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

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Running title: Anxiolytic-like effects of chrysin and flavone

Abstract

Chrysin (5,7-dihydroxyflavone), a nutraceutical flavonoid present in diverse plants, has a backbone structure shared with the flavone backbone, with additional hydroxyl groups that confers its antioxidant properties and effects at the $GABA_A$ receptor complex. However, whether these effects are due to the hydroxyl groups is unknown. Here we report the effects of chrysin or the flavone backbone (1 mg/kg) in rats subjected to the elevated plus-maze and the locomotor activity test, as well as in the zebrafish evaluated in light/dark model. Chrysin, but not flavone, increased entries and time in the open arms of the elevated plus-maze, as well as time on white compartment of the light/dark model in zebrafish. These effects were comparable to diazepam, and were devoid of motor effects in both tests, as well as in the locomotor activity test. On the other hand, flavone decreased risk assessment in the light/dark test but increased rearing in the locomotor activity test in rats, 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 flavone include more of a mechanism of action in which in addition to its action at the $GABA_A$/benzodiazepine receptor complex also could be involved its free radical scavenging abilities, which require specific research. **Preprint:** https://doi.org/10.1101/575514; **Data and scripts:** https://github.com/lanec-unifesspa/chrysin

Keywords: Chrysin; Flavone; Anxiety; Rat; Zebrafish

Introduction

Flavonoids are polyphenolic compounds present in diverse plants (Ghosh & Scheepens, 2009; Krafczyk et al., 2009) and have been considered as nutraceuticals due to they are part of the human diet (Marventano et al., 2017) and produce diverse pharmaceuticals actions. Also, they are able to cross the blood-brain barrier and modify the brain function (Johnston et al., 2015). Particularly, the flavonoid chrysin (5,7-dihydroxyflavone) has been 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). Chrysin is a natural flavonoid present in honey, propolis and diverse plant extracts (German-Ponciano et al., 2018), and is one of the most studied polyphenolic nutraceuticals (Chadha et al., 2017). Chrysin is used as a nutraceutical as a "testosterone boosting agent" (a claim that is probably very exaggerated; Gambelunghe et al., 2003), but there is some preclinical evidence that this molecule ameliorates indices of hepatic and renal functioning in diabetic animals (Ramanathan & Thekkumalai, 2014; Yamamoto, 2014). There is also preclinical evidence that this flavonoid exerts anxiolytic-like effects (Wolfman et al., 1994; Salgueiro et al., 1997; Zanoli et al., 2000; Rodríguez-Landa et al., 2019).

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 flavone backbone, 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 & Augusti, 1995). However, no studies have directly compared the effects of similar doses of chrysin and the flavone backbone on the potential therapeutic effects reported at preclinical research.

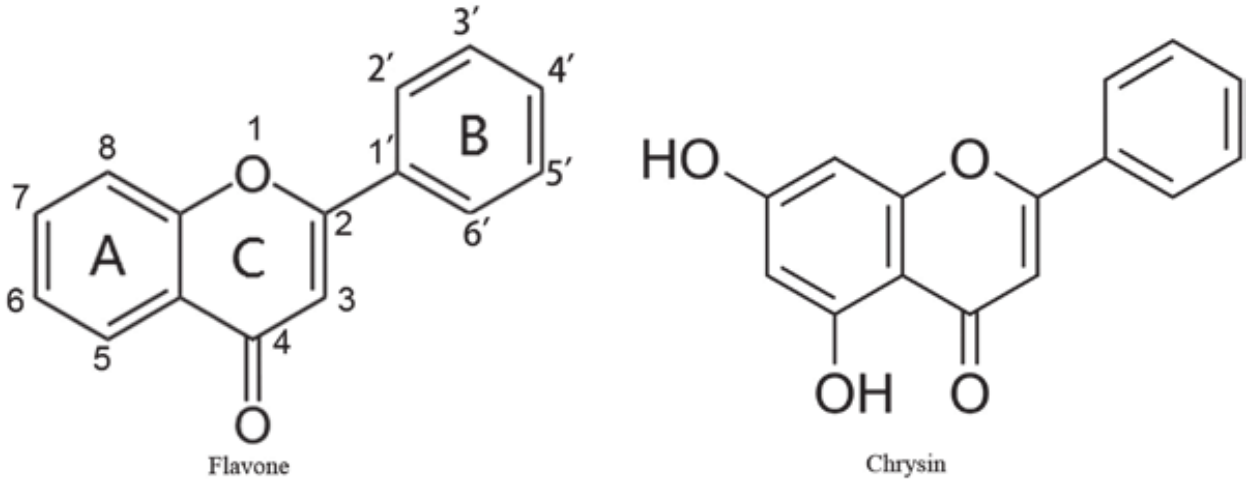

Figure 1: Chemical structures of flavone and chrysin.

Chrysin (either isolated from *Passiflora Coerulea, Passiflora incarnata, Matricaria Chamomilla* or as a synthetic drug) exerts anxiolytic-like effects through action on the $GABA_A$ receptors without the associated side effects (i.e. sedation, amnesia, myorelaxation) 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 the flavone backbone in preclinical models of anxiety-like behavior.

In addition to mammalian model organisms used to study the anxiolytic-like effects of diverse substances, non-mammalian models have been developed to explore the potential therapeutic effects of drugs in a short time (Norton & Bally-Cuif, 2010). The use of zebrafish in preclinical research in the psychopharmacology field has increased recently. Biobehavioral assays of anxiety and screening tests for anxiolytic-like effects, 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 (Bencan et al., 2009; Gebauer et al., 2011; Maximino et al., 2011; Schaefer et al., 2015) 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 fish, rodents, and humans (Gerlai, 2014).

The aim of the present study was to compare the effects of the nutraceutical flavonoid chrysin and the flavone backbone (the basic structure of the flavone without the hydroxyl radical groups), using the LDT in zebrafish, as well as the elevated plus-maze (EPM) and locomotor activity test in rats. 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 chrysin, 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 & methods

Animals

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 25ºC ± 1ºC with *ad libitum* 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 Research Council (US)

Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011) ethical recommendations. All efforts were made to minimize animal discomfort during the study.

Zebrafish. 48 adult unsexed 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; [https://dx.doi.org/10.17504/protocols.io.rupd6vn]). Recommendations in the Brazilian legislation (Diretriz brasileira para o cuidado e a utilização de animais para fins científicos e didáticos - DBCA. Anexo I. Peixes mantidos em instalações de instituições de ensino ou pesquisa científica, 2017) were followed to ensure ethical principles in animal care and throughout experiments.

Drugs and treatments

Chrysin (CAS #480-40-0; Sigma-Aldrich, C80105) and flavone (CAS #525-82-6; Sigma-Aldrich, F2003) were dissolved in 5% dimethyl sulfoxide (DMSO) and injected *i.p.* For all drugs, the dose was 1 mg/kg, a dose of chrysin shown to be anxiolytic in elevated plus-maze (Wolfman et al., 1994). Thirty-two rats were assigned to four independent groups (n = 8/group). The vehicle group received 5% DMSO solution (Golden Bell Reactivos, México City, México) in which chrysin and diazepam were prepared; a second group (CHR) received 1 mg/kg chrysin; a third group (FLAV) received 1 mg/kg flavone; and a fourth group (DZP) received 2 mg/kg diazepam (CAS #439-14-5; Tocris, 2805; German-Ponciano et al., 2020) as a pharmacological control of anxiolytic activity. Sixty minutes after the injection, the rats were evaluated in the elevated plus maze and then in the locomotor activity test. The time elapsed between injection and the behavioral test has previously demonstrated anxiolytic-like effects of both chrysin and diazepam at the

doses that were uesed in present study (Taiwo et al., 2012; Rodríguez-Landa et al., 2019; Cueto-Escobedo et al., 2020; German-Ponciano et al., 2020).

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, including vehicle (5% DMSO). The injection volume was 1 μL/0.1 g of body weight, and animals were injected intraperitoneally (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.

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 "+" configuration, and it was situated in an illuminated room at 40 lux. The open arms were 50 cm length × 10 cm width, and the closed arms were 50 cm length × 10 cm width, with 40 cm-high walls. The entire maze was elevated 50 cm above the floor (Walf & Frye, 2007). 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 during 5 min:

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);
6. *Time spent in head-dipping* (s);
7. *Number of head-dipping events* (N);
8. *Time spent in stretched-attend posture* (SAP; s);

9. *Number of SAP events* (N).

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

Locomotor activity test

Each rat was individually placed in the locomotor activity cage (44 cm length × 33 cm width × 20 cm height). The floor of the cage was delineated into twelve 11 cm × 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* (crossings; a crossing was considered when the rat passed from one square to another with its hind legs);
2. *Time spent rearing* (rearing was considered when the rat emitted a vertical position relative to the cage floor);
3. *Time spent grooming* (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.

Light/dark test

The light/dark preference (scototaxis) assay was performed as described elsewhere (Maximino, 2018 [https://10.17504/protocols.io.srfed3n]; Maximino et al., 2010b). 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) 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 a 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 ± 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:* the time spent in the white half of the tank (*s*);
2. *Transitions to white:* the number of entries in the white compartment made by the animal throughout the session;
3. *Entry duration:* the average duration of an entry (time on white / transitions);
4. *Erratic swimming:* defined as the number of zig-zag, fast, and unpredictable swimming behavior of short duration;
5. *Freezing:* the duration of freezing events (*s*), defined as complete cessation of movements with the exception of eye and operculum movements;
6. *Thigmotaxis:* the duration of thigmotaxic events (*s*), defined as swimming in a distance of 2 cm or less from the white compartment's walls;
7. *Frequency of risk assessment:* 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 > 0.95.

Statistical analysis

Data were analyzed using Asymptotic General Independence Tests, a permutation-based analogue of one-way ANOVA, followed by pairwise permutation tests, analogous to pairwise t-tests with *p*-values adjusted for the false discovery rate. Analyses were made in R (R Core Team, 2019), using the package 'coin' (Hothorn et al., 2006). Normality was not assumed, and thus no specific test for normality was performed; however, this type of analysis is resistant to deviations from the assumptions of the traditional ordinary-least-squares ANOVA, and is robust to outliers, thus being insensitive to distributional assumptions (such as normality) (Mangiafico, 2015). This test is based on the permutation of a statistic maxT, the maximum of several competing univariate statistics. 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 in zebrafish.

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

Elevated plus-maze

In the elevated plus-maze (Figure 2A), animals made more entries in the open arms (Figure 2B; maxT = 2.4407, p = 0.05) after the treatment with chrysin (p = 0.02) and

diazepam (p = 0.02) in comparison to the control group. No effect was found for entries in the closed arm (Figure 2C; maxT = 1.0295, p = 0.63) or total entries (Figure 2D; maxT = 1.5331, p = 0.36). A significant effect was found for percentage of entries in the open arms (Figure 2E; maxT = 2.7848, p = 0.02), but differences disappeared after adjusting *p*-values in *post-hoc* contrasts. A significant effect was found for time spent in the open arms (Figure 2F; maxT = 2.6129, p = 0.03), this variable was increased after treatment with chrysin (p = 0.02) and diazepam (p = 0.01). No effects were found for time spent head-dipping (Figure 2G; maxT = 1.3025, p = 0.51) or number of head-dipping events (Figure 2H; maxT = 0.853, p = 0.8). A significant treatment effect was found for time spent in SAP (Figure 2I; maxT = 2.5326, p = 0.04), but meaningful differences disappeared after adjusting *p*-values in *post-hoc* contrasts. Finally, a significant effect was found for SAP frequency (Figure 2J; maxT = 3.2229, p = 0.01), with flavone (p = 0.02) and diazepam (p = 0.01) reducing SAP.

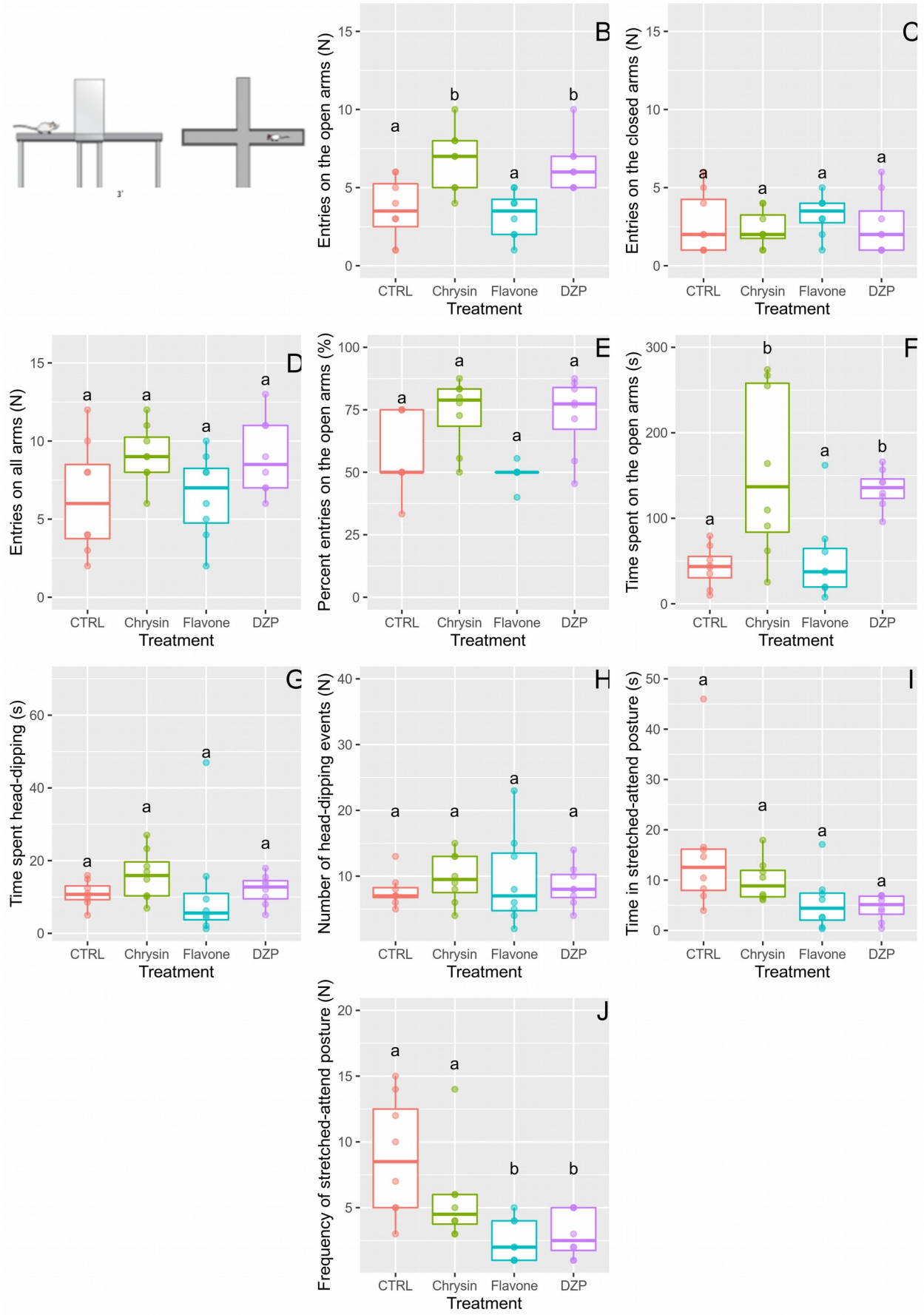

Figure 2: Effects of chrysin, flavone, and diazepam on the elevated plus-maze. A. Scheme of the apparatus. B. Entries on the open arms. C. Entries on the closed arms. D. Entries on all arms. E. Percent entries on the open arms. F. Time spent on the open arms. G. Time spent in headdipping. H. Number of head-dipping events. I. Time spent in stretched-attend posture (SAP). J. Frequency of SAP 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 (p≤0.05).

Locomotor activity test

In the locomotor activity test (Figure 3A), none of the treatments modified the number of squares crossed (Figure 3B; maxT = 1.6296, p = 0.31) and grooming behavior (Figure 3C; maxT = 1.5029, p = 0.38); but significant differences were founded in rearing (Figure 3D; maxT = 4.0090, p = 0.01). *Post-hoc* test revealed that flavone backbone increased this variable (p = 0.01) respect to the control group.

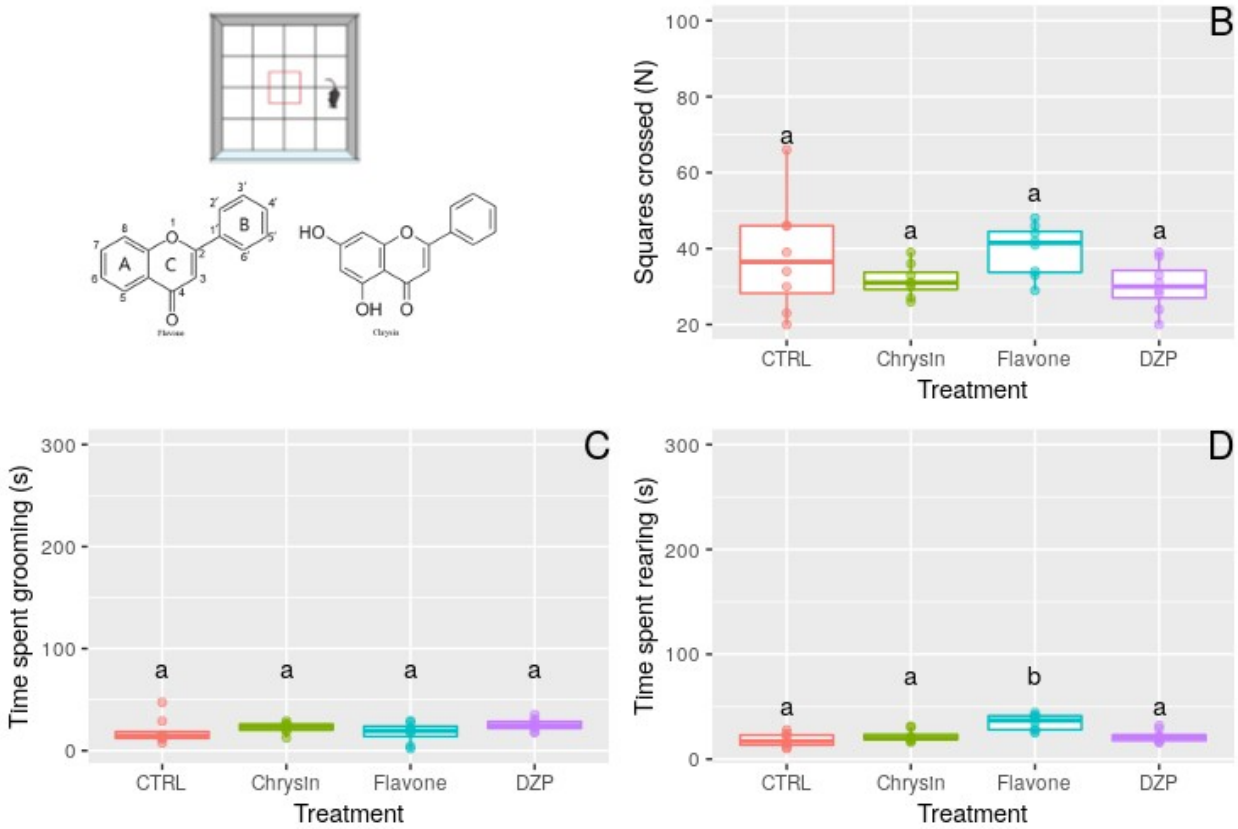

Figure 3: Effects of chrysin, flavone, and diazepam on the locomotor activity test. A. Scheme of the apparatus and test molecules. B. Squares crossed. C. Time spent in grooming. D. Time spent in rearing. For variables with the same letter, the difference is not statistically significant. Likewise, for variables with a different letter, the difference is statistically significant (p≤0.05).

297 **Light/dark test**

298 In the light/dark test (Figure 4A), chrysin (p = 0.02) and diazepam (p = 0.02), but not the
299 flavone backbone (p = 0.14), significantly increased (maxT = 3.7331, p = 0.01) the time
300 spent into the white compartment (Figure 4B) compared to the control group. No
301 significant changes were detected in the duration of entries (maxT = 2.0981, p = 0.12)
302 or transitions (maxT = 0.6707, p = 0.89) to the white compartment (Figures 4C and 4D,
303 respectively), associated with the treatments.

304 On the other hand, the flavone backbone (p = 0.02), but neither chrysin (p = 0.06) nor
305 diazepam (p = 0.06), significantly (maxT= 2.6404, p = 0.03) reduced risk assessment
306 (Figure 4E) in relation to the control group. Diazepam (p = 0.031), but not chrysin (p =
307 0.60) or flavone (p = 0.50), increased erratic swimming (Figure 4F; maxT = 3.9468, p =
308 0.01). No significant changes were observed in thigmotaxis (Figure 4G; maxT = 1.4946,
309 p = 0.39), or freezing duration (Figure 4H; maxT = 2.3935, p = 0.06) between
310 treatments.

311

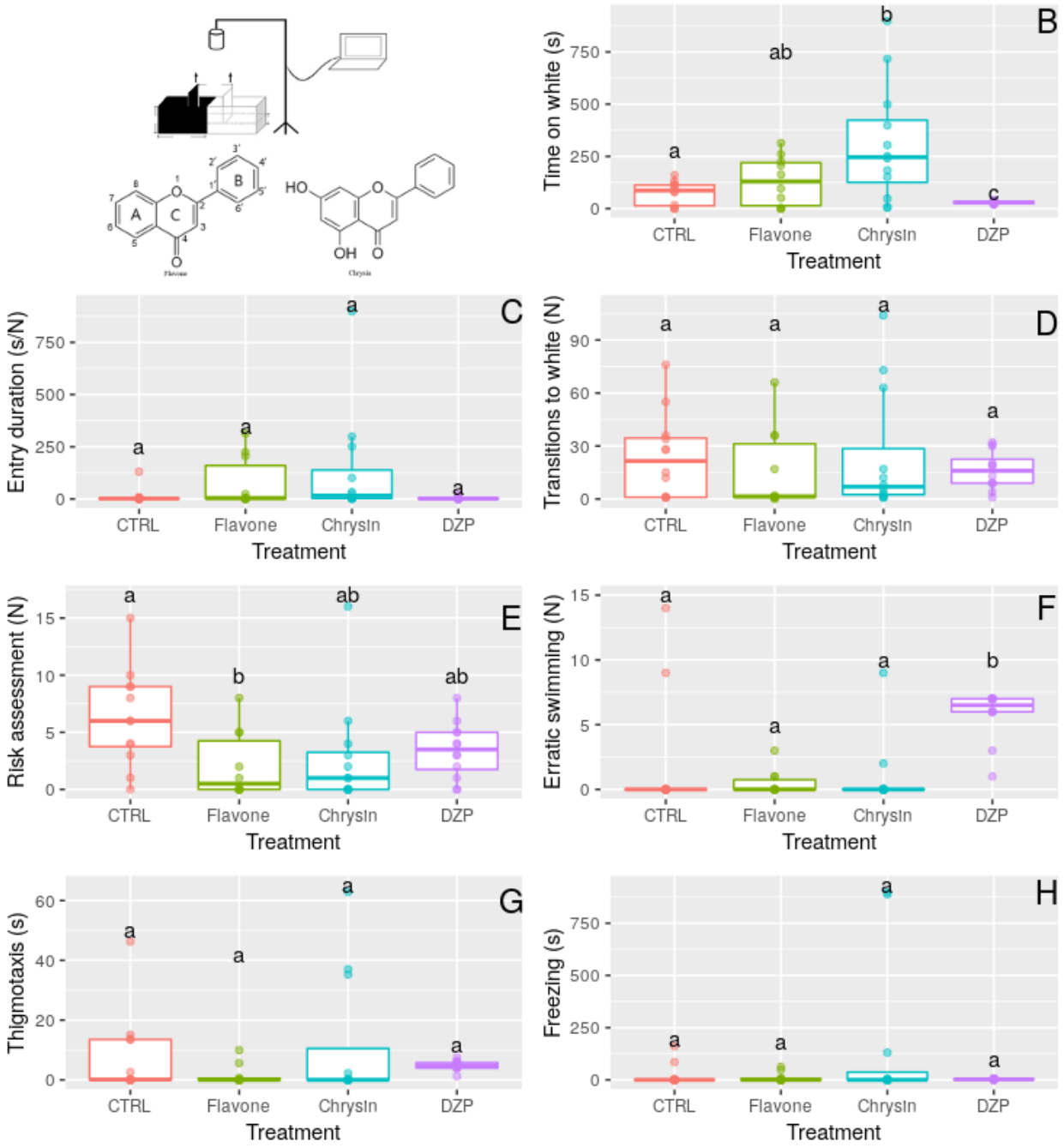

Figure 4: Effects of chrysin, flavone, and diazepam on the light/dark test. A. Scheme of the apparatus and test molecules. B. Time spent on the white compartment. C. Duration of entries on the white compartment. D. Transitions to white compartment. E. Frequency of risk assessment events. F. Frequency 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 (p≤0.05).

313 **Discussion**

314 The present study demonstrated that 1) the anxiolytic-like effect of the flavonoid chrysin
315 in the rat EPM and LDT, without locomotor effects, seems to be associated with
316 hydroxyl groups substituted in 5 and 7 positions of the A ring in the flavone backbone;
317 and 2) the flavone backbone is devoid of anxiolytic-like effects in the rat EPM in rats, but
318 decreases risk assessment in the LDT in zebrafish and rearing in the locomotor activity
319 test. These results contribute to the knowledge that positions of hydroxyl in the flavone
320 backbone are important structural characteristic to produces its pharmacological action
321 on the central nervous system, and suggest that a comparative approach is best suited
322 to detect conserved drug effects across species that are more likely to be shared with
323 humans.

324 In the present experiments, after chrysin treatment the number of entries into and time
325 spent into the open arms were increased, without locomotor effects either in the EPM or
326 in the locomotor activity test, an effect that is consistent with anxiolysis and is
327 comparable to DZP. The effects of chrysin in the EPM have been shown before.
328 Wolfman et al. (1994) showed that chrysin (1 mg/kg), isolated from *Passiflora coerulea*,
329 exerts an anxiolytic-like effect in the EPM. We have previously shown that chrysin (2
330 and 4 mg/kg) blocked the anxiogenic-like effects of long-term ovariectomy in female
331 rats, which were attributed to the activation of $GABA_A$ receptors, as they were blocked
332 by pretreatment with picrotoxin (Rodríguez-Landa et al., 2019).

333 Similar effects were observed in the LDT, a biobehavioral assay that is based in the
334 preference of zebrafish for dark environments (Serra et al., 1999) and has been
335 validated pharmacologically as a model to screening diverse anxiolytic drugs (Maximino
336 et al., 2011; Magno et al., 2015). In this sense, the flavonoid chrysin increased the time
337 spent in the white compartment in comparison to the control group. This variable is the
338 main endpoint of the LDT (Maximino et al., 2011; Lima-Maximino et al., 2018), also
339 clinically effective anxiolytic drugs that target different sites of the $GABA_A$ receptor, like
340 diazepam and other benzodiazepines, can increase it (Gebauer et al., 2011; Maximino

341 et al., 2011; Magno et al., 2015), which has been considered as an anxiolytic-like effect
342 at preclinical-research.

343 However, the flavone backbone did not modify the time spent into the open arms in the
344 EPM neither the time spent in the compartment in the LD, in relation to the control
345 groups. The basic flavone moiety (backbone) has a hydrogen bond donor site, lipophilic
346 pockets, and an electron rich site (Marder & Paladini, 2002), which are thought to be
347 important for the binding of both flavonoids and 1,4-benzodiazepines to the central
348 benzodiazepine (BZD) site at $GABA_A$ receptors (Marder & Paladini, 2002). Since the
349 chrysin hydroxyl groups are located in one region of the flavone basic ring, this could be
350 important for the steric interactions associated with binding, and therefore chrysin could
351 bind to BZD sites with a higher affinity than flavone (Marder & Paladini, 2002; Huen et
352 al., 2003). This could explain the wider anxiolytic-like effect of chrysin, but not flavone
353 backbone, in the present study.

354 In addition to the effects of chrysin and flavone on the BZD site, another potential
355 mechanism is a free radical scavenging effect: chrysin, like most flavonoids, exerts
356 powerful antioxidant effects, because it possesses highly reactive hydroxyl groups in its
357 basic structure, which confers a potent free radical scavenging activity (Panche et al.,
358 2016). It has been reported that the generation of free radicals promote the inactivation
359 of the enzyme glutamate decarboxylase (responsible for the synthesis of GABA),
360 leading to a decrease of GABA concentrations and facilitating excitatory
361 neurotransmission (Singh et al., 2014), and thus the scavenging potential of chyrsin
362 could enhance the GABAergic neurotransmission (Harvey, 2015). On another hand, it
363 has been reported that chrysin increases testosterone levels (Ciftc et al., 2011), which
364 could be associated to its aromatase inhibitor activity (Campbell & Kurzer, 1993). In this
365 sense, Fernández-Guasti et al. (2005) demonstrated that testosterone administration
366 exerts an anxiolytic-like effect in male Wistar rats due to the $GABA_A$-benzodiazepine
367 receptor modulation by testosterone. This suggest that the flavonoid chrysin can exert
368 more than one effect in the $GABA_A$/benzodiazepine site complex.

369 In the other hand, chrysin did not alter risk assessment behavior in the present
370 experiments, a variable that has been shown to be sensitive to non-benzodiazepine
371 treatments as well as to benzodiazepines (Maximino et al., 2014; Lima-Maximino et al.,
372 2018). In a multivariate approach, risk assessment has been shown to co-vary with
373 other variables in the LDT, forming a separate cluster from thigmotaxis and time on
374 white (Maximino et al., 2014); thus, risk assessment is sensitive to anxiolytic treatments,
375 but represents a different dimension or factor in the multivariate structure of zebrafish
376 behavior in the LDT.

377 Surprisingly, the flavone backbone decreased risk assessment in the LDT and SAP in
378 the rat EPM, and increased rearing in the locomotor activity test. These outcomes are
379 paradoxical; effects on rearing are difficult to interpret, but it has been suggested (Lever
380 et al., 2006) that rearing is associated with environmental novelty, with a possible
381 cognitive function in information-gathering for escape behavior. However, risk
382 assessment in the LDT and SAP in the EPM can be interpreted as having an
383 information-gathering function as well, suggesting different mechanisms for rearing, on
384 the one hand, and risk assessment behavior in anxiety tests, on the other. Taken
385 together, these results suggest that, although not able to reduce the avoidance of
386 potentially adverse environments such as the open arm of the EPM or the light
387 compartment of the LDT tank, the flavone backbone is able to reduce risk assessment
388 behavior. Risk assessment has been suggested as a cognitive component of anxiety-
389 like behavior (Blanchard et al., 2011), and is more sensitive than spatio-temporal
390 measures (time spent on open arms) in the EPM to detect effects of serotonergic
391 anxiolytics (Griebel et al., 1997).

392 Finally, the present study compared the effect of a single dose of chrysin (1 mg/kg)
393 *versus* the flavone backbone. We have previously reported other doses (2 and 4 mg/kg)
394 of this flavonoid with anxiolytic-like effect in the EPM (Rodríguez-Landa et al., 2019).
395 However, that study used female Wistar rats with long-term ovariectomy, while in this
396 work male Wistar rats were used. In other work, we demonstrated that, in male Wistar
397 rats, only 1 mg/kg, but no 0.5 neither 2 mg/kg, of chrysin exerted an anxiolytic-like effect
398 in the EPM in male Wistar rats (German-Ponciano et al., 2020). Thus, we used a single

399 dose of chrysin in agreement with the ethical recommendations of the 3R´s principles in
400 order to reduce the number of animals used in this study (Russell and Burch, 1959).
401 Nevertheless, future work will be required in order to compare the possible dose-
402 response curves of chrysin *versus* flavone backbone.

403 Overall the results of the present study show that, in zebrafish and rats, the anxiolytic-
404 like effect of the flavonoid chrysin might be due to more than one effect in the
405 $GABA_A$/benzodiazepine receptor complex including its antioxidant effect, which could be
406 related with the presence of the hydroxyl groups in its basic structure. However,
407 additional work is required to determine the molecular mechanism through this
408 nutraceutical compound and other flavonoids exert their potential therapeutic to
409 ameliorate anxiety symptoms at preclinical research.

410

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