuses and methods.

Rat elevated plus-maze The rats were separately evaluated in the elevated plus maze, and then in the locomotor activity test. On the testing day, the rats were brought to the experimental room at 11:00 AM and left for 1 h to acclimate to the novel surroundings. The behavioral tests began at 12:00 AM. The apparatus consisted of two opposite open and closed arms set in a 'plus' configuration, and it was situated in an illuminated room at 40 lux. The open arms were 50 cm length x 10 cm width, and the closed arms were 50 cm length x 10 cm width, with 40 cm-high walls. The entire maze was elevated 50 cm above the floor (Figure 2A). To evaluate the effects of the

treatments, the rats were individually placed in the center of the maze, facing an open arm. The following variables were scored:

1. *Number of entries into the open arms* (N);
2. *Number of entries into the closed arms* (N);
3. *Total number of entries* (open arms + closed arms, N);
4. *Percentage of open arm entries* (Proportion of entries made in the open arms in relation to the total number of entries)
5. *Time spent on the open arms* (s);

After the elevated plus maze test, the rats underwent the locomotor activity test. Approximately 2 min elapsed between tests.

Rat locomotor activity test. Each rat was individually placed in the locomotor activity cage (44 cm length x 33 cm width x 20 cm height; Figure 3A). The floor of the cage was delineated into twelve 11 cm x 11 cm squares to evaluate spontaneous locomotor activity, grooming, and rearing for 5 min. At the beginning of the test, the rat was gently placed in one of the corners of the cage. The following variables were scored:

1. *Number of squares crossed* (N); a crossing was considered when the rat passed from one square to another with its hind legs;
2. *Time spent rearing* (s); rearing was considered when the rat emmited a vertical posture relative to the cage floor
3. *Time spent grooming* (s); including paw licking, nose/face grooming, head washing, body grooming/scratching, leg licking, and tail/genital grooming

Digital video cameras (Sony DCR-SR42, 40 optical zoom, Carl Zeiss lens) were installed above each apparatus (elevated plus maze, and locomotor activity test) to record activity. Two independent observers measured the behavioral variables using ex profeso software to record the number and time (in seconds) of each evaluated behavioral variable until $> 95\%$ agreement was reached among the observers. After each test session, the apparatus was carefully cleaned with a 10% ethanol solution to remove the scent of the previous rat, which can influence spontaneous behavior of the subsequent rat.

Zebrafish light/dark test. The light/dark preference (scototaxis) assay was performed as described elsewhere (Maximino et al., 2010b; Maximino, 2018). Briefly, 30 min after injection animals were transferred individually to the central compartment of a black/white tank (15 cm height X 10 cm width X 45 cm length; Figure 4A) for a 3-min acclimation period, after which, the doors which delimit this compartment were removed and the animal was allowed to freely explore the apparatus for 15 min. While the whole experimental tank was illuminated from above by an homogeneous light source, due to the reflectivity of the apparatus walls and floor average illumination (measured just above the water line) above the black compartment was 225 ± 64.2 (mean $\pm$ S.D.) lux, while in the white compartment it was 307 ± 96.7 lux. The following variables were recorded:

1. *Time spent on the white compartment* (s), the time spent in the white half of the tank;

2. *Transitions to white* (N), the number of entries in the white compartment made by the animal throughout the session;
3. *Entry duration* (s), the average duration of an entry (time on white / transitions);
4. *Erratic swimming* (N), defined as the number of zig-zag, fast, and unpredictable swimming behavior of short duration;
5. *Freezing duration* (s), defined as complete cessation of movements with the exception of eye and operculum movements;
6. *Thigmotaxis* (s), defined as swimming in a distance of 2 cm or less from the white compartment's walls;
7. *Risk assessment* (N), defined as a fast (¡1 s) entry in the white compartment followed by re-entry in the black compartment, or as a partial entry in the white compartment (i.e., the pectoral fin does not cross the midline).

A digital video camera (Samsung ES68, Carl Zeiss lens) was installed above the apparatus to record the behavioral activity of the zebrafish. Two independent observers, blinded to treatment, manually measured the behavioral variables using X-Plo-Rat 2005 (`https://github.com/lanec-unifesspa/x-plo-rat`). Inter-observer reliability was at least > 0.85.

Statistical analysis.

Data were analyzed using one-way ANOVAs with permutation tests, followed by pairwise permutation tests with p-values adjusted for the false discovery rate. Analyses were made in R (R Core Team, 2018), using the package 'coin' (Hothorn et al., 2006). Outliers were detected using an a priori rule based on median absolute deviation (MAD) of time on white (the main endpoint of the LDT) or time in the open arms (the main endpoint of the EPM), with values above or below 3 MADs being removed (Leys et al., 2013). Using this procedure, one outlier was removed from the flavone-treated group.

Open science practices.

Hypotheses and methods were not formally pre-registered. Data and analysis scripts can be found at a GitHub repository (`https://github.com/lanec-unifesspa/chrysin`).

Results

Rat elevated plus-maze.

In the rat elevated plus-maze (Figure 2A), a main effect of treatment was found for entries in the open arms (Figure 2B; maxT = 2.4407, p = 0.05), with chrysin (p = 0.024 in relation to control) and diazepam (p = 0.023 in relation to controls) increasing this variable. No effect was found for entries in the closed arm (Figure 2C; maxT = 1.0295, p = 0.6392) or total entries (Figure 2D; maxT = 1.5331, p = 0.3679). A significant effect was found for percentage of entries in the open arms (Figure 2E; maxT = 2.7848, p = 0.02047), but differences disappeared after adjusting p-values in post-hoc contrasts. Finally, a significant effect was found for time spent in the open arms (Figure 2F; maxT = 2.6129, p = 0.03373), with chrysin (p = 0.0257) and diazepam (p = 0.0027) increasing this variable.

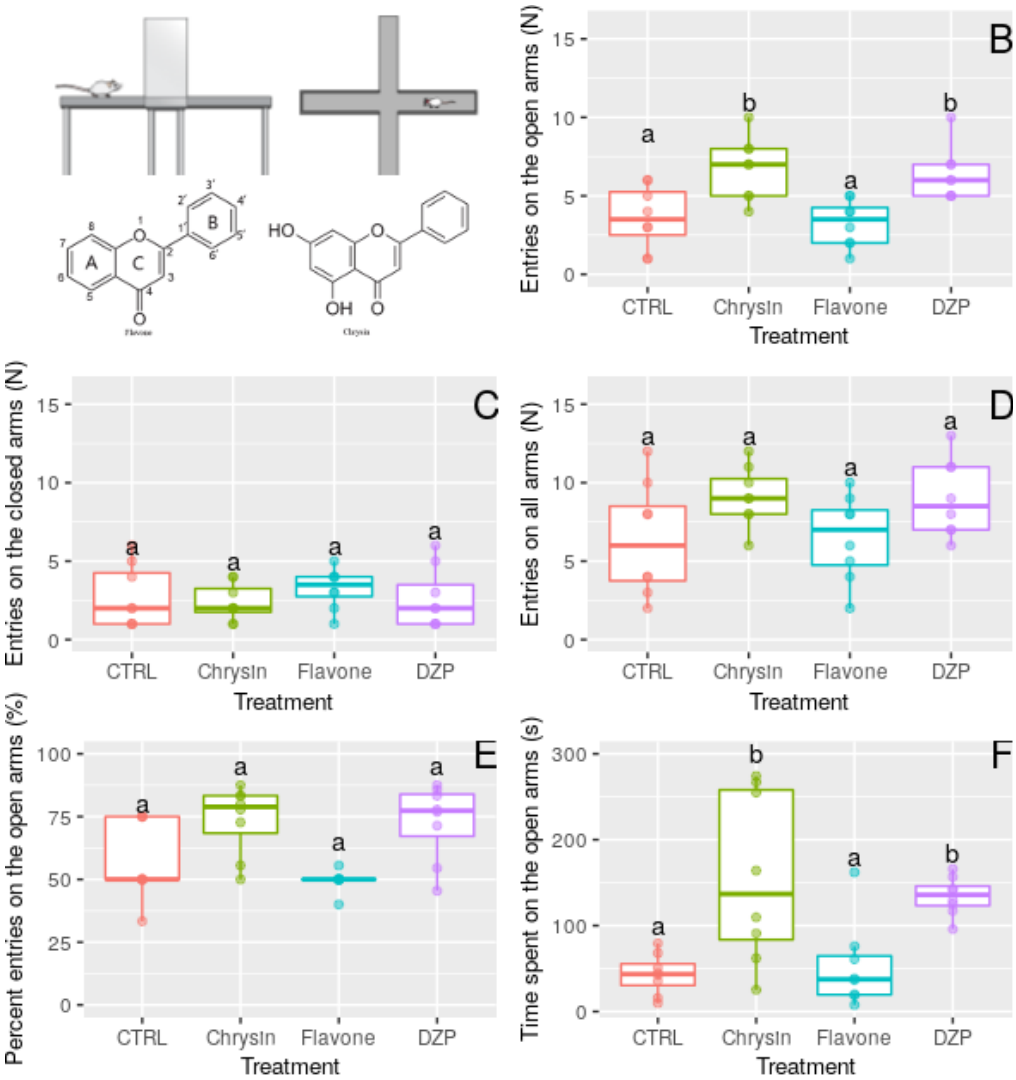

Figure 2. Effects of chrysin, flavone, and diazepam on the rat elevated plus-maze. **A** Scheme of the apparatus and test molecules. **B** Entries on the open arms. **C** Entries on the closed arms. **D** Entries on all arms. **E** Proportion of entries on the open arms in relation to all entries. **F** Time spent in the open arms. For variables with the same letter, the difference is not statistically significant. Likewise, for variables with a different letter, the difference is statistically significant.

Rat locomotor activity test.

In the rat locomotor activity test (Figure 3A), none of the treatments altered the number of squares crossed in the arena (Figure 3B; maxT = 1.6296, p = 0.3139). Grooming (Figure 3C; maxT = 1.5029, p = 0.3861) was not changed; a significant effect was found for rearing (Figure 3D; maxT = 4.009, p = 0.0002), with the flavone backbone increasing it (p = 0.0089 in relation to controls).

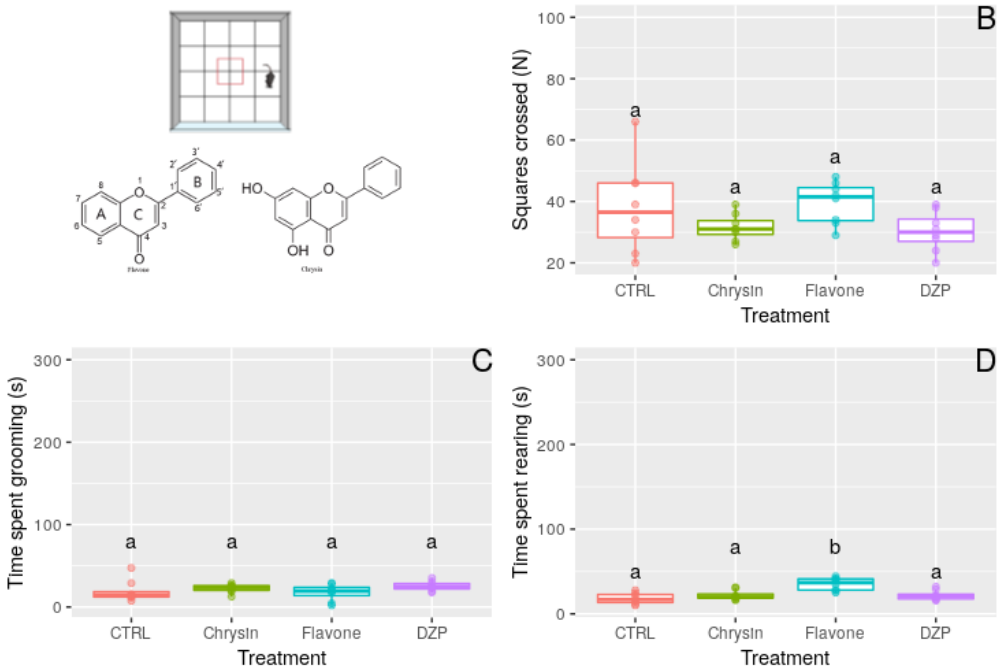

Figure 3. Effects of chrysin, flavone, and diazepam on the rat locomotor activity test. **A** Scheme of the apparatus and test molecules. **B** Squares crossed. **C** Grooming. **D** Rearing. For variables with the same letter, the difference is not statistically significant. Likewise, for variables with a different letter, the difference is statistically significant.

Zebrafish light/dark test.

In the zebrafish light/dark test (Figure 4A), chrysin ($p = 0.0208$) and diazepam ($p = 0.0208$), but not the basic structure of the flavone ($p = 0.1487$), significantly increased ($maxT = 3.7331$, $p = 0.0007321$) the time spent into the white compartment (Figure 4B) in relation to the control group. No significant changes were detected in the duration of entries ($maxT = 2.0981$, $p = 0.1243$) or transitions ($maxT = 0.67072$, $p = 0.891$) to the white compartment (Figures 4C and 4D, respectively), associated with the treatments.

On the other hand, the basic structure of flavone ($p = 0.0201$), but not chrysin ($p = 0.06649$) nor diazepam ($p = 0.05843$), significantly ($maxT= 2.6404$, $p = 0.03119$) reduced risk assessment (Figure 4E) in relation to the control group. Diazepam ($p = 0..319$), but not chrysin ($p = 0.6088$) or flavone ($p = 0.5098$), increased erratic swimming (Figure 4F; $maxT = 3.9468$, $p = 0.002846$). No significant changes were observed in thigmotaxis (Figure 4G; $maxT = 1.4946$, $p = 0.3913$), or freezing duration (Figure 4H; $maxT = 2.3935$, $p = 0.06058$) between treatments.

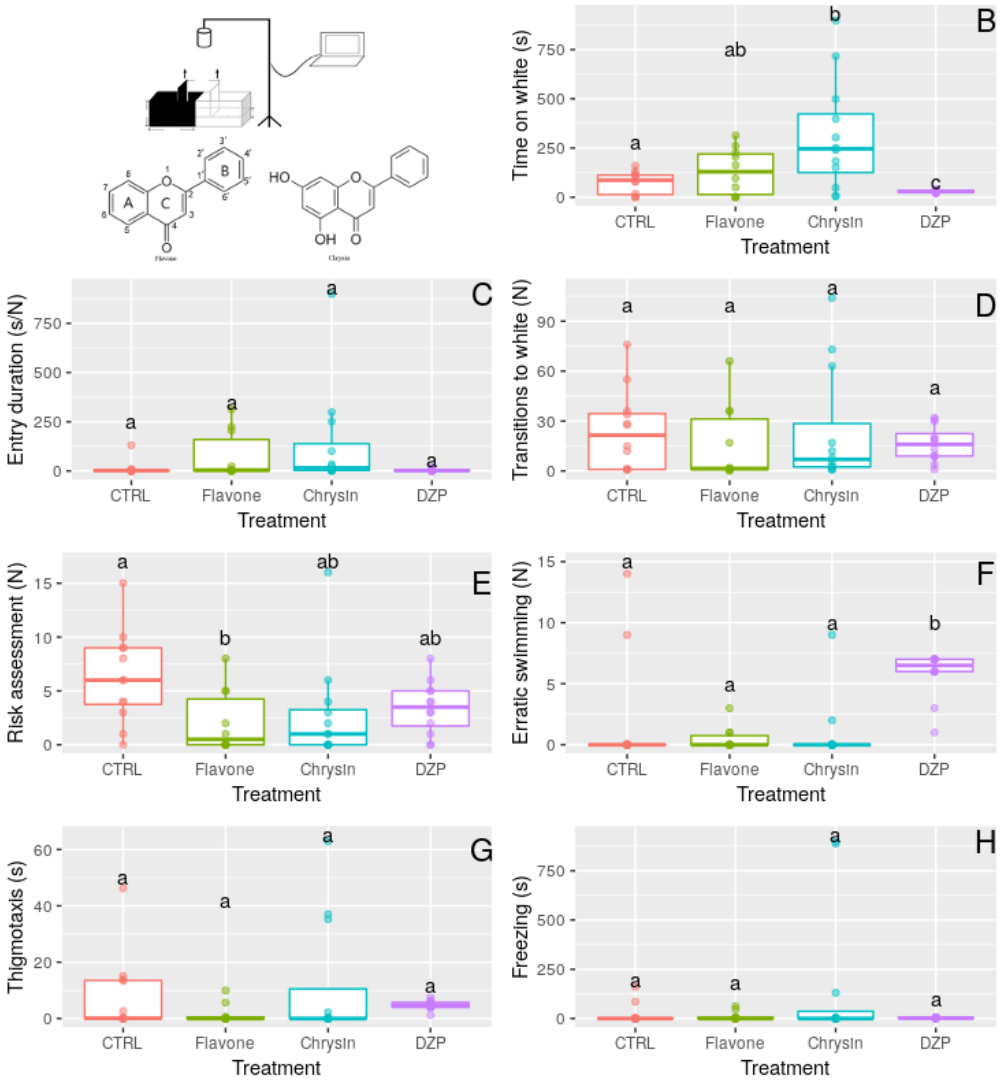

Figure 4. Effects of chrysin, flavone, and diazepam on the zebrafish light/dark test. **A** Scheme of the apparatus and test molecules. **B** Time spent on the white compartment. **C** Duration of entries on the white compartment. **D** Entries the white compartment. **E** Frequency of risk assessment events. **F** Frequency of erratic swimming events. **G** Time spent in thigmotaxis. **H** Duration of freezing events. For variables with the same letter, the difference is not statistically significant. Likewise, for variables with a different letter, the difference is statistically significant.

Discussion

The present study demonstrated that 1) the anxiolytic-like effect of the flavonoid chrysin in the rat EPM and zebrafish LDT, without locomotor effects, seems to be associated with hydroxyl groups substituted in 5 and 7 positions of the A ring in the basic structure of flavone; and 2) the basic structure of flavone is devoid of anxiolytic-like effects in the rat EPM, but increases risk assessment in the zebrafish LDT and rearing in the rat locomotor activity test. These results contribute to the knowledge that positions of hydroxyl in the basic structure of flavones are an important

structural characteristic to produces its pharmacological action on the central nervous system, and suggest that a comparative approach is best suited to detect conserved drug effects across species that are more likely to be shared with humans.

In the present experiments, chrysin increased the number of entries into the open arms and in the time spent on the open arms without locomotor effects either in the EPM or in the locomotor activity test, an effect that is consistent with anxiolysis and is comparable to DZP. The effects of chrysin in the EPM have been shown before. Wolfman et al. (1994) showed that chrysin (1 mg/kg) increased the number of entries into the open arms and in the time spent on the open arms, while a higher dose was needed to increase the number of head-dippings in the holeboard. Brown et al. (2007), however, failed to observe behavioral effects of chrysin (2 mg/kg) in the EPM. We have previously shown that chrysin (2 and 4 mg/kg) blocked the anxiogenic-like effects of long-term ovariectomy in female rats (Rodríguez-Landa et al., 2019), a model of menopause in rats. These last effects were attributed to the activation of $GABA_A$ receptors, as they were blocked by pretreatment with picrotoxin (Rodríguez-Landa et al., 2019).

Similar effects were observed in the zebrafish LDT, a biobehavioral assay that is based in the preference of zebrafish for dark environments (Serra et al., 1999). This behavior reflects the conflict between a motivation for staying in a "safe" place (i.e. dark compartment) and an innate motivation to explore novel, potentially risky environments (Magno et al., 2015). The zebrafish LDT has been validated pharmacologically as a model to screening diverse anxiolytic drugs (Maximino et al., 2011; Magno et al., 2015). Clinically effective anxiolytic drugs that target different sites of the $GABA_A$ receptor, like diazepam and other benzodiazepines, increase the time spent in the light compartment of the apparatus (Gebauer et al., 2011; Maximino et al., 2011), which has been considered as an anxiolytic-like effect at preclinical-research. Moreover, drugs which indirectly target the central benzodiazepine site at $GABA_A$ receptors - such as FGIN-1-27, a drug that promotes neurosteroidogenesis by activating the translocator protein 18 kDa (TSPO) - have been shown to produce an anxiolytic-like effect in the zebrafish LDT (Lima-Maximino et al., 2018).

In the present study, the flavonoid chrysin increased the time spent in the light compartment in comparison to the control group; however, the basic structure of the flavone did not modify this variable. This variable is the main endpoint of the LDT, and is altered by benzodiazepines and TSPO agonists (Sackerman et al., 2010; Stewart et al., 2010; Maximino et al., 2011; Lima-Maximino et al., 2018). This is concordant with effects observed in the rat EPM. However, chrysin did not alter risk assessment behavior in the present experiments, a variable that has been shown to be sensitive to non-benzodiazepine treatments as well as to benzodiazepines (Maximino et al., 2014; Lima-Maximino et al., 2018). In a multivariate approach, risk assessment has been shown to co-vary with other variables in the LDT, forming a separate cluster from thigmotaxis and time on white (Maximino et al., 2014); thus, risk assessment is sensitive to anxiolytic treatments, but represents a different dimension or factor in the multivariate structure of zebrafish behavior in the LDT.

Surprisingly, the flavone backbone decreased risk assessment in the LDT and increased rearing in the locomotor activity test. These outcomes are paradoxical; effects on rearing are difficult to interpret, but it has been suggested (Lever et al., 2006) that rearing is associated with environmental novelty, with a possible cognitive function in information-gathering for escape behavior. However, risk assessment in the LDT can be interpreted as having an information-gathering function as well, and the opposite effects of the flavone backbone on both outcomes are difficult to understand. More behavioral work on the functions of rearing and risk assessment are needed to disentangle this issue.

It has been reported that the generation of free radicals promote the inactivation of

the enzyme glutamate descarboxilase (responsable for the synthesis of GABA), leading to a decrease of GABA concentrations and facilitating excitatory neurotransmission (Singh et al., 2014). Since chrysin, like most flavonoids, exert powerful antioxidant effects, because it possesses highly reactive hydroxyl groups in its basic structure, this confers a potent scavenging free radicals activity (Panche et al., 2016). The aforementioned could promote the GABAergic neurotransmission enhancing, similar to clinically effective benzodiazepine drugs (Harvey, 2015). Moreover, chrysin has been reported to bind to benzodiazepine sites (Medina et al., 1990) and to inhibit aromatase (Campbell and Kurzer, 1993), suggesting that this flavonoid can exert more than one effect in the $GABA_A$/benzodiazepine receptor complex.

Overall the results of the present study show that, in zebrafish and rats, the anxiolytic-like effect of the flavonoid chrysin could be related with the presence of the hydroxyl groups in its basic structure. However, additional work is required to determine the molecular mechanism through the flavonoid chrysin and others flavonoids exert their potential therapeutic to ameliorate anxiety symptoms at preclinical research.

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