The biologically active compound of *Withania somnifera (L.) Dunal,* Docosanyl Ferulate, is endowed with potent anxiolytic properties but devoid of typical benzodiazepine-like side effects

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Running Title: Anxiolytic properties of Docosanyl Ferulate

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Abstract

Background: Clinical and experimental studies support the therapeutic potential of Withania somnifera (WS) (L.) Dunal on anxiety disorders. This potential is attributable to components present in different plant extracts; however, the individual compound(s), endowed with specific anxiolytic effects and potential modulatory activity of the GABAA receptor complex (GABAAR), have remained unidentified until the recent isolation, from a WS methanol root extract, of some GABAAR-active compounds, including the long alkil-chain ferulic acid ester, Docosanyl Ferulate (DF).

Aims: This study was designed to assess whether DF (0.05, 0.25, and 2 mg/kg), similarly to Diazepam (2 mg/kg), may exert anxiolytic effects, whether these effects may be significantly blocked by the benzodiazepine antagonist, Flumazenil (10 mg/kg), and whether DF may lack of some of the benzodiazepines' typical motor, cognitive and motivational side effects.

Methods: The following behavioral paradigms, Elevated Plus Maze, Static Rods, Novel Object Recognition, Place Conditioning and potentiation of ethanol-induced Loss of Righting Reflex were applied on male CD-1 mice.

Results: Similarly to Diazepam, DF exerts anxiolytic effects, blocked by Flumazenil. Moreover, at the full anxiolytic dose of 2 mg/kg, DF lacks of typical benzodiazepine-like side effects on motor and cognitive performances and on place conditioning. Moreover, DF also fails to potentiate ethanol's (3 g/kg) depressant activity at the ethanol-induced Loss of Righting Reflex paradigm.

Conclusions: These data point to DF as an effective benzodiazepine-like anxiolytic compound that, in light of its lack of motor, mnemonic and motivational side effects, could be a suitable candidate for the treatment of anxiety disorders.

Conflict of interest statement

Authors have no conflict of interests to disclose and confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Keywords

Anxiety; Diazepam; Docosanyl Ferulate; Flumazenil; $GABA_A$ receptors; *Withania somnifera*

Introduction

Withania somnifera (WS) (L.) Dunal is a medicinal plant originall**y included in** ~~of~~ the Ayurvedic Indian Traditional System of Medicine **and** presently also broadly used in western countries. Its curative properties, attributed to the constituents that take part in the composition of its phyto-therapeutic complex extend from neuroprotective to anti-inflammatory activities (Dar et al., 2015). Moreover, both clinical (Pratte et al., 2014) and experimental (Kaur et al., 2017; Kaur and Kaur,

2017) evidence justifies also *WS's* long-standing reputation as an effective anxiolytic treatment. In this regard, modulation of GABA neurotransmission is of fundamental importance in pharmacotherapeutic perspective (Ngo and Vo, 2019). Notably, experimental evidence supports the possibility that some constituents of *WS* could target the $GABA_A$ receptor ($GABA_AR$) (Bassareo et al., 2019; Bhattarai et al. 2010; Orrù et al., 2014; Mehta et al., 1991; Ruiu et al., 2013). In keeping with these studies, we recently detected, in a methanolic extract of the roots of *WS,* some secondary metabolites with affinity for this receptor. In particular, the long alkyl chain ferulic acid ester, Docosanyl Ferulate (DF), showed the highest modulatory activity on the $GABA_AR$ in rat brain slices (Sonar et al., 2019).

Based on this evidence, we designed the present in vivo study aiming to investigate whether this molecule could have anxiolytic effects. We therefore performed a series of assays on mouse behavioral models using Diazepam (DZP)**, a positive allosteric modulator of GABAAR (Nutt and Blier, 2016),** as Benzodiazepine's (BDZ) reference-compound. The potential DF's anxiolytic effects were investigated in an Elevated Plus Maze (EPM) test **(Lister, 1987; Pellow et al. 1986; Rodgers and Johnson, 1995)** and**,** in order to confirm the BDZ-like mechanism of DF effects in the EPM test, we used the BDZ antagonist Flumazenil (FMZ) **(Razavi et al. 2017)**. Moreover, although BDZs are among the most prescribed psychiatric medications, they are also classified as addictive drugs (Tan et al., 2011) and their beneficial effects are restrained by adverse motor and cognitive side effects (Roth et al., 1984; Rowlett et al., 2004). Hence, we also investigated whether DF shares **with** BDZ~~'~~s **their** most typical side effects such as motor and mnemonic impairments as well as addictive potential. To this end, we verified whether DF could impair motor abilities in a Static Rods test **(Deacon, 2013)**; subsequently, we evaluated the performance of DF- and DZP-treated mice in a Novel Object Recognition (NOR) test **(Costa et al. 2014)** and, in order to evaluate if, similarly to DZP (Acquas et al., 1989; Spyraki et al., 1985), DF presents addictive potential, we also tested whether DF, at its full anxiolytic dose, elicits place conditioning **(Tzschentke, 2007)**.

Finally, while BDZs themselves are quite safe medications, an extremely high risk of generating adverse reactions is related to the interaction with other depressant substances including ethanol, the most abundant constituent of alcoholic drinks. Indeed, ethanol and BDZs share the ability to interact with the $GABA_AR$ complex and, for this reason, ethanol increases the misuse of BDZs and BDZs-related cases of overdose (Linnoila, 1990; Votaw et al., 2019). Accordingly, we evaluated whether, similarly to Diazepam, DF is endowed with the property to potentiate ethanol-induced Loss of Righting Reflex (LORR) **(Correa et al., 2001; Slater et al., 2016)**.

Materials and methods

Animals

Adult male CD-1 mice (22-24 g, Charles River, Calco, Italy) (n=336) were housed in groups of eight per cage, under a 12:00/12:00 h light/dark cycle (lights on at 08:00 a.m.) with food (Mucedola Srl, Settimo Milanese (Milan) Italy) and water available *ad libitum*. All the experiments were carried out during the light phase, between 09:00 and 18:00 h. On the day of the experiment mice, kept in their home cages, were carried in the experimental room where they had 1 h of habituation before the experiments' start. The total numbers of mice were n=116 for the EPM, n=49 for the Static Rods, n=43 for the NOR, n=40 for the Place Conditioning and n=88 for the LORR. All the experimental procedures were performed in accordance with the Principles of laboratory animal care, with the guidelines and protocols approved by the European Union (2010/63/UE L 276 20/10/2010) and with the approval (1177/2016) of the local Committee. Every possible effort was made to minimize animal suffering and discomfort and to reduce the number of experimental subjects.

Drugs administration

DF (0.05, 0.25 and 2 mg/kg) (Fig. 1), synthetized (purity >98% by HPLC) according to Sonar et al. **(2019)** ~~[11]~~, DZP (2 mg/kg) (FIS, Altemantecchio, Vicenza, Italy) and FMZ (10 mg/kg) (gift from Hoffmann-La Roche, Basel, Switzerland), dissolved in Tween 80 (Sigma-Aldrich, Milan, Italy) and suspended in isotonic saline (NaCl 0.9% w/v) were administered at 10 ml/kg of volume injection. Vehicle consisted in the same volume of Tween 80 and isotonic saline used to dissolve the drugs. Ethanol (3 g/kg, 10 ml/kg volume injection) (Sigma-Aldrich, Milan, Italy) was diluted (37% v/v) with isotonic saline. All drugs were administered intraperitoneally (IP). **Based on previous literature the doses of DZP, FMZ and ethanol were selected in agreement with, respectively, Löw et al., (2000), Razavi et al., (2017) and Slater et al., (2016).**

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~~***Fig. 1*** ~~Chemical structure of Docosanyl Ferulate, bioactive secondary metabolite isolated from a methanolic extract of the roots of WS (Sonar et al., 2019).~~

Elevated Plus Maze

The EPM consisted of a central platform (5 x 5 cm, W x L), two open arms (5 x 25 cm, W x L) aligned perpendicularly to two closed arms (5 x 25 cm, W x L) at an height of 40 cm from the ground. For these experiments two different protocols, 1 and 2, have been followed. In protocol 1 mice were selected randomly and assigned to one of the **following** groups: **VEH (vehicle 10 ml/kg)** (n=12), DF 0.05 **(DF 0.05 mg/kg)** (n=16), DF 0.25 **(DF 0.25 mg/kg)** (n=16), DF 2 **(DF 2 mg/kg)** (n=12), DZP **(DZP 2 mg/kg)** (n=10). **After** ~~V~~vehicle or drugs were administered**,** ~~and~~ mice **were** put back in their home cages **and** ~~After~~ 30 min **later**~~, mice~~ were tested individually by being placed in the centre of the maze facing an open arm**.** ~~and their~~ **The** spontaneous activity **of mice** was automatically recorded

for 5 min during which the experimenter left the room. After each experiment the apparatus was cleaned with 10% denatured ethanol allowing some time for evaporation before testing the following mouse.

In protocol 2 mice were selected randomly and assigned to one of the following groups: VEH/VEH (vehicle/vehicle) (n=12), FMZ/VEH (Flumazenil 10 mg/kg/vehicle) (n=10), VEH/DF 2 (vehicle/Docosanyl Ferulate 2 mg/kg) (n=12), FMZ/DF (Flumazenil 10 mg/kg/Docosanyl Ferulate 2 mg/kg) (n=16). After 15 min from pre-treatment (VEH or FMZ), mice were administered VEH or DF 2~~. After~~ **and** 30 min **later** mice were tested following protocol 1.

~~Time spent in the open arms and number of entries in the open arms were recorded.~~ **In agreement with Lister (1987) and Pellow et al. (1986), data were calculated as the percentage of time spent on the open arms (time on open arms divided by time on open arms + time in closed arms) and, in agreement with Rodgers and Johnson (1995), as the percentage of closed arm entries (number of entries into open arms divided by number of entries into open arms + number of entries into closed arms).** The analysis was performed by an experimenter blind to treatments on the videos recorded during the tests. Mice that fell from the maze have been discarded from the analysis.

Motor Coordination

Motor Coordination has been measured with the Static Rods test according to Deacon (2013) with minor changes. In particular, we tested motor coordination in 4 static rods of progressively narrower diameters (25, 20, 15, and 10 mm). Mice were selected randomly and assigned to one of the following groups: ~~CG~~**VEH** (n=11), DF 0.05 (n=9), DF 0.25 (n=10), DF 2 (n=10), DZP (n=9). Vehicle or drugs were administered and 30 min later each mouse was tested individually following Deacon (2013).

Novel Object Recognition

The effects of DF on antegrade memory have been evaluated by the NOR test according to Costa et al. (2014). Mice were selected randomly and assigned to one of the groups: ~~CG~~**VEH** (n=9), DF 0.05 (n=8), DF 0.25 (n=9), DF 2 (n=9), DZP (n=8). Mice of ~~CG~~**VEH**, DF or DZP groups were administered 30 min before the acquisition phase. On the test day, the time spent exploring the novel and the familiar object were recorded and subsequently analysed in blind. Data are expressed as time spent exploring the novel object out of total exploring (novel + familiar) time.

Place conditioning

The apparatus consisted of two rectangular Plexiglas boxes (48L x 20W x 30H cm) separated by a guillotine door, placed in a sound-proof room with a constant light of 37.5 Lux (ELD 9010 Luxmeter, Eldes Instruments, Italy) provided by a 40W lamp placed above each compartment. Different visual and tactile cues distinguished the two compartments: vertically striped black and white walls and

white smooth floor for one compartment (A), and horizontally striped black and grey walls and fine grid floor for the other compartment (B). The spontaneous preference was randomly distributed between compartments (55% for compartment A and 45% for compartment B) (one-way ANOVA: $F_{(3.36)}$=0.32). Experiment consisted of three phases. During the first phase (pre-test, day 1), the guillotine door was kept raised and each mouse was placed randomly in one compartment and given access to both compartments of the apparatus for 15 min (900 sec.). The time spent in one compartment was recorded and taken as indication of spontaneous preference. During the second phase (conditioning, days 2-5), mice of the experimental groups ~~CG~~**VEH** (n=10), DF 0.05 (n=10), DF 0.25 (n=10)~~,~~ **and** DF 2 (n=10) were administered either vehicle or DF and returned to their home cages for 30 minutes. At the end of this period mice were exposed for 30 min to the given compartment. On the same day, 8 h later, mice of all groups were administered vehicle and, after 30 min, exposed to the opposite compartment. The sequence of administrations of mice of DF groups was alternated in the following days so that on consecutive days mice did never receive DF and vehicle administrations in the same order. During the third phase (post-conditioning test, day 6), 24 h after the last conditioning session, the guillotine door was kept raised and the time spent by each mouse in the drug-paired compartment out of 15 min was recorded. The conditions of the post-conditioning test were identical to those of the pre-conditioning test. Performances at the pre- and post-conditioning tests were videotaped and subsequently analysed in blind. A statistically significant difference between the time spent during pre- and post-conditioning tests (side preference shift) of the drug group with respect to that of the vehicle group was taken as indication of the development of place conditioning.

Loss of righting reflex

The interactions between ethanol and DF or DZP were tested through the evaluation of the ethanol-induced LORR in 88 adult CD1 mice **following Correa et al. (2001) with some modifications and Slater et al. (2016)**. Mice were casually selected and assigned to the experimental **following** groups**:** ~~CG~~**VEH** (n=17), DF 0.05 (n=19), DF 0.25 (n=22), DF 2 (n=18)~~,~~ **and** DZP (n=12). After habituation in the experimental room, mice were administered DF or vehicle and put back in their home cage for 25 min. At this time, ethanol (3 g/kg) was administered, and mice were placed individually in an empty **plexiglass** cage in order to be evaluated. The time necessary to lose the righting reflex after ethanol administration was measured and considered as "latency" (max 20 min). If ethanol succeeded in inducing the LORR, the animal was instantly placed supine on a V-shape plastic apparatus **(with the two faces forming a 45° angle)** (4 x 4 x 10 cm, H x W x L) ~~with the two faces forming a 45° angle~~. Each mouse was carefully monitored and the length of the LORR was measured (max 300 sec). The effect was considered over if the mouse raised its back and touched the V-shape apparatus with its paws. The percentage of animals in which ethanol succeeded in inducing the LORR was also measured.

Statistical analyses

The statistical analyses were performed using StatSoft (v. 8.0, StatSoft Inc., Tulsa (OK), USA). One-way ANOVA, followed by Newman-Keuls post-hoc test, was applied in the EPM~~,~~ **and** the NOR **tests to determine significant effects of treatments with DF or DZP and to verify the absence of statistical differences among the spontaneous preferences in the Place Conditioning experiments** ~~and, the Place Conditioning tests, to determine significant effects of treatments~~. **Two-way ANOVA, followed by Duncan's post-hoc test, was used, in agreement with Gonzalez et al. (1996), to verify the effects of pre-treatment (FLM) and treatment (DF) and their interaction in the EPM tests; repeated measures two-way ANOVA was applied on the Place Conditioning experiments to assess the effects of treatment.** Non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparisons, and Fisher's exact test were applied in the Static Rods and LORR tests. Effects were considered statistically significant when $p<0.05$.

Results

DF exerts DZP-like anxiolytic effects blocked by Flumazenil

Fig. 2A represents the time spent in open arms during the EPM test. One-way ANOVA revealed a significant effect of treatment ($F_{(4, 61)}$=~~8.397~~**12,51**, p<0.0001). Post-hoc analysis using Newman-Keuls multiple comparison test revealed that DF, at 0.25 and 2 mg/kg, significantly **and dose-dependently** increases the time spent in open arms, in comparison to vehicle ~~(CG~~**VEH**~~)~~ **and DF 0.05 groups**. Also, as expected, DZP significantly increases the time spent in open arms in comparison to ~~CG~~**VEH group**. Interestingly, **Newman-Keuls test also revealed that** DF ~~0.25 and~~ 2 did not differ significantly from DZP group on this measure. Fig. 2B represents the **effects of treatments on** number of entries in the open arms. One-way ANOVA revealed a significant effect of treatment ($F_{(4, 61)}$=**6.41**~~10.66~~, p<0.0001). Post-hoc analysis using Newman-Keuls multiple comparison test revealed that DF **0.25 and** 2 significantly increases the time spent in open arms, in comparison to ~~CG~~**VEH and DF 0.05. Intriguingly, both doses did not differ from DZP group (p>0.05).** ~~On this parameter, DZP-administration determines statistically significant effects compared to each group.~~ Fig. 2C **represents the number of entries in closed arms. One-way ANOVA failed to reveal any significant effect of treatment. Figs.**~~and~~ 2D **and 2E** show the effects of **pre-treatment with** FMZ on DF 2-dependent time spent in open arms and number of entries in open arms, respectively. **Two-way ANOVA revealed significant effects of pre-treatment ($F_{time(1,46)}$=7.14, p<0.0001; $F_{entries(1,46)}$=7.41, p<0.0001) and treatment ($F_{time(1,46)}$=6.02, p<0.0001; $F_{entries(1,46)}$=22.45, p<0.0001) and a significant pre-treatment by treatment interaction ($F_{entries(1.46)}$=5.03, p<0.0001); Duncan's post-hoc test revealed that pre-treatment with FMZ fully reversed the anxiolytic effect of DF (p<0.05 for FMZ/DF vs VEH/DF on both time and entries). Two-way ANOVA, moreover, failed to reveal any significant effect of pre-treatment or treatment on number of closed arms entries (Fig. 2F).** ~~One-way ANOVA revealed a significant effect of treatment (2C: $F_{(3,46)}$=12.53, p<0.0001; 2D: $F_{(3,46)}$=4.287, p<0.01). Post-hoc analysis using Newman-Keuls multiple comparison test revealed that pre-treatment with FMZ significantly prevents the effect of DF 2 on both parameters.~~

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~~**Fig. 2:** Effects of DF and DZP (A-B) and effects of pre- treatment with FMZ on the anxiolytic effects of DF 2 (C-D) on the EPM. **A:** time in open arms, expressed as % of CG group (100%). **B:** number of entries in open arms, expressed as % of CG group (100%). **C:** time in open arms, expressed as % of VEH/VEH group (100%). **D:** number of entries in open arms, expressed as % of VEH/VEH group~~

~~(100%). Values are expressed as mean + SEM. **A-B:** §p<0.05 vs CG; *p<0.05 vs DF 0.05; °p<0.05 vs DF 0.25; #p<0.05 vs DF 2. **C-D:** §p<0.05 vs VEH/VEH; ±p<0.05 vs FMZ/VEH; °p<0.05 vs DF 2.~~

Unlike DZP, DF does not impair motor coordination

Fig. 3 shows the effects of treatment with DF and DZP on Orienting (A) and Total Transit (B) Time at the Static Rods test. Non-parametric Kruskal-Wallis test revealed that the treatments have a significant effect on both parameters on the 25, 20, and 15 mm diameter rods ($p<0.05$). Post-hoc analysis using Dunn's multiple comparison test revealed that DF at all doses tested is devoid of significant effect on performances on each rod ($p>0.05$) as compared with ~~CG~~**VEH** group; in contrast, DZP, compared with ~~CG~~**VEH** group, significantly increases orienting time on the 25, 20, and 10 mm rods ($p<0.05$) and total transit time on the 25 and 20 mm rods ($p<0.05$).

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~~**Fig. 3:** Effects of DF on motor coordination evaluated on four progressively narrower horizontal static rods (diameter= 25, 20, 15 and 10 mm). **A:** time (sec) spent by mice orienting themselves of 180° from initial position. **B:** time (sec) of orientation plus time spent to cross the rod. Values are expressed as mean + SEM. §p<0.05 vs CG**VEH**. ±p<0.05 vs DF 0.05. °p<0.05 vs DF 0.25. #p<0.05 vs DF 2.~~

Unlike DZP, DF does not impair mnemonic performances

Fig. 4 shows the effects of the treatment with DF and DZP on the performance of mice at the NOR test. ~~One~~**Two**-way ANOVA revealed a significant effect of **object ($F_{(4,77)}$=137,76; p<0.0001) but not** treatment ($F_{(4,77}$~~$_{39)}$~~=**0.001**~~8.852, p<0.0005~~) ~~on the time spent exploring the novel object.~~**and a significant treatment by object interaction ($F_{(4,77)}$=13,55; p<0.0001).** Post-hoc analysis using Newman-Keuls multiple comparison test revealed that **VEH- and DF-treated groups spent significantly more time exploring the novel object in comparison to the familiar objects (p<0.05). Accordingly, novel object exploration time of DZP-treated group was significantly lower in comparison to VEH- and to DF-treated groups (p<0.05).** ~~DZP significantly reduces this parameter (p<0.05 vs CG and vs DF at all doses) but, conversely, disclosed no significant differences between DF, at all doses tested, and CG group (p>0.05).~~

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~~**Fig. 4:** Effects of DF and DZP at the NOR test. The histograms represent the time (expressed as % of total exploration time) exploring the familiar and the novel object. Values are expressed as mean + SEM. §p<0.05 vs CG**VEH**; *p<0.05 vs DF 0.05; $^{\circ}$p<0.05 vs DF 0.25; $^{\#}$p<0.05 vs DF 2.~~

DF fails to elicit Place Conditioning

Fig. 5 shows the time spent in the drug-associated compartment by mice ~~treated with vehicle (CG~~**of the VEH-**~~) or~~ **and** DF (0.05, 0.25, and 2 mg/kg)**-treated groups** during conditioning. ~~One~~**Repeated measures two-**way ANOVA revealed that treatment with DF, at every dose tested, is devoid of significant effects on place conditioning (p>0.05).

~~*Figure 5 has been removed from here*~~

Unlike DZP, DF fails to potentiate ethanol-induced LORR

Fig. 6 shows the effects of treatment with DF and DZP on duration of (A) and latency to (B) ethanol (3 g/kg)-induced LORR. Non-parametric Kruskal-Wallis test revealed that medians are significantly different (p<0.001). Post-hoc analysis using Dunn's multiple comparison test indicates that treatment with DF, at every dose tested, has no significant effect with respect to the ~~CG~~**VEH** group (p>0.05). In contrast, DZP significantly increased the time of**,** and decreased latency to**,** LORR with respect to ~~CG~~**VEH** and DF 0.05, 0.25, and 2 mg/kg-treated groups (p<0.05). Moreover, Fig. 6C shows that in 100% of DZP-treated mice ethanol succeeded in potentiating the LORR (p<0.05), while the percentage of DF-treated mice does not statistically differ from that of ~~CG~~**VEH** (p>0.05).

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~~**Fig. 6:** Effects of DF and DZP on ethanol-induced LORR. A: duration (sec) of LORR. B: time (min) to LORR (latency). C: proportion of subjects in which ethanol induced/did not induce LORR, expressed as % of the total number of mice. Values are expressed as mean + SEM. §p<0.05 vs CG; *p<0.05 vs DF 0.05; $^{\circ}$p<0.05 vs DF 0.25; $^{\#}$p<0.05 vs DF 2.~~

Discussion

Evidence of anxiolytic properties and of a GABA mimetic profile of *WS* extracts is consistently present in the literature (Bassareo et al., 2019; Bhattarai et al., 2009; Mehta et al., 1991; Orrù et al., 2013), although no individual constituents of *WS* have yet been recognised as responsible of these effects. The present study provides the first behavioral pharmacological characterization of DF, a long alkyl chain ferulic acid ester recently isolated from *WS* and found able to enhance the $GABA_AR$ inhibitory postsynaptic currents in rat hippocampal acute slices with an IC50 value of 7.9 μM (Sonar et al., 2019). The present multilevel behavioral evaluation was conducted *in vivo* in male CD-1 mice, in order ascertain whether DF is endowed with an anxiolytic profile.

The results at the EPM test disclose that DF exerts anxiolytic effects that appear clearly mediated through DF's modulation of the $GABA_AR$ complex activity by interacting with the BDZ binding site since the BDZ competitive antagonist, FMZ (10 mg/kg), completely blocks DF's effects at the EPM (Fig. 2). **Moreover, since the number of closed arms entries in this model measures the effects of treatments on locomotor activity and exploration (Rodgers and Johnson, 1995), the present results also reveal that ~~indeed~~ DF is devoid of both ~~locomotor~~ inhibitory or stimulatory properties on this behavioral component, thus further pointing out its mere anxiolytic action as revealed in the EPM paradigm.**

Remarkably, BZDs are known to carry negative side-effects on motor coordination, cognition and motivation (Roth et al. 1984; Tan et al. 2011). Hence, we also extended our investigations on DF pharmacological profile to assess whether**,** at ~~the~~ **anxiolytic-exerting** doses ~~at which it exerts marked anxiolytic activity~~, it could also carry the typical BZD's adverse effects. Strikingly, we found that DF, unlike DZP, lacks of the property of impairing motor coordination (Fig. 3)~~,~~ **and** anterograde memory (Fig. 4)**.** ~~and of eliciting place conditioning (Fig. 5)~~. Moreover, also in contrast with DZP's ability to **exert reinforcing properties in the place conditioning procedure (Acquas et al. 1989; Spiraky et al. 1895; Tzschentke, 1998) and to** potentiate the ethanol-induced LORR **(Fig. 6)**, DF at full anxiolytic doses fails **to elicit place conditioning (Fig. 5) and** to enhance ethanol's depressant properties at this assay (Fig. 6). **In the present study, in application of the 3R principle and based on previous literature (Acquas et al. 1989; Spiraky et al. 1895, Tzschentke, 1998) we did not repeat the DZP groups in the place conditioning experiments.**

A possible explanation to interpret the differences between DF and DZP, could be that in spite of their common site of action at the BDZ site of the $GABA_AR$, DF interacts with $GABA_AR$ whose subunits have a different stoichiometry than those responsible of mediating the adverse effects of DZP. In this regard, previous pharmacological and behavioural studies found a correlation between BZDs' effects and $GABA_AR$ α subunit isoforms (Tan et al., 2011), with the outcome of the anxiolytic effect seemingly being mediated mostly by α_2-containing $GABA_ARs$ (Löw et al., 2000). Accordingly,

we speculate that DF may exert its keen anxiolytic properties by selectively binding to α_2-containing $GABA_AR$s, thus avoiding undesired side effects mediated by other α $GABA_AR$s subunits (Biggio et al., 2001). ~~To validate this hypothesis~~ **F**~~f~~urther **studies, in particular including** ~~with~~ **a model of** ~~conditioned~~ **Fear Conditioning (Curzon et al. 2009) and other models of anxiety (Bailey et al. 2009) will also contribute to validate this hypothesis. Moreover,** functional and electrophysiological studies will **also** have to be performed perhaps ~~also~~ in order to **explain** ~~understand the reason~~ why DF, unlike DZP, fails to potentiate ethanol's depressant activity (LORR).

In conclusion, this study points out, for the first time, a single possible $GABA_AR$/BZD-acting effector of the anxiolytic properties of *WS* and, also, suggests the possibility of ferulic acid esters to efficiently interact with the $GABA_AR$ to induce BDZs'-like anxiolytic effects. Overall, DF shows a promising pharmacological profile worth of future studies toward its suggestion as safe, selective and anxiolytic compound devoid of critical side effects that could reduce its compliance and manageability.

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482

Figures and Figures' legends

Figure 1

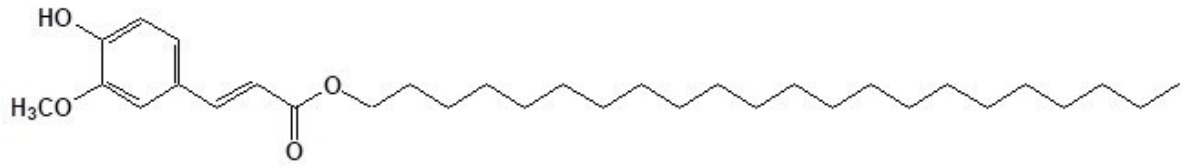

Fig. 1: Chemical structure of Docosanyl Ferulate, bioactive secondary metabolite isolated from a methanolic extract of the roots of WS (Sonar et al. 2019).

Figure 2

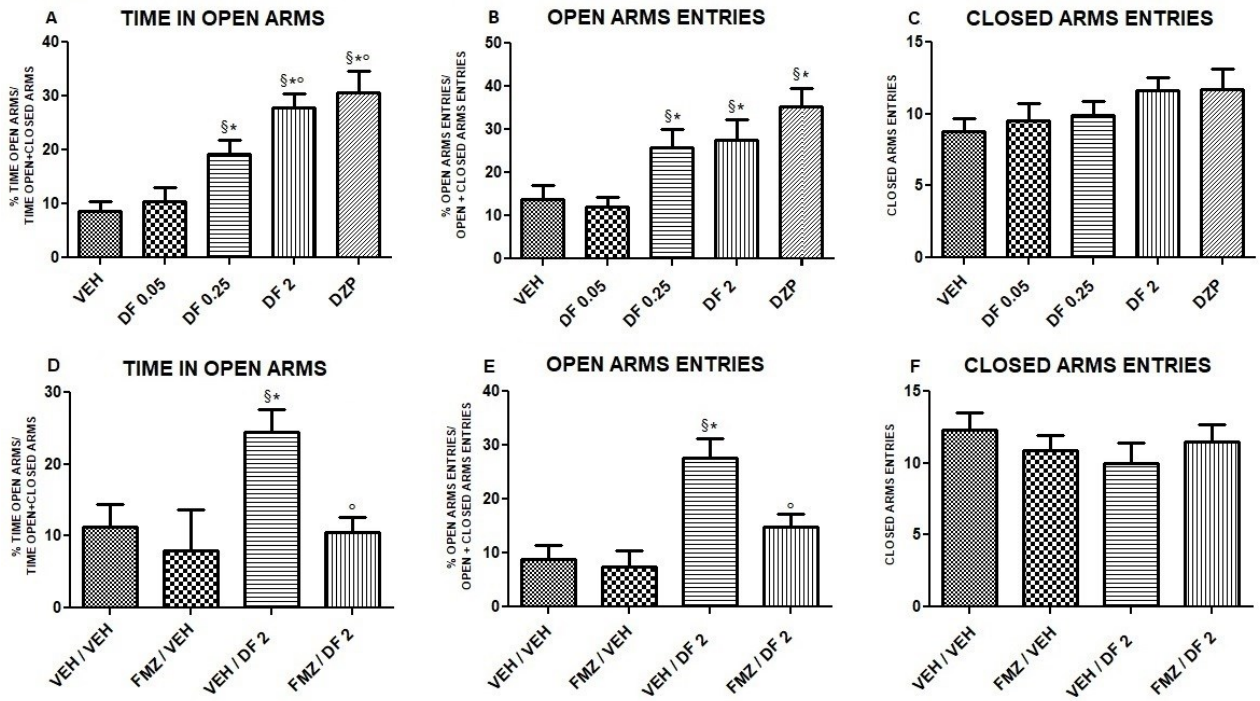

Fig. 2: Effects of treatment with DF and DZP (A-C) and effects of pre- treatment with FMZ on the effects of DF 2 mg/kg (D-F) on the EPM. A and D: % time in open arms (time in open arms divided by time in open arms + time in closed arms), expressed as % of VEH group (100%). B and E: % number of entries in open arms(entries in open arms divided by entries in open arms + entries in closed arms), expressed as % of VEH group. C and F: number of entries in closed arms. Values are expressed as mean + SEM. A-B: $^{\S}p<0.05$ vs VEH; $^{*}p<0.05$ vs DF 0.05; $^{\circ}p<0.05$ vs DF 0.25; D-E: $^{\S}p<0.05$ vs VEH/VEH; $^{*}p<0.05$ vs FMZ/VEH; $^{\circ}p<0.05$ vs DF 2.

Figure 3

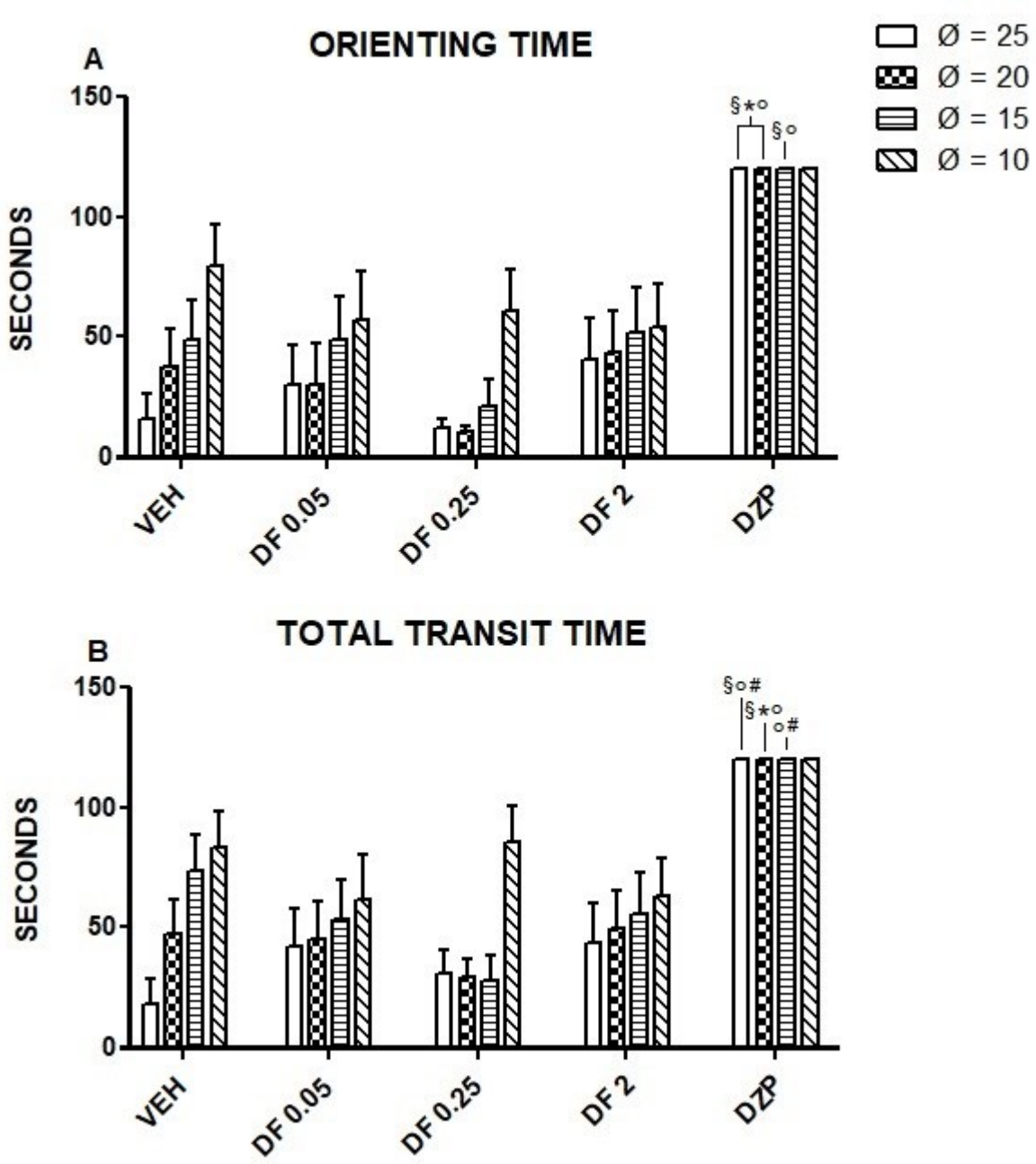

Fig. 3: Effects of DF on motor coordination evaluated on four progressively narrower horizontal static rods (diameter= 25, 20, 15 and 10 mm). A: time (sec) spent by mice orienting themselves of 180° from initial position. B: time (sec) of orientation plus time spent to cross the rod. Values are expressed as mean + SEM. $^{\S}p<0.05$ vs VEH. $^{*}p<0.05$ vs DF 0.05. $^{\circ}p<0.05$ vs DF 0.25. $^{\#}p<0.05$ vs DF 2.

Figure 4

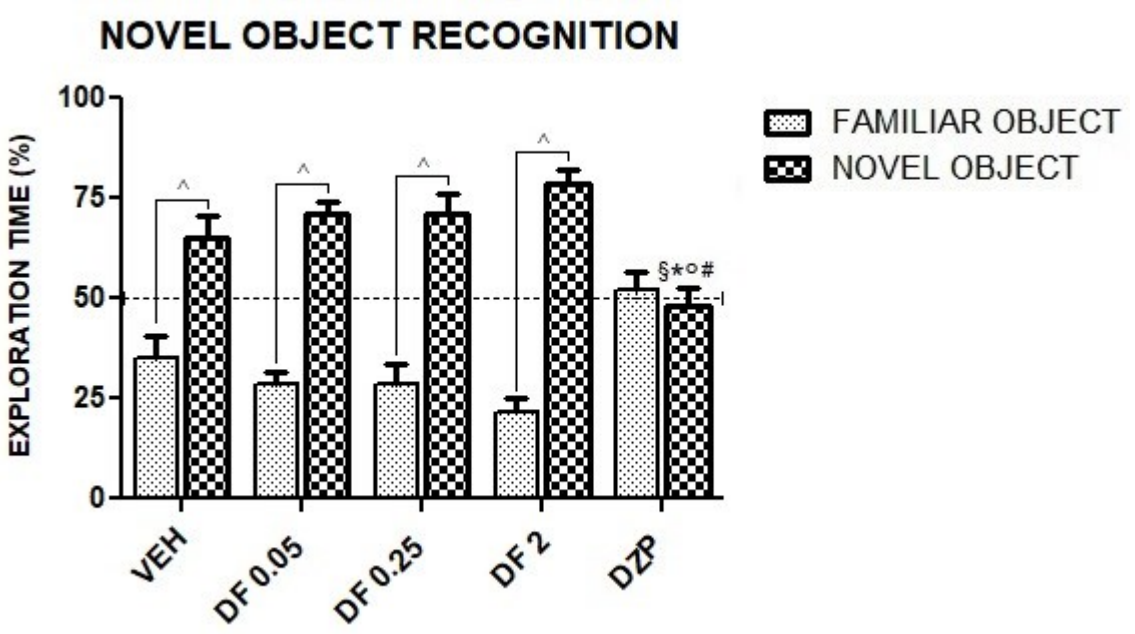

Fig. 4: Effects of DF and DZP at the NOR test. The histograms represent the time (expressed as % of total exploration time) exploring the familiar and the novel object. Values are expressed as mean + SEM. $^{\wedge}p<0.05$ vs familiar object; $^{\S}p<0.05$ vs VEH; $^{*}p<0.05$ vs DF 0.05; $^{\circ}p<0.05$ vs DF 0.25; $^{\#}p<0.05$ vs DF 2.

Figure 5

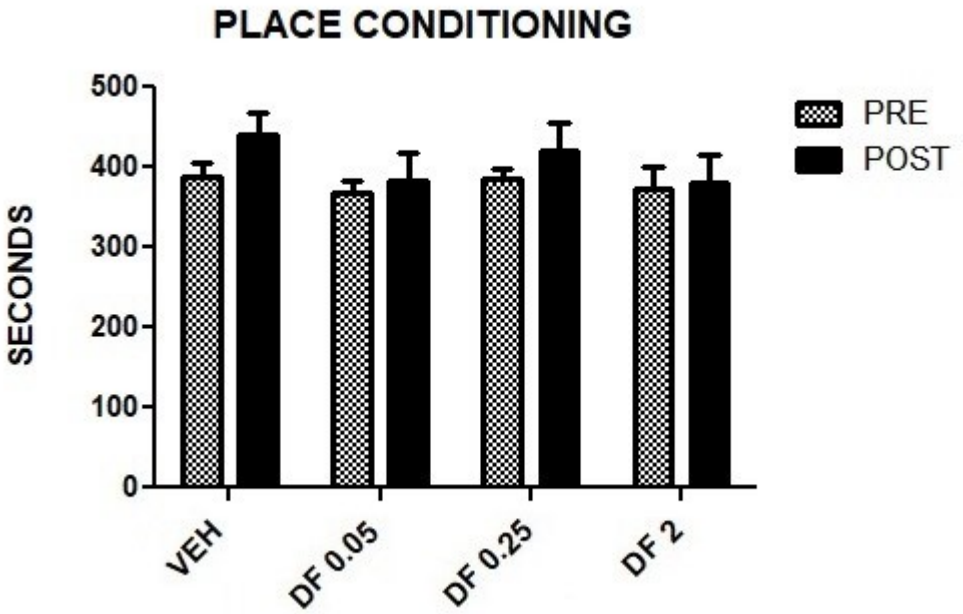

Fig. 5: Effects of DF on place conditioning. Histograms represent the time (sec) in the drug-paired compartment, before (pre) and after (post) conditioning. Values are expressed as mean + SEM.

Figure 6

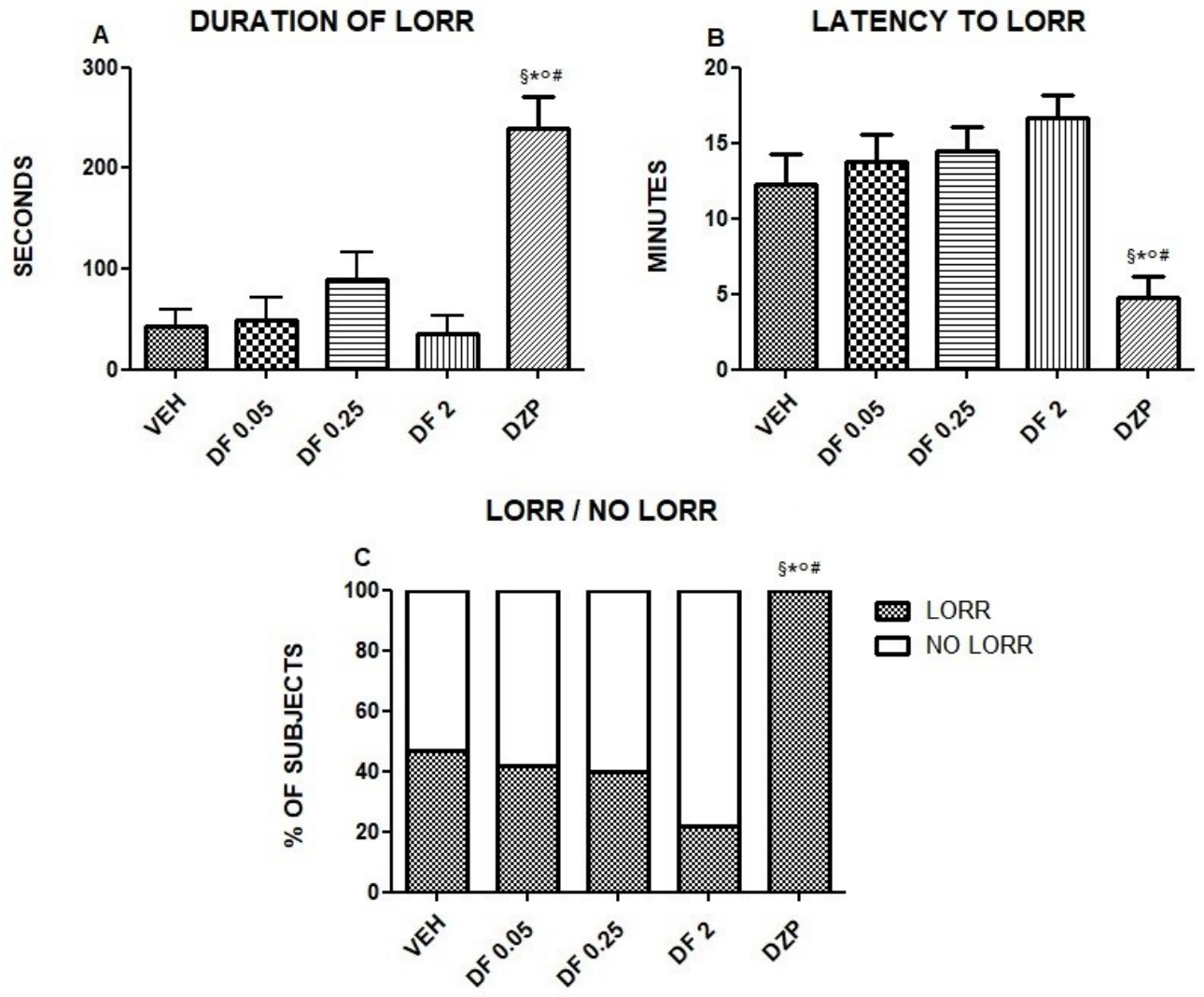

Fig. 6: Effects of DF and DZP on ethanol-induced LORR. A: duration (sec) of LORR. B: time (min) to LORR (latency). C: proportion of subjects in which ethanol induced/did not induce LORR, expressed as % of the total number of mice. Values are expressed as mean + SEM. $^{\S}p<0.05$ vs VEH; $^{*}p<0.05$ vs DF 0.05; $^{\circ}p<0.05$ vs DF 0.25; $^{\#}p<0.05$ vs DF 2.