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Broad-spectrum *Cannabis* oil alleviates behavioral symptoms associated with stress-related anxiety and depression in mice

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Running title: Cannabis oil for mood disorders

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Abstract

Background: Posttraumatic stress disorder (PTSD) is a psychiatric condition that manifests through a broad range of symptoms and shares several phenotypes with anxiety and depression. Refractory PTSD affects 10 – 30% of the patients and highlights the need for alternative pharmacotherapy. The suggested involvement of the endocannabinoid system (ECS) with the emotional processes has enlightened the use of *Cannabis* sp. Then, this study aimed to evaluate the therapeutic effects of a broad-spectrum Cannabis oil on anxiety- and depressive-like behaviors triggered by stressors from combined nature. In addition, this study investigated the effect of the oil on central cannabinoid receptor 1 and serum levels of cytokines, chemokines, and growth factors. **Methods:** Mice were randomized into five groups (vehicle; *Cannabis* oil; fluoxetine; single oral dose) and submitted to acute restraint and chronic unpredictable stress. Then, they were behaviorally assessed in the elevated plus-maze test (EPMT), forced swimming test (FST), splash test (ST), and open field test (OFT). The tetrad cannabinoid assay evaluated the central effect of the oil. Serum biomarkers levels were measured by a multiplex bead-based assay. **Results:** *Cannabis* oil (0.1 mg/kg, p.o.) significantly reduced the anxiety-like behavior in EPMT in the acute restraint stress model ($p < 0.05$) as compared to vehicle. Moreover, compared to the vehicle, *Cannabis* oil significantly reverted the despair and anhedonic-like behaviors in FST ($p < 0.05$) and ST ($p < 0.05$), respectively, in chronically stressed mice. Yet, compared to vehicle, therapy with *Cannabis* oil did not induce cannabinoid-tetrad ($p < 0.0001$); downregulated granulocyte-macrophage colony-stimulating factor (GM-CSF; $p < 0.01$) and advanced glycation end-products (RAGE; $p < 0.0001$); and upregulated vascular endothelial growth factor (VEGF; $p < 0.01$) serum levels. **Conclusion:** Altogether, our data suggest the potential of the broad-spectrum *Cannabis* oil to improve symptoms related to anxiety and depression caused by traumatic events.

Keywords: anxiety; broad-spectrum *Cannabis* oil; *Cannabis* sp.; depression; posttraumatic stress disorder.

1. Introduction

Globally, the knowledge of the real prevalence of posttraumatic stress disorder (PTSD) is unclear.[1] It has been estimated that 61% to 80% of individuals are going to experience some type of traumatic event during their lifetime.[2] From those individuals, approximately 5% to 10% will develop PTSD.[3] This psychiatric ailment affects the self and social functions of individuals by a broad range of symptoms, including the involvement of cognition, emotion, and mood.[4] In these people, PTSD is frequently comorbid with depression and anxiety. According to the National Epidemiologic Survey on Alcohol and Related Conditions, 59% and 35.2% of those who met the criteria for PTSD also met the criteria for anxiety and depression, respectively.[5]

Although there are significant proportions of people suffering from PTSD and its comorbidities, such as depression and anxiety, the molecular mechanisms underlying the pathophysiology are still poorly understood. The current knowledge suggests alterations in neurogenesis, neurohormonal, and neurotransmitter functioning.[6] Another aspect that has attracted attention is the probable involvement of the immune system and its dysregulation in PTSD[7-9] and PTSD-related disorders – anxiety and depression.[10] Then, a better understanding of the molecular basis of PTSD is worth for future development of diagnosis, prognosis, and therapeutic strategies.

Thinking about new pharmacotherapy options is particularly important if considering the challenges faced by patients with the available drugs. To date, the US Food and Drug Administration (FDA) has only approved two selective serotonin reuptake inhibitors for the treatment of PTSD: sertraline and paroxetine.[11] Off-label pharmacotherapy includes fluoxetine and venlafaxine.[12] However, 10 – 30% of the patients are still refractory to the conventional prescriptions,[13] highlighting the demand for alternative strategies that are safe and have good tolerability. In this scenario, the endocannabinoid system (ECS) has emerged

as a promising pharmaceutical target for the modulation of the synaptic transmission involved with cognition, stress responses, and emotional stability,[14] besides its connection with the immune system and neurogenesis.[15] Consequently, the therapeutic role of *Cannabis* sp. has attracted more attention.

Among the more than 400 compounds identified in *Cannabis* sp. so far, delta-9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD) are the most studied phytocannabinoids. The psychomimetic effects of *Cannabis* sp. have been attributed mainly to Δ9-THC, which is an agonist for central cannabinoid receptor type 1 (CB1R). CBD, instead, does not elicit euphoria.[16] Indeed, CBD is known for its pharmacological properties, which include analgesic and anti-inflammatory actions.[17] Additionally, this phytocannabinoid has proved to have neuroprotective, anxiolytic, antipsychotic, antiemetic, and antioxidant properties through a multi-target mechanism.[18-21] Despite all the pieces of evidence suggesting the effectiveness of *Cannabis* sp. on psychiatric disorders, the FDA has only approved this compound for children who suffer from Lennox-Gastaut Syndrome and Dravet syndromes.[22,23] It is worth mentioning that the approved drugs consist of derivatives of isolated phytocannabinoids, which are way different from *Cannabis* oil. According to the extraction techniques, broad-spectrum *Cannabis* oil can be almost free of Δ9-THC but contains all the phytochemicals found in the plant, including terpenes, flavonoids, and other phytocannabinoids, such as CBD.[24] This composition is said to contribute to the synergistic effects of *Cannabis* sp. and might be an alternative treatment for complex psychiatric diseases.

Animal models are widely used to research new treatments for PTSD. It helps to comprehend the molecular basis of the disease and, consequently, identify potential targets for new drugs or even drug repositioning. Using animals in pre-clinical research is also a strategy for screening new potential drugs to treat PTSD.[25] Recently, Deslauriers *et al*.[26] have

reviewed >600 articles to examine the ability of current rodent models to probe biological and behavioral phenotypes of PTSD. The authors have evaluated several paradigms, including the restraint stress and the chronic unpredictable stress, for their efficacy in stimulating PTSD-like constructs (learned fear and extinction, avoidance, reduced motivation/reward, arousal, and cognitive deficits) in addition to biological and physiological phenotypes associated with PTSD. All the reviewed paradigms produced lasting effects on general depression- and/or anxiety measures.[26] That said, behavioral tests are methodological tools that represent the best approach to measure anxiety- and depressive-like phenotypes in a PTSD model.[27]

Thus, considering the high prevalence of PTSD; the patients' refractory to medical treatment; and the suggested therapeutic potential of *Cannabis* sp. on psychiatric illness, this study aimed to evaluate in mice the effects of a broad-spectrum *Cannabis* oil on anxiety- and depressive-like behaviors triggered by stressors from combined nature. Further, it was investigated the central effects of the oil on CB1R, as well as its influence on the serum levels of cytokines, chemokines, and growth factors.

2. Experimental Section

2.1. Broad-spectrum Cannabis oil

The broad-spectrum *Cannabis* oil was produced and analyzed by the Brazilian Association ABRACE (Associação Brasileira de Apoio Cannabis Esperança, Paraíba - Brazil) that is enrolled with the National Register of Legal Entities (CNPJ) under the number 23.877.015/0001-38. The chromatographic analysis reported a CBD:Δ9-THC proportion of 11:1 and total cannabinoids of 40.2% (Figure 1). Regarding the microbiological assessment, the oil was under the current quality parameters.

[Insert Figure 1 near here]

2.2. Animals

A total of 120 male Swiss mice (30-50 g, 50–90 days of age) were provided by the breeding unit of the Universidade Federal de Santa Catarina (UFSC). The animals (maximum of 12 mice group-housed in clear-transparent plastic cages with dust-free sawdust bedding) were housed under regulated conditions that included a 12-h-light/-dark cycle (artificial light at 07:00 a.m. to 07:00 p.m.), controlled temperature (22 ± 2 °C), and standard food and water *ad libitum*. All of the tests were conducted between 8:00 a.m. and 5:00 p.m., and the animals were acclimatized to the laboratory settings for at least 1 h before testing. Each animal was used only once throughout the experiments. They were randomly assigned before treatment or behavioral evaluation. All efforts were made to minimize their suffering and reduce the number of animals required for the experiments. The experiments herein described were reported in compliance with the ARRIVE guidelines[28-30] and approved by the Animal Ethics Committee of the UFSC (CEUA-UFSC) under protocol 7176240920. All the experimental procedures were conducted according to the guidelines of CONCEA and CEUA/UFSC, based on the 3R's principles: replacement, reduction, and refinement. A blind operator performed both the nociception assessments and the statistical analysis.

2.3. Experimental design

Broad-spectrum *Cannabis* oil (0.1, 1, 3 and 10 mg/kg) and fluoxetine (10 mg/kg), a selective inhibitor of serotonin used as the positive control, were dissolved in medium-chain triglycerides (MCT) (Vitafor®, Araçoiaba da Serra, São Paulo, Brazil) and saline, respectively, and administered by oral gavage (p.o.). Fluoxetine has been used and proved to be effective on pre-clinical models of PTSD[27,31,32] and clinical studies.[33,34]

The chronic protocol was also featured with an additional group treated continuously with *Cannabis* oil at 0.1 mg/kg (p.o.; 1x/administration; 5-day treatment). This extra group

aimed to verify if repeated treatment could result in a long-lasting anxiolytic-/antidepressant-like effect**.** All drugs were prepared right before the treatment, and the volume of the administration was 10 ml/kg, 1 h before the constraint stress or immediately after the last stressful stimulus (14-day protocol). The choice of the doses used was based on pilot experiments (Supplemental Data) or on previous data described in the literature.[35-37]

2.3.1. Acute restraint stress-induced paradigm

Five groups of randomized mice were tested through this protocol: vehicle, fluoxetine (10 mg/kg, p.o., 1x/administration), or broad-spectrum *Cannabis* oil (0.1, 3, and 10 mg/kg, p.o., 1x/administration), as shown in Figure 2A. For the acute restraint stress-induced protocol, mice were first maintained inside their home cages, with free access to water and food, during the drug administration and the period before the restriction (1 h). Then, each animal was removed from the group-house and introduced into a fenestrated punctured plastic tube (18 cm × 4 cm) placed in a horizontal, so the normal orientation of the animal's body was kept. The animal remained in that position for 7 h, with all physical movements restrained but without any pain. No food or water was offered to the animal during the 7-hour period. After release, the animals waited for 40 min before being behaviorally assessed.[38-41] The researcher was blind for the treatments, and the behavioral tests were manually scored.

2.3.2. Chronic unpredictable stress (CUS) paradigm

Five groups of randomized mice were evaluated: vehicle, fluoxetine (10 mg/kg, p.o., 1x/administration), or broad-spectrum *Cannabis* oil (0.1, 1 and 3 mg/kg, p.o., 1x/administration), as shown in Figure 2B. This protocol was also featured with an additional group that was continuously treated with *Cannabis* oil at 0.1 mg/kg (p.o.) – administered in

the last 5 days of the protocol. The stressful stimuli were designed to maximize the unpredictable nature of the stressor. Therefore, several stressors varying in duration and time were randomly applied for 14 days, and food and water were offered *ad libitum.* As soon as mice were exposed to the stressor, they returned to their home cage and were kept under laboratorial standard conditions. Mice received the aforementioned treatments on the 14th and last day of the protocol and were behaviorally assessed 24 h after the treatment.[42,43]

Briefly, the CUS paradigm consisted of exposure, once daily, to one of the following aversive stressors: **restraint** – mice were placed into a plastic tube (18 cm × 4 cm) sealed at the extremities and properly perforated to promote air circulation. This stimulus occurred on the 1st and 7th days of the protocol; **forced swimming** – mice were into a cylindrical recipient (10 cm × 25 cm) containing 19 cm of water on the 6th day of the protocol; **cold bath** – mice were placed inside a group-house with 2 cm of water on the 2nd and 9th days of the protocol; **wet wood shaving** – wood shavings were wet with 400 ml of water, and the group-house was tilted at 45° angle. This stimulus occurred on the 3rd and 12th days of the protocol; **footshock** – test took place at the passive avoidance apparatus (Insight® – Ribeirão Preto, São Paulo, Brazil). Mice were placed on the bars and received paw shocks (0.7 mA; 0.5 s/min) every 30 sec for 3 min on the 8th, 10th, and 14th days of the protocol; **tail compression** – on the 4th and 13th days, the tail compression stimulus was conducted by positioning a clothespin 1 cm from the base of the animal's tail. Figure 2B summarizes all the steps aforementioned.

[Insert Figure 2 near here]

2.4. Behavioral tests

2.4.1. Forced swimming test

The FST assessed antidepressant-like behavior and followed the method described by Porsolt *et al.*[44] Each one of the animals was placed into a transparent cylindrical tank (30 cm

× 20 cm) with 15 cm of water at a temperature of 22-25 ºC. The test was conducted for 6 min, with a habituation period of 2 min. The antidepressant-like effects of the treatments were assessed in the function of the latency to immobility.[45,46] The FST was only executed in the chronic protocol.

2.4.2. Splash test

The splash test (ST) was used to measure the anhedonia-like state. It consisted of squirting a sucrose solution (200 µl, 20%) on the dorsal coat of the animals. Because of the high viscosity of sucrose in this concentration, the animals initiate the self-cleaning behavior, a typical symptom of anhedonia used to a depression diagnosis. After squirting, the latency to grooming was recorded for 5 min as an indicator of self-care and motivational behavior.[27,47] The ST was only executed in the chronic protocol.

2.4.3. Elevated plus-maze test

The elevated plus-maze test (EPMT) assessed anxiety-like behavior in mice. The apparatus comprises two open arms (36 cm × 5 cm) and two closed arms (36 cm × 5 cm × 18 cm) that are connected to a central platform (5 cm × 5 cm) and elevated 50 cm from the ground. For the test, an animal per time was placed at the center of the platform, facing toward the closed arm, and allowed to move through the arms. The time spent in the open arms and the number of entries made into the open arms were recorded for 5 min. Subsequently, the percentage of time spent in open arms was calculated from the total spent there divided by the total time spent in both open and closed arms. The percentage of entries made into the open arms was given from the total entries made into these arms divided by the total entries made in both open and closed arms.[48]

2.4.4. Open field test

The open-field test (OFT) evaluated whether the animals had any locomotor impairment during the experimental protocols and treatments. Each one of the mice was placed in the center of an acrylic box (30 cm × 30 cm) that possess nine square areas equally divided (Insight® – Ribeirão Preto, São Paulo, Brazil). The crossing number (scored by the number of segments crossed with the four paws) was used to assess locomotor activity. The test lasted 5 min per animal, and the apparatus was cleaned with a solution of ethanol 10% after each test to avoid clues and smells from the predecessor animal.[49]

2.5. Cannabinoid-induced tetrad

The classical cannabinoid-induced tetrad is a preclinical model that evaluates the "safety-pharmacology" of new cannabinoid-related molecules.[50] The main purpose of the test was to monitor the central effects of broad-spectrum *Cannabis* oil on cannabinoids by measuring the following parameters: spontaneous locomotor activity, rectal temperature, catalepsy, and antinociception.[51] Four groups were assessed for each parameter aforementioned: vehicle (MCT); broad-spectrum *Cannabis* oil (3 or 30 mg/kg, p.o.); or WIN 55,212 (1.5 mg/kg, i.p. – a potent cannabinoid receptor agonist). Tests were conducted every 1 h, during the period of 6 h following the drug or vehicle administration.

Spontaneous locomotor activity was evaluated using the rotarod apparatus (Ugo Basile, Italy). It was fixed at a rotational speed of 4 revolutions per minute (rpm). Before the experiment, mice had been trained for 60 sec on two consecutive days. Latencies were determined in the case the animals had fallen off the apparatus.

Core temperatures were measured using a clinic digital thermometer (BD Basics, New Jersey, USA) that was lubricated with intimate gel before inserting it into the rectum to a constant depth of 3 cm.

To measure catalepsy mice were hung by their frontal paws from a plastic stem (12 cm of diameter) fixed horizontally at a height of 2-3 cm, allowing them to stay standing. This test measured the time that the animal spent moving and touching the bottom of the box. The test cut-off point was 180 sec.

To evaluate antinociception, the tail-flick apparatus with a fixed temperature of 45 °C. The test had a cut-off point of 15 sec.

2.6. Serum biomarkers

Blood was collected from each animal on the 15th and last day of CUS protocol, and serum was separated by centrifugation (5500 × g for two 15-min cycles) and stored until further analysis. Briefly, 50 µl of the sample was processed with a multiplex bead-based assay (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. The assay determined the serum levels of different cytokines, chemokines, and growth factors [for instance, monocyte chemoattractant protein-1 (MCP-1), eotaxin (EOTX), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin- (IL-) 1β, IL-4, IL-17A, IL-33, IL-2, IL-6, IL-17E, vascular endothelial growth factor (VEGF), macrophage inflammatory protein (MIP)-1α, keratinocytes-derived chemokine (KC), intercellular adhesion molecule (ICAM), and receptor for advanced glycation end products (RAGE)]. Measurements and analysis were performed by the Luminex platform (Luminex® 100/200™ System, Texas, EUA). Each multiplex immunoassay was performed in quintuplicate, and results were expressed as pg/ml serum.

2.7. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM) of 4 – 10 animals/group. A statistical comparison of the data was performed by one- or two-way

ANOVA followed by Bonferroni to multiple comparisons *post hoc* test. $P < 0.05$, 0.01, and 0.001 were considered significant. Statistical analyses were performing using GraphPad Prism 8.2.1 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Effects of single administration doses of broad-spectrum Cannabis oil on acute restraint stress-induced behaviors in the EPMT and OFT

The EPMT assessed the anxiolytic effect of the treatment with a single dose of broad-spectrum *Cannabis* oil (0.1, 3, and 10 mg/kg, p.o.) during acute restraint stress-induced behaviors. As shown in Figure 3A, the oil significantly increased the time spent in open arms at 0.1 mg/kg as compared to vehicle ($p < 0.05$). Figure 3B shows that the more entries made into the open arms by the animals from the groups treated with *Cannabis* oil were not significantly different from the vehicle ($p > 0.05$). Finally, no changes were observed in the locomotor activity ($p > 0.05$) in the OFT (Figure 3C*)*, confirming that the anxiolytic effect of *Cannabis* oil occurs independently of any motor changes. Compared to the positive group, no significant changes were observed.

[Insert Figure 3 near here]

3.2. Effects of the treatment with broad-spectrum Cannabis oil on CUS-induced behaviors

As shown in Figure 4A, the treatment with broad-spectrum *Cannabis* oil at 0.1 mg/kg significantly diminished the latency to immobility in the FST as compared to the vehicle group ($p < 0.05$), similarly to the effect seen in the fluoxetine group ($p < 0.05$). Moreover, Figure 4B showed that mice treated with *Cannabis* oil (0.1, 1, and 3 mg/kg) started grooming significantly faster than animals treated with vehicle ($p < 0.05$) in the ST. Relevantly, the OFT (Figure 4C) showed no change in the locomotor behavior of the animals ($p > 0.05$).

Regarding the EPMT (Figure 4D, E) no significant differences were observed in the time spent in the open arms ($p > 0.05$), although mice treated with *Cannabis* oil (0.1 mg/kg) significantly demonstrated more entries into the open arms ($p < 0.01$). Finally, mice under CUS protocol were continuously treated with broad-spectrum *Cannabis* oil (0.1 mg/kg) for 5 days and then behaviorally compared to vehicle and fluoxetine groups, although no significant changes were observed (data not shown).

[Insert Figure 4 near here]

3.3. Broad-spectrum Cannabis oil did not induce cannabinoid-like effects on tetrad assay

The effects of treatment with broad-spectrum *Cannabis* oil (3 and 30 mg/kg, p.o.) on locomotion, nociception, catalepsy, and body temperature are shown in Figure 5. As expected, WIN 52,212-2 (1.5 mg/kg, i.p.), a potent CB1R agonist, induced cannabinoid-like effects during tetrad assay. This agonist reduced significantly the locomotor activity ($p < 0.0001$; Figure 5A), increased the threshold sensitivity ($p < 0.0001$, Figure 5B), induced catalepsy (< 0.0001; Figure 5C), and decreased the body temperature ($p < 0.0001$; Figure 5D) as compared to the vehicle. Otherwise, no significant behavior changes were observed following the oral administration of *Cannabis* oil ($p > 0.05$).

[Insert Figure 5 near here]

3.4. Serum biomarkers

Table 1 shows the cytokines, chemokines, and growth factors levels following a single dose or continuous treatment with broad-spectrum *Cannabis* oil (0.1 mg/kg, p.o.) during CUS protocol. Serum levels of GM-CSF were significantly reduced ($p < 0.01$) when compared to vehicle, after single-dose administration. Under continuous treatment and compared to the vehicle group, serum levels of VEGF were significantly upregulated ($p <$

0.01), and RAGE levels were significantly downregulated ($p < 0.0001$). No statistically significant differences were found for the other measurements. The level of IL-4 could not be detected in the assay.

[Insert Table 1 near here]

4. Discussion

The present results showed that broad-spectrum *Cannabis* oil reduced the anxiety-like behavior triggered by the acute restraint paradigm. The oil also reverted despair and anhedonic-like behaviors triggered by unpredictable stress. Importantly, cannabinoid-like effects were not observed at the doses of 3 and 30 mg/kg of *Cannabis* oil during tetrad assay. Lastly, oral administration of the oil downregulated the serum levels of GM-CSF and RAGE, whereas VEGF serum levels were found to be upregulated.

Cannabis sativa, a botanical plant with a millenary history of medicinal use, and its phytocannabinoids have been under investigation by their potential effects on a wide range of conditions, including psychiatric illnesses.[52] Stressful episodes influence homeostasis by changing the physiological and neurobehavioral profiles throughout adaptive processes. Stress is one of the external causes of anxiety and depression, the two of the most common psychiatric illnesses, and is known for playing critical roles in the pathophysiology of these conditions.[48,53] Interestingly, the involvement of the ECS with emotional processing has gained attention in the last few years, suggesting the use of *Cannabis* sp. as a therapeutic alternative for the treatment of symptoms related to PTSD.[52]

In the first set of experiments, mice were treated with broad-spectrum *Cannabis* oil and then submitted to the acute restraint stress protocol. Substantial findings have supported this model as able to evoke PTSD-like constructs, including depressive- and anxiety-like symptoms.[26,38,53-56] Moreover, this protocol has been employed to screen the therapeutic

potential of drugs to manage mood symptoms related to PTSD since behavioral changes in mice can be monitored. Herein, we found that *Cannabis* oil (0.1 mg/kg; p.o.), rich in CBD, showed an anxiolytic effect while it did not change depressive-like symptoms. Previously, Resstel *et al.*[57] also demonstrated that a single dose of CBD (10 mg/kg, i.p.) increased the percentage of open arm entries in the EPMT of rats that have had their movements restrained. This anxiolytic action was attributed to the activation of 5-HT1A receptors. Yet, a recent study showed that mice exposed to traumatic brain injury had the anxiety- and depressive-like behaviors reestablished by a commercially available 10% CBD oil.[58] Contradicting these data and the study of Sales *et al.*[59], which demonstrated the antidepressant-like effect of CBD (10 mg/kg, i.p.) on mice in the FST, we found that *Cannabis* oil was ineffective to revert the depressive-like symptom in the FST. It is worth mentioning that those studies evaluated depressant-like symptoms triggered by other protocols as compared to ours. Yet, we are comparing the results of isolated CBD with a broad-spectrum oil, even though the pieces of evidence suggest that there is a positive contribution from the combination of phytocannabinoid and other molecules, such as terpenes, also called as "entourage effect".[60]

Otherwise, neuropsychiatric alterations related to anxiety- and depressive-like behaviors are also described as consequences of exposure to chronic unpredictable stress.[43,61,62] In the second set of experiments, different types of stressors applied to mice for 14 days significantly altered the parameters related to latency to immobility in the FST and grooming behavior in the ST. In rodents, a reduced persistence of swimming and a sucrose indifference are commonly associated with depressive-like symptoms.[39] Since we observed an improvement of the anhedonia and depressive patterns, we could interpret these data as a consequence of the treatment with broad-spectrum *Cannabis* oil. Previously, isolated CBD (10 mg/kg; i.p.) had exerted pro-hedonic effects on rats subjected to chronic unpredictable

mild stress by increasing their sucrose preference.[39] Still, other reports demonstrated that CBD isolated (30 and 200 mg/kg) significantly diminished depressive-like behavior in mice during FST.[63,64] Taken together, one may conclude that *Cannabis* and *Cannabis* derivatives reduce depressant-like behaviors in rodents in a large range of dosages.

When evaluating the potential psychoactive effects of cannabinoids, the tetrad assay is very useful to characterize their biological activity. Mainly, the cannabinoid tetrad reveals cannabimimetic effects related to those elicited by CB1R agonist Δ9-THC.[65] Our findings showed no effects of broad-spectrum *Cannabis* oil at 3 and 30 mg/kg in the tetrad assay, confirming the analytical parameters of quality attested by the supplier (CBD:Δ9-THC proportion of 11:1 and total cannabinoids of 40.2%). CBD is well known for its low affinity by CB1R and as expected, it does not activate the cannabinoid tetrad.[66] Our findings are in accordance with these previous data since the *Cannabis* oil we tested is rich in CBD, but it is not the only compound. Thus, the psychoactive effects on mood-related symptoms appear to be independent of CB1R activation, although further experiments are needed to confirm this hypothesis.

Earlier experiments have suggested the psychoactive action of phytocannabinoids, mainly CBD, throughout the 5-HT1A signaling pathway.[57,67] Besides, the pieces of evidence suggesting the anti-inflammatory properties of *Cannabis*[68,69] contributed to the increasing interest in its therapeutic potential in mood disorders. Earlier experiments have demonstrated that GM-CSF levels are downregulated with phytocannabinoids treatment.[70,71] We also found a significant decrease of GM-CSF serum levels following a single dose administration of broad-spectrum *Cannabis* oil (0.1 mg/kg, p.o.). This chemokine plays a critical role in regulating leukocyte counts,[72] of which are elevated in PTSD patients.[73,74] Another finding of our study was the significant downregulation of RAGE following continuous treatment with broad-spectrum *Cannabis* oil (0.1 mg/kg, p.o.). To the light of our current knowledge, there

are no previous reports regarding the influence of *Cannabis*, or any isolated phytocannabinoid, upon RAGE expression. This receptor is known for its ability to recognize danger-associated molecular patterns (DAMPs) that can be released on a larger scale because of psychological and physical stress.[75] A recent review showed that several DAMPs, including the high mobility group box-1 (HMGB-1), may trigger depressive-like behaviors in the stress-induced depression model.[76] HMGB-1 is a well-known ligand of RAGE and, interestingly, a prospective study had associated the high plasma levels of HMGB-1 with the more likely to develop PTSD.[77] Taken together, the accumulating data suggest the role of the HMGB-1/RAGE signaling in mood processes, and the effects observed in our study suggest that *Cannabis* may mediate immunomodulatory effects through this cellular pathway.

Last, but not least, the continuous treatment with broad-spectrum *Cannabis* oil (0.1 mg/kg, p.o.) significantly upregulated the serum levels of VEGF. In different cells and tissues, VEGF plays key roles in physiologic vascular homeostasis but is also associated with the molecular pathogenesis of tumor growth and metastasis.[78] Previously, Wheal *et al.*[79] showed the increase of the circulating levels of VEGF in ZDF diabetic rats treated with CBD, while Leishman *et al.*[80] showed a significant lowering of the mRNA encoding for VEGF in mice following acute Δ9-THC administration (3 mg/kg; i.p.). We are not aware of any studies that have investigated the effects of *Cannabis* oil, and not only isolated phytocannabinoids, on VEGF levels in stress-induced mice, thus justifying additional investigation.

5. Conclusions

In summary, our data showed the potential of broad-spectrum *Cannabis* oil to change behavioral patterns related to stress-induced anxiety- and depressive-like symptoms. Importantly, the oil did not trigger psychomimetic effects related to CB1R activation, as

shown by the tetrad assay. Moreover, our data suggest the potential of the oil to regulate biomarkers involved with inflammation and angiogenesis, although further investigations are required to confirm these hypotheses.

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Tables

Table 1. Cytokines, chemokines, and growth factors serum levels following single and continuous treatment with broad-spectrum *Cannabis* oil (0.1 mg/kg, p.o.).

Measurement	Vehicle	Fluoxetine	Single Dose	Continuous Treatment	P-value
MCP-1	281.5 (12.42)	294.8 (14.27)	340.8 (28.86)	294.9 (24.54)	0.2354
EOTX	255.7 (35.97)	249.9 (16.86)	282.1 (11.32)	256.7 (16.64)	0.7447
GM-CSF	4.525 (0.10)	4.289 (0.10)	4.018 (0.09)**	4.304 (0.08)	0.0081
IL-1β	49.76 (6.89)	51.49 (8.62)	45.40 (9.03)	44.98 (7.39)	0.9244
IL-4	ND	ND	ND	ND	-
IL-17A	7.769 (1.55)	6.971 (0.95)	10.55 (2.43)	10.37 (3.43)	0.6031
IL-33	54.86 (1.57)	47.57 (2.10)	56.12 (2.88)	52.04 (2.75)	0.0775
VEGF	12.88 (0.37)	12.77 (0.65)	13.66 (0.54)	22.26 (4.06)**	0.0075
MIP-1α	0.2138 (0.04)	0.2583 (0.08)	0.3688 (0.08)	0.3550 (0.03)	0.1943
KC	65.79 (3.41)	54.06 (2.27)	60.59 (1.82)	68.68 (6.25)	0.0590
ICAM	25450 (2138)	24210 (900.7)	20710 (1517)	22360 (1420)	0.1734
IL-2	3.630 (0.13)	3.423 (0.27)	3.718 (0.22)	3.208 (0.15)	0.3012
IL-6	3.370	4.430 (1.12)	5.810 (1.22)	7.030	0.1596
IL-17E	5.296 (5.296)	5.296 (5.296)	8.406 (5.369)	15.84 (8.925)	0.6196
RAGE	36.40 (1.97)	18.13 (2.12)*	36.62 (1.18)	20.19 (2.98)***	<0.0001

Measurements were in ng/ml. Values are reported as mean ± SEM. *$p < 0.05$, $p < 0.01$, and ***$p < 0.001$ *versus* vehicle (one-way ANOVA followed by Bonferroni *post-hoc* test). ND: not detected.

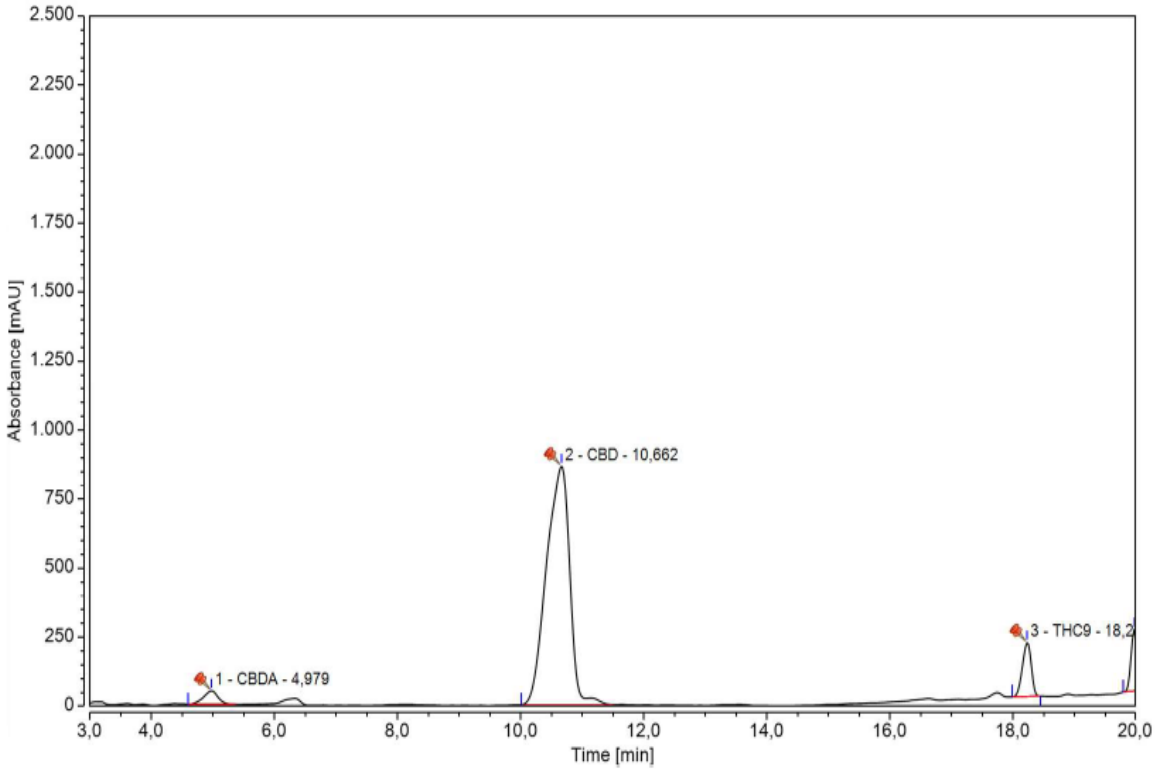

Figure 1. High-performance liquid chromatography (HPLC) analysis of broad-spectrum *Cannabis* oil. THC (retention time = 18.2 min); CBD (retention time = 10.6 min). THC: tetrahydrocannabinol; CBD: cannabidiol.

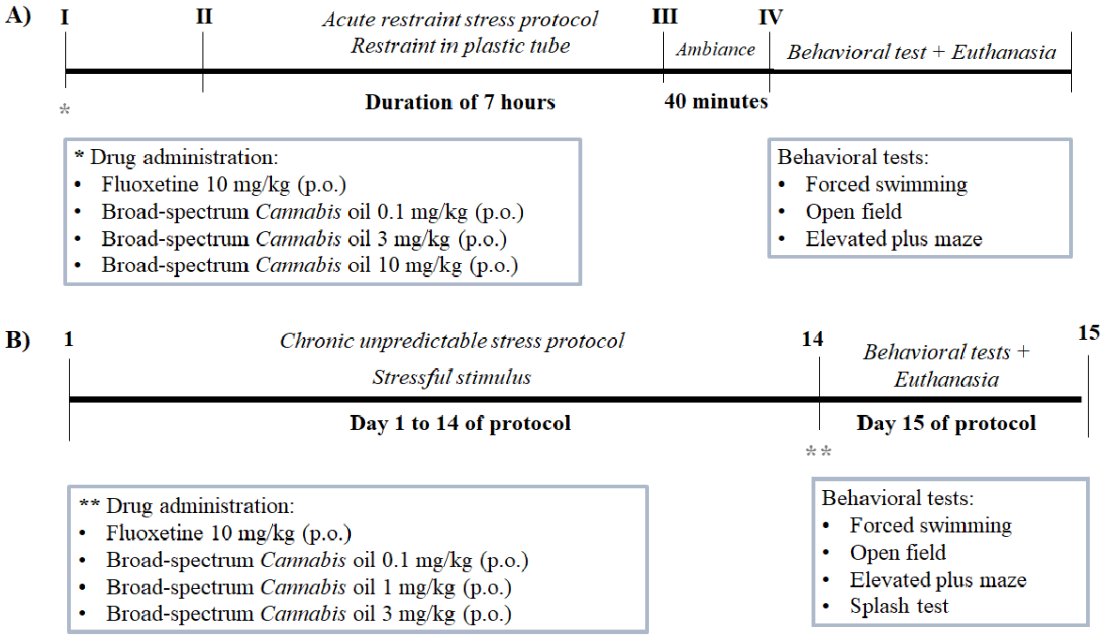

Figure 2. Experimental design. (A) The acute restraint stress-induced protocol was conducted in four different stages as illustrated above (I-IV), which included the drug administration 1 h before the restraint, the containment in tubes for 7 h, the ambiance of 40 min, and the behavioral tests followed by euthanasia. Behavioral tasks were evaluated by the elevated plus-maze and open field tests. (B) The chronic unpredictable stress (CUS) protocol, applied for 15 days, was divided into periods of stressful stimuli and behavioral tests/euthanasia. The stressful stimuli consisted of containment, forced swimming, cold bath, wet wool shavings, shock, and tail compression in alternated days and hours, always unpredictably. In this protocol, the drug was administered on the last day (14) or daily for the group under continuous treatment. Behavioral tasks were assessed by the forced swimming (FST), open field (OFT), elevated plus-maze (EPMT), and splash (ST) tests.

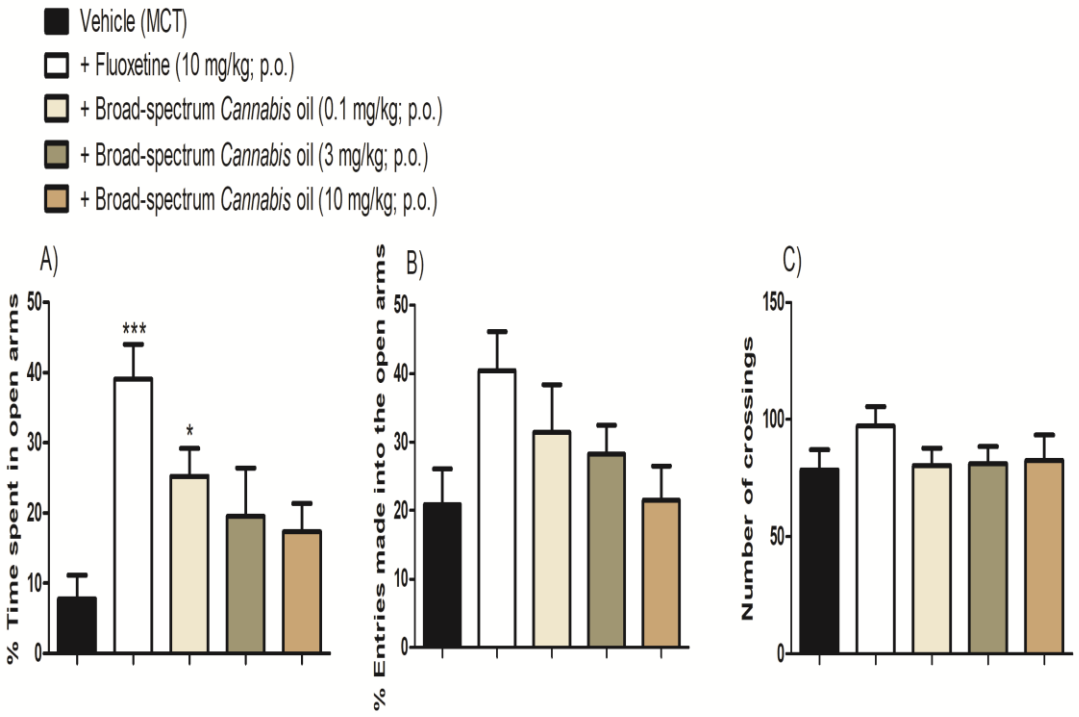

Figure 3. Effect of the treatment with broad-spectrum *Cannabis* oil (0.1, 3, and 10 mg/kg, p.o.) or fluoxetine (10 mg/kg, p.o.) on mice induced to acute restraint stress and submitted to EPMT (A and B) and OFT (C). Values are expressed as mean ± SEM of 7-10 animals *per* column. ***p < 0.001 *versus* vehicle (one-way ANOVA followed by Bonferroni *post-hoc*-test). EPMT: elevated plus-maze test; OFT: open field test.

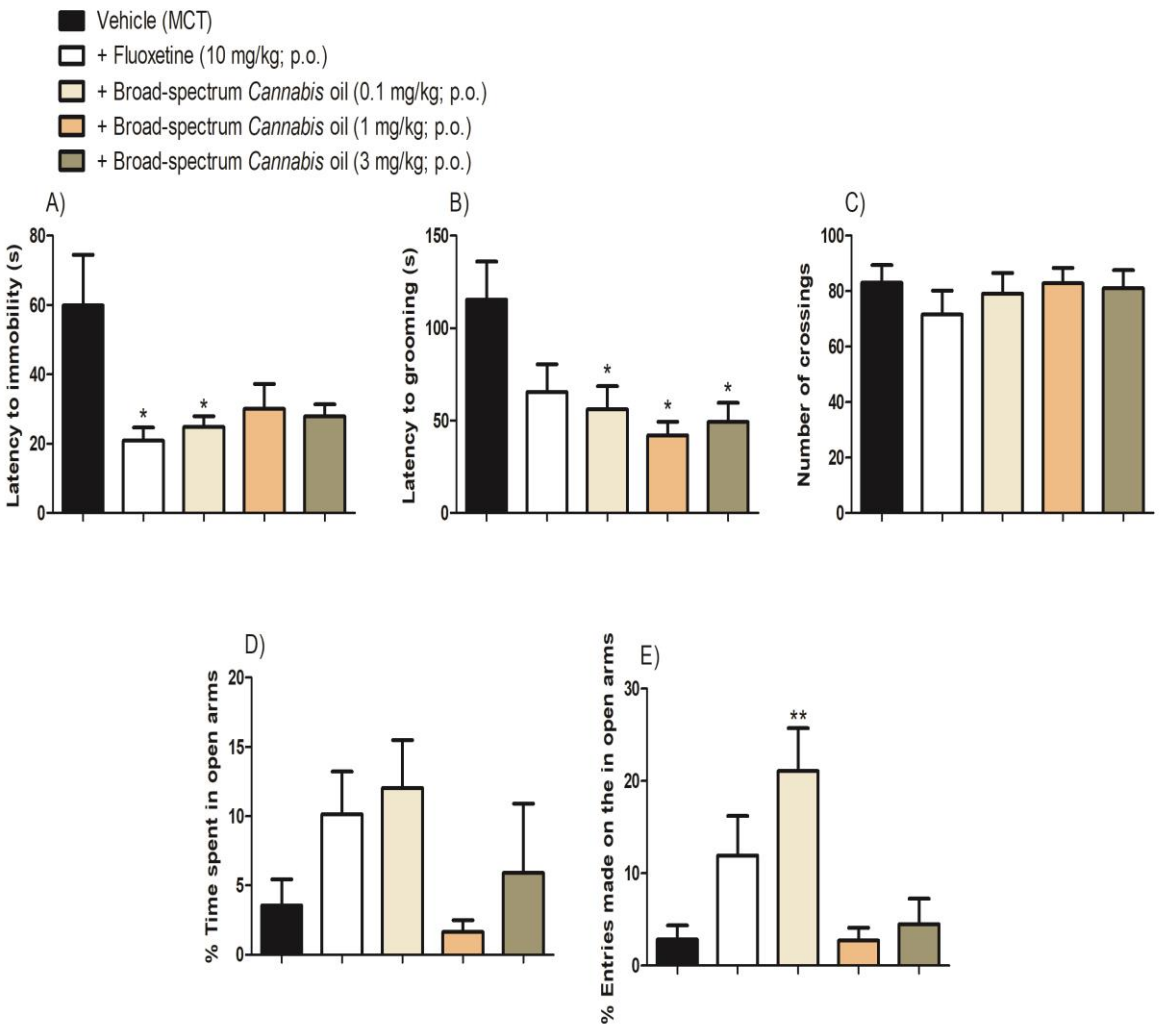

Figure 4. Effects of the treatment with broad-spectrum *Cannabis* oil (0.1, 1 and 3 mg/kg; p.o.) or fluoxetine (10 mg/kg; p.o.) on mice chronically stressed by unpredictable stressors and submitted to the FST (A), ST (B), OFT (C), and EPMT (D and E). Treatments were administered 24 h before behavioral assessments. Values are expressed as mean ± SEM of 7-10 animals *per* column. *$p < 0.05$ and **$p < 0.01$ *versus* vehicle (one-way ANOVA followed by Bonferroni *post-hoc*-test). FST: forced swimming test; ST: splash test; OFT: open field test; EPMT: elevated plus-maze test.

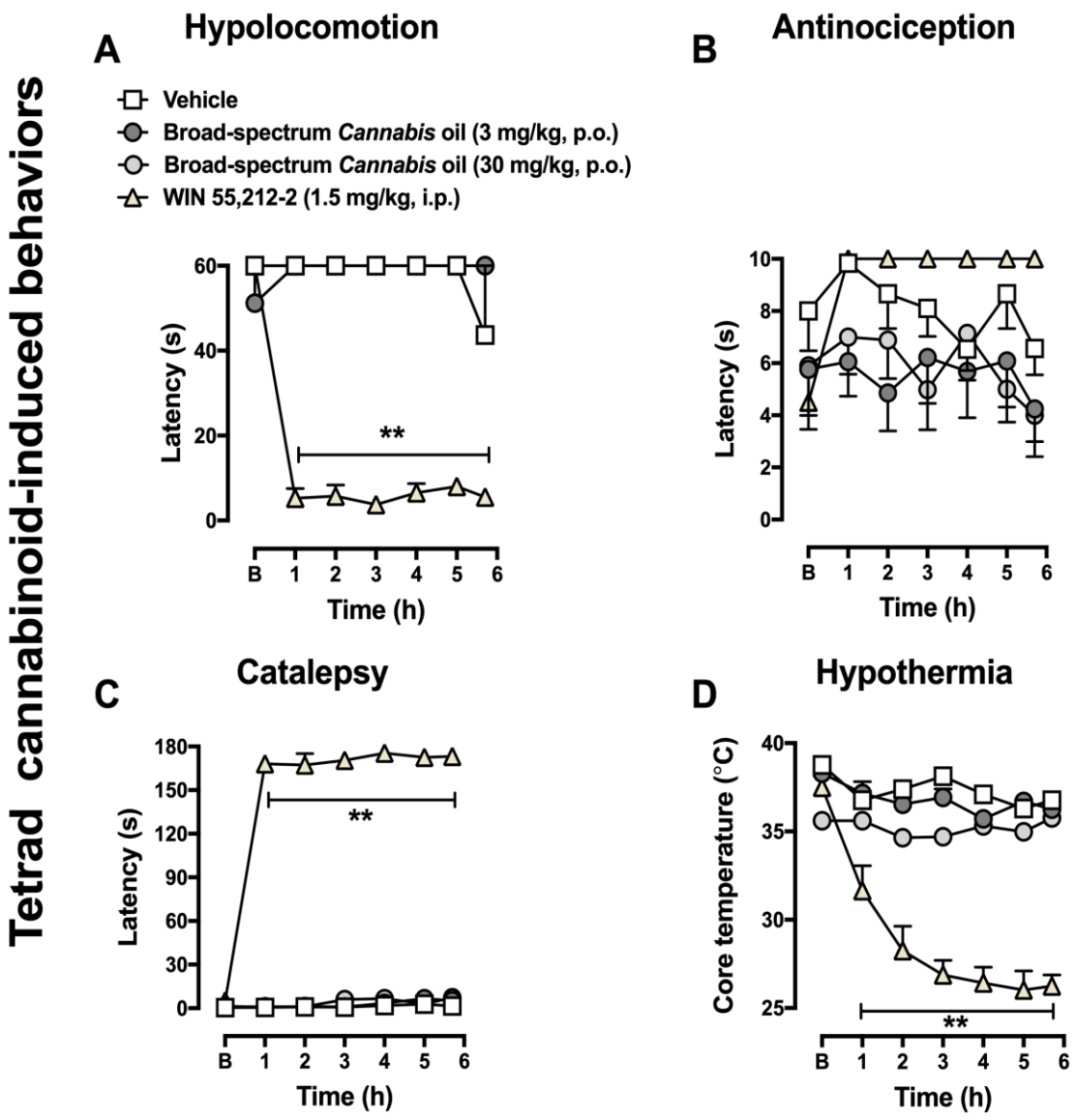

Figure 5. Evaluation of the treatment with broad-spectrum *Cannabis* oil on cannabinoid tetrad assay. The tests included locomotor activity (A), threshold hyperalgesia (B), catalepsy-like behavior (C), and thermal body measurement (D). WIN 52,212-2 (1.5 mg/kg, i.p.) – a potent cannabinoid receptor agonist – was used as the positive control. Data are presented as mean ± SEM of 4-5 mice per group. **p < 0.001 *versus* naïve (two-way ANOVA followed by Bonferroni *post-hoc*-test). B: baseline withdrawal threshold refers to the evaluation performed before treatment.