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Cannabidiol and cannabigerol effect on cognitive deficit induced by intracerebroventricular administration of amyloid beta 42 in experimental Alzheimer's disease model

Arda Can Aydin[1], Ayse Nur Hazar-Yavuz[1], Naziye Ozkan[2], Sarfraz Ahmad[3]*, Rezzan Gulhan[4], Levent Kabasakal[1] & Hatice Kubra Elcioglu[1]*

[1]Department of Pharmacology, Faculty of Pharmacy, Marmara University, Istanbul 34854, Turkey

[2]Department of Medical Services and Techniques, Vocational School of Health Services, Marmara University, Istanbul 34854, Turkey

[3]AdventHealth Medical Center, Orlando, FL 32804, USA

[4]Department of Medical Pharmacology, Faculty of Medicine, Marmara University, Istanbul 34854, Turkey

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Alzheimer's disease (AD) causes amyloid beta (Aβ) plaque formation in regions such as the cerebral cortex and hippocampus, which have a cognitive function. Besides oxidative stress, neuroinflammation and acetylcholine, the degeneration of glutamatergic pathways in individuals with AD causes acetylcholine accumulation in the cortex and hippocampus, leading to the formation of Aβ plaque. Herein, we investigated the effects of cannabidiol (CBD) and cannabigerol (CBG) which are *Cannabis sativa* components on AD-like cognitive deficit induced by intracerebroventricular (*icv*) administration of $A\beta_{1-42}$. Sprague Dawley rats were divided into four groups: i) Control, ii) Alzheimer, iii) Alzheimer+CBD, and iv) Alzheimer+CBG. The AD model was induced by icv injection of $A\beta_{1-42}$ and then CBD and CBG treatments were administrated for 2 weeks. Open field test, passive avoidance test, and Morris' water maze test were performed, and on 15th day, the rats were decapitated. Hippocampus and cerebral cortex were removed from the brain, and levels of interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) were measured by ELISA, and $A\beta_{1-42}$ expression was evaluated immunohistochemically. There was no significant difference between the groups in the parameters evaluated by the open field test. In passive avoidance and Morris's water maze tests, both CBD and CBG enhanced the learning-memory functions impaired by AD. CBD and CBG treatments successfully reduced the levels of TNF-α and IL-1β in AD. Immunohistochemical analysis revealed decreased expression of $A\beta_{1-42}$ in CBD and CBG treatment groups. CBD and CBG treatments improved learning and memory deficits in the $A\beta_{1-42}$ induced AD model. We implicate that these experimental findings would lead to better avenues for targeted studies on *C. sativa* (a natural product of herbal origin and its components) that can potentially be developed for AD treatment.

Keywords: Alzheimer's disease, Rat model, Cannabidiol, Cannabigerol, Inflammatory cytokine, Amyloid beta

Alzheimer's disease (AD) is a neurodegenerative disease discriminated by progressive dementia. According to data from the World Health Organization (WHO), about 50 million people worldwide are known to have dementia, and this number is predicted to exceed 82 million by the year 2030 and 152 million by the year 2050[1]. The economic cost of dementia is also quite high, and it is thought that the cost of dementia worldwide is one trillion U.S. dollars, and this cost will reach two trillion dollars by the year 2030[1]. AD is characterized by the accumulation of extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles that cause neuroinflammation and oxidative stress, leading to the formation of neurotoxicity that strengthens neurodegeneration and eventually leads to cognitive decline[2]. Glutamate, which causes oxidative stress in AD, is the primary excitatory neurotransmitter in cortical and hippocampal cells and has been reported to be responsible for 75% of the transmission in the central nervous system (CNS). As a result of $A\beta_{1-40/1-42}$ oligomerization and the development of intracellular oxidative stress, glutamate is oxidized and over-produced. Increasing glutamatergic activity further raises Aβ formation, causing neurodegeneration[3,4]. Glutamate, when present in excess, causes oxidative stress in AD, making it the primary contributor.

*Correspondence:
Phone: +1 407 312 2766 (Mob.)
E-mail: kubra.elcioglu@marmara.edu.tr (HKE);
sarfraz.ahmad@adventhealth.com (SA)

Acetylcholinesterase inhibitors such as donepezil, galantamine and rivastigmine; and N-methyl D-aspartate receptor antagonist (memantine) are used for the treatment of AD for years; but unfortunately learning and memory dysfunctions cannot be prevented completely in the progression of the disease with these drugs[5]. Apart from these drugs, for the first time since the year 2003, another drug (*viz*., aducanumab) received accelerated approval from the U.S. Food and Drug Administration (FDA) in 2021, followed by lecanemab in 2023. In addition, aducanumab is the first treatment for the presence of Aβ plaques in the brain, which is the underlying pathology of AD[6]. Despite these available treatments, new drug studies are ongoing because they are not sufficient to treat the disease and have many side effects. In addition to these modern medicines, about 80% of the population in developing countries use herbal medicines. Plants are utilized as a source of biologically active chemical substances and often these substances lead to the development of new drugs[6]. Herbal medicines also provide an alternative to high-priced medicines and health applications[7].

The use of cannabis (*Cannabis sativa*) dates back to ancient times. Studies about *C. sativa*, especially in the last 100 years or so, have provided the elucidation of the structures and pharmacological mechanisms of the chemical agents (cannabinoids) of it[8]. Nevertheless, there are a limited number of studies in the peer-reviewed literature on the effects of *C. sativa* and its effective compounds on AD, and most of them are not sufficient enough to illuminate with its mechanism(s) of action[9].

In the peer-reviewed literature, it has been reported that cannabinoids play a neuroprotective role by preventing excitotoxicity, calcium flow, inflammation, and decrease neuronal damage in the brain during ischemic attacks[10-12]. It has been stated that these agents have effects related to various pathological conditions and ageing processes, including synaptic plasticity[13], neuroinflammation[14], neurodegeneration[15], regulation of sleep cycle[16], and immune function[17]. It has also been shown to have roles in epilepsy[18], traumatic brain injury[19], stroke[20], multiple sclerosis[21], Parkinson's disease[22], Huntington's disease[23], amyotrophic lateral sclerosis[24], and AD[25,26].

Cannabidiol (CBD) is a non-psychotropic cannabinoid whose beneficial effects are known in AD[27]. In animal models, CBD reduces anxiety, agitation, psychosis, aggression, depression, pain, oxidative stress, inflammation, and reorganizes impairment in learning and memory function[28]. The mechanism of action of CBD in neurodegenerative diseases has also been described in previous studies. According to these studies, CBD, a multi-target molecule, has neuroprotective and anti-psychotic properties through cannabinoid receptor (CB1) receptor, anti-inflammatory and neuroprotective through CB2, and anti-inflammatory, neuroprotective, neurogenesis, anti-apoptotic properties through peroxisome proliferator activating receptor gamma (PPAR-γ) receptor. Moreover, it is reported that it has anti-depression, anxiolytic and antiemetic effects with 5-hydroxytryptamine 1A (5-HT1A), anti-inflammatory effects with adenosine A2A, and anti-inflammatory and analgesic effects with transient receptor potential cation channel subfamily vannilloid member 1 (TRPV1, capsaicin receptor).

In addition, cannabidiol received approval from the FDA in 2018 for the treatment of pediatric epilepsy. The antiepileptic effects of CBD can again be explained by its multi-target nature. First, although CBD does not promote direct activation of CB1R, indirect activation of this receptor is the result of increased anandamide levels due to the actions of CBD (FAAH blockade and anandamide cellular uptake inhibition). It is thought that this effect may protect against seizures by suppressing neuronal hyperactivity. Moreover, CBD appears to prevent CB1R changes in limbic brain structures and promotes the control of neuronal activity. CBD may also increase extracellular adenosine levels through equilibrative nucleoside transporter (ENT)-1 blockade. This effect allows activation of A1 receptors by adenosine, resulting in antiseizure effects. It is possible that the antiseizure effects of CBD are due in part to its effects on the dopaminergic system, as these changes have been implicated as a possible pathophysiological mechanism of epilepsy. Another important target is the G protein-coupled receptor (GPR55), which regulates glutamate release and modulates neuronal excitability through a calcium-dependent mechanism. Therefore, antagonism of GPR55 receptors by CBD may reduce glutamate release and have antiseizure activity. Additionally, abnormalities in GABAergic transmission have been associated with the development of epilepsy. CBD has been reported to be a positive allosteric modulator of $GABA_A$ receptors, possibly enhancing GABAergic inhibitory

transmission and explaining the additional antiseizure effects of CBD. Regarding TRPV1 channels, their activation increases neuronal excitability and consequently facilitates seizures. Although CBD has agonistic effects on TRPV1 channels, it induces rapid desensitization of these channels, which may indirectly reduce neuronal excitability and explain the antiseizure effects of CBD. Finally, T-type calcium channels and voltage-gated sodium channels are involved in the regulation of cell excitability and are therefore implicated in epilepsy. CBD has been shown to cause inhibition of these channels, effects that may mediate the antiseizure effects of CBD.

Cannabigerol (CBG) is another non-psychotropic cannabinoid that has been shown to have neuroprotective effects in experimental Huntington's disease[29]. Since CBG is a weak CB1 receptor agonist, it does not have a psychotropic effect, but it is known to exhibit antioxidant and anti-inflammatory properties. CBG is a partial agonist at CB2 receptors, a potent α2-adrenoceptor agonist, and a moderate 5-HT1A receptor antagonist, as well as interacting with various TRP isoforms, including TRPV1 and 2-channel. CBG has been found particularly effective in models of neurodegenerative disease, Huntington's disease. It targets many aspects of the disease, including gene expression, alleviation of motor symptoms, reduction of microglial activation, and attenuation of the inflammatory response. Microglial-mediated neuroinflammation, which is involved in the pathophysiology of Huntington's disease, is suppressed by the activation of CB2 receptors in the use of CBG. However, it is also thought to act through activation of PPARγ, which has a significant effect on microglial activation independent of the CB1 and CB2 receptor. It is stated that it can protect neurons by its effects on microglial activation and by secreting various trophic factors as well as anti-inflammatory cytokines such as IL-4 and IL-10[30]. It is also worth noting that the effects of CBG on Alzheimer's disease have not been investigated in any *in vitro* or *in vivo* studies. *In silico* analysis reports that CBG has the ability to protect cells, eliminate intraneuronal Aβ, reduce oxidative damage, and protect them from loss of energy or trophic support[31].

Studies have reported that the endogenous cannabinoid system is a promising therapeutic target for treating neurodegenerative disorders associated with AD[32]. It is known that the $A\beta_{1-42}$ induced AD model is effective in causing cognitive disorders similar to the pathology developing in the brain of AD[33]. Herein, we investigated the effects of CBD and CBG (glutamate receptor blockers, which are known to have antioxidant and anti-inflammatory effects) on learning and memory skills in rats with $A\beta_{1-42}$ induced AD model utilizing open field, passive avoidance, and Morris' water maze tests. In addition to the cognitive functions, their effects on neuroinflammation were investigated biochemically. Furthermore, Aβ plaques in the brain were also examined by immunohistochemistry.

Materials and Methods

Chemicals

$A\beta_{1-42}$, CBD, and CBG were purchased from Sigma-Aldrich, Inc. (St. Louis, Missouri, USA). Biomarkers' Enzyme-linked immunosorbent assay (ELISA) kits were procured from Bioassay Technology Laboratory, Korain Biotech Co. Ltd. (Shanghai, China).

Animal and ethical approval

Adult male/female Sprague Dawley rats (220-260 g) were obtained from Marmara University Experimental Animal Application and Research Center (Istanbul, Turkey). During the experimental study, the rats were kept in a controlled room with humidity 50%, temperature 25±2 ^{o}C, and 12:12 h dark/light cycle with free access to water and food. This study is approved by the Marmara University, Animal Experiments Local Ethics Committee (Permission Number: 50.2019.mar).

Experimental design

The rats were randomly divided into the following groups each containing eight rats: i) Control Group (healthy rats were given unilaterally *icv* citrate buffer on days 1-15), ii) Alzheimer Group [Alzheimer rats were given unilaterally *icv* $A\beta_{1-42}$ (2.2. nmol) in 10 µL volume on day 1 and *icv* citrate buffer on days 1-15], iii) Alzheimer+CBD Group [Alzheimer + CBD rats were given unilaterally *icv* $A\beta_{1-42}$ (2.2. nmol) in 10 µL volume on day 1 and *icv* CBD (100 ng) on days 1-15], and iv) Alzheimer+CBG Group [Alzheimer rats were given unilaterally *icv* $A\beta_{1-42}$ (2.2. nmol) in 10 µL volume on day 1 and *icv* CBG (100 ng) on days 1-15]. On day 15, the last day of the study, all rats were sacrificed.

Induction of Alzheimer's disease

In this study, $A\beta_{1-42}$ peptide was used as a neurotoxic agent to create an AD model in rats. On

the day of the surgical operation, all rats were anaesthetized by intraperitoneal administration of ketamine (100 mg/kg) and xylazine (5 mg/kg). After the anaesthetic agent was administered, the scalp of the anaesthetized rats was shaved, and their heads were fixed to the stereotaxic device (Harvard Apparatus, Massachusetts, United States). After the incision was made on the scalp of the rats with a scalpel, bregma was determined. Taking bregma as the reference point in the skull, a hole was drilled unilaterally (right) in the lateral ventricle corresponding to the following coordinates: 0.8 mm downwards from bregma, 1.6 mm to the right and 4 mm inward in the skull. Cannulas were placed in the opened holes with the help of acrilic. After the animals were placed in single cages, they were left to recover for 3 days, with access to food and water.

Before $A\beta_{1-42}$ peptide (2.2 nmol/10 µL) was administered (i.c.v.) to Alzheimer model rats, $A\beta_{1-42}$ peptide was incubated at 37°C for 72 h to ensure the formation of fibrillar structures. After 3 days, $A\beta_{1-42}$ peptide was injected into a single lateral ventricle (right ventricle) (AP: −0.8 mm, ML: −1.6 mm, and DV: −4.0 mm) at a rate of 1 µL/min using a Hamilton microsyringe (26 G) (microsyringe pump) was injected and the syringe was left in the designated area for 5 min after application. Rats in the control group were given 10 µL physiological saline i.c.v. at the same rate and volume. was injected. Treatment applications were started the day after $A\beta_{1-42}$ application. The treatments were administered icv with the above-mentioned doses using a cannula[34].

Behavioural tests

All behavioural tests were performed 30 min after the administration of the treatment agents and between 09:00 am and 12:00 noon.

Open field test

The open field test was applied on the 8th day of the study. The locomotor activities of the rats were evaluated by the open field test, which was carried out in a 50x50x30 cm box made of black plexiglass. The experiment was started by placing the rats in the corner of the box, and the movements of the rats were recorded with a camera for 5 min. The number of squares crossed and rearing were used to assess the rats' locomotor activity[35].

Passive avoidance test

During the 9th-10th days of the study, a passive avoidance test was applied. The apparatus consists of two different compartments (20x20x30 cm of each), light and dark, separated by a wall with a passage way between them. One of the compartments was illuminated with the light. Two consecutive days were implemented, i.e., training and test. In the training day, each rat was placed in the individually light compartment and when it moved to the dark compartment, an electric shock was applied to its feet over the grid floor (0.5 mA electric shock was applied for 3 second). The rat was quickly removed and placed in its cage. The test day was carried out after 24 h and the rats were put in the light compartment again. After being placed in the light compartment, the latency time to the dark compartment was recorded. The test of rats that did not enter the dark compartment within 300 second, was terminated and the latency time was accepted as 300 second[36].

Morris water maze test

Morris water maze test was performed between the 11th and 15th days of the study. The tank consisted of a circular tank with a diameter of 160 cm and a height of 35 cm. The tank was divided into four equal quadrants with two imaginary vertical intersecting diameters. It was to include an escape platform measuring 10x10x25 cm in the same colour as the environment in the tank (to avoid incorrect results related to the image). During the test, this platform was kept on the same quadrant of the tank and 1.5 cm below the water surface. Rats were slowly dropped off at a starting point on the edge of a quadrant with their faces pointing towards the wall. Unable to find the escape platform within 75 second, the rats were politely directed to the platform and kept on it for 20 second, enabling them to learn the platform using clues. The rats were given four attempts for four consecutive days during which stage the time taken to reach the platform was evaluated. On day 5, the platform was removed from the tank, and a probe trial was carried out to calculate how long the rats had spent on the quadrant where the platform was previously located. The rats were released to swim freely for 60 second. This test was considered a measure of spatial learning and memory. The data was recorded with a video camera[36].

Biochemical analysis

The experimental rats were sacrificed after all the tests were completed. Tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) levels were measured in the tissue homogenates obtained from the hippocampus and cortex of rats utilizing ELISA tests

in accordance with the manuscfacturer's protocols (Bioassay Technology Laborator, Korain Biotech Co. Ltd., Shanghai, China)[37].

Immunohistochemical analysis

$A\beta_{1-42}$ immunoexpression was evaluated by polymer-based horseradish peroxidase (HRP) immunohistochemistry (IHC) staining. For this, 3 µm thick sections were taken from formalin-fixed paraffin-embedded (FFPE) tissues to positively charged slides and kept in an oven at 37ºC for overnight. Then, de-paraffinization was completed in three separate xylenes for 5 min each. The slides were hydrated by soaking in two separate 96% ethanol for 10 min. Endogenous peroxidase activity in the tissues was suppressed for 20 min with 3% hydrogen peroxide prepared in methanol. After washing the sections with distilled water, they were treated with citrate buffer solution (pH 6.0) for 20 min in a 200 W microwave oven for antigen recovery. The slides were cooled at room temperature for 20 min and washed for 5 min in two separate phosphate buffer solutions (PBS; pH 7.4). Protein blockade (EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit; ab236466, Abcam, Cambridge, UK) was applied to the tissues to prevent non-specific staining. The blocking solution was removed, and the sections were incubated for 30 min at room temperature by dropping a 1:500 dilution of monoclonal $A\beta_{1-42}$ (NBP2-13075; Novus, UK) antibody. The sections washed with two separate PBS for 5 min were first dripped with mouse specific reagent and left for 10 min. After washing with PBS, HRP-polymer was applied (EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit; ab236466, Abcam, Cambridge, UK) and incubated for 15 min. The sections were washed with two separate PBS for 5 min and kept in 3,3'-diaminobenzidine (DAB) chromogen for 5 min. Nuclear counterstaining was performed with Mayer Hematoxylin for 1 min and the slides were dehydrated with 96% ethanol and covered with a capping agent. To detect specific binding in staining, the negative control was also studied simultaneously. In the tissue section used as negative control, all steps of the method except the primary antibody stage were applied, and no staining was observed in this tissue.

$A\beta_{1-42}$ immunoexpression was evaluated semi-quantitatively in the cortex and hippocampus areas under light microscopy (Olympus CX41; Feasterville, PA, USA). Scoring according to the staining intensity was: 0: no staining, 1: weak staining, 2: moderate staining, and 3: strong staining. Photographs were taken from the sections using the 'My Camera Digital/Imaging System' at 10x magnification.

Statistical analysis

The results of the tests were analyzed by one-way analysis of variance (ANOVA) and two-way ANOVA followed by *Tukey*'s method as a post-test and represented as mean ± standard error of the mean (SEM). *P*-values < 0.05 were considered significant. Data analysis were performed using GraphPad Prism 6.5 software (San Diego, CA, USA).

Results

Open field test

When the number of squares crossed (Fig. 1A) and the number of rearings (Fig. 1B) were examined, we observed that there was no significant difference between the control and experimental groups.

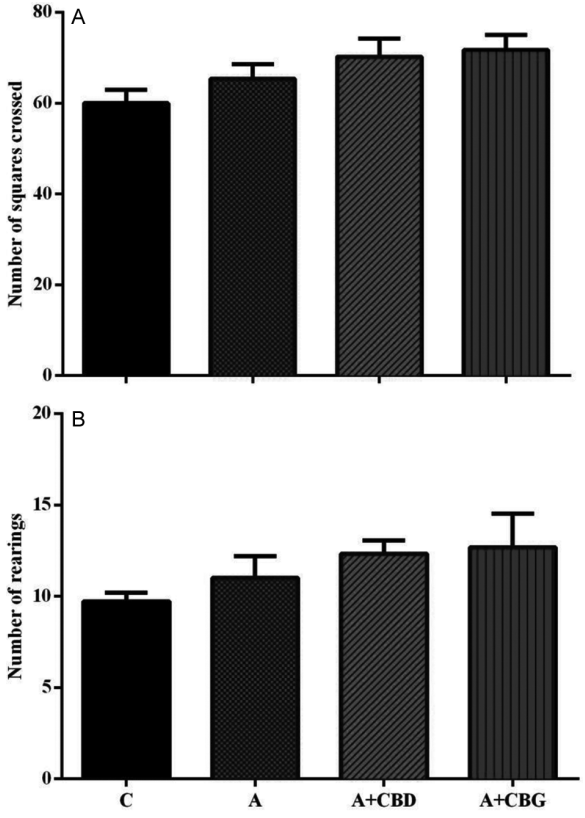

Fig. 1 — (A) Determination of the number of squares crossed in open field test, and (B) the number of rearings in open field test. Values are expressed as mean SEM (n=8). One-way ANOVA and Tukey's post hoc tests were used for statistical analysis. *Abbreviations:* A = Alzheimer; C = Control; SEM = standard error of the mean; ANOVA = analysis of variance; CBD = cannabidiol; CBG = cannabigerol.

Passive avoidance test

Latency time to the dark compartment was decreased in the Alzheimer group as compared to the Control group (P<0.01) (Fig. 2). When the experimental treatment groups were examined, we observed that there was an increase in the latency time to the dark compartment in the Alzheimer+CBD group as compared to the Alzheimer group (P<0.05). The CBG treatment did not affect the reduction in Alzheimer group (Fig. 2).

Morris' water maze test

While there was no significant difference between the Control and experimental Alzheimer groups on the 1st and 2nd days, the Alzheimer group on the 3rd and 4th days had a longer time to reach the platform as compared to the Control group (P<0.001), as shown in Fig. 3. When the Alzheimer+CBD group was compared with the Alzheimer group, the duration was shortened on the 3rd (P<0.001) and 4th (P<0.01) days. The Alzheimer+CBG group also shortened the latency time on the 1st (P<0.05), 3rd (P<0.001), and 4th (P<0.001) days as compared to the Alzheimer group (Fig. 3).

When the time spent in the target quadrant given was examined, it was observed that the Alzheimer group shortened this time as compared to the Control group (P<0.01). The CBD and CBG expertimental treatments were able to increase/reverse the decrease in Alzheimer group (P<0.05) (Fig. 4).

Biochemical analysis

Interleukin-1β activity

Interleukin-1β (IL-1β) levels in the samples obtained from the brain tissues of rats used in the experiments were determined utilizing the ELISA kits. When looking at the hippocampus results, an increase in the IL-1β levels was observed in Alzheimer group as compared to the Control group (P<0.01), while the CBD and CBG treatments were able to reverse this increase (P<0.01) (Fig. 5A). When the cerebral cortex IL-1β levels were examined, an increase was observed in the Alzheimer group as compared to the Control group (P<0.01), and the CBD and CBG experimental treatments eventually reduced this increase (P<0.05) (Fig. 5B).

Tumour necrosis factor-α activity

The levels of tumour necrosis factor-α (TNF-α) in the cerebral cortex and hippocampus specimens were

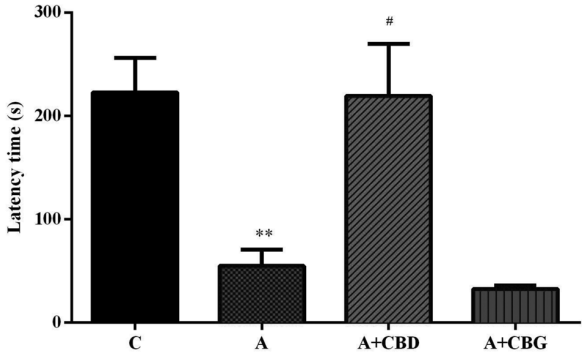

Fig. 2 — Determination of the latency time (sec) in the passive avoidance test. Values are expressed as mean ± SEM (n=8). One-way ANOVA and Tukey's post hoc tests were used for statistical analysis. **P<0.01 compared to Control group, #P<0.05 compared to Alzheimer group. *Abbreviations:* A = Alzheimer; C = Control; SEM = standard error of the mean; ANOVA = analysis of variance; CBD = cannabidiol; CBG = cannabigerol.

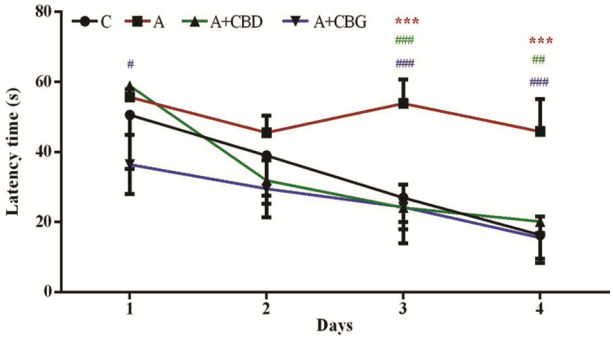

Fig. 3 — Determination of the latency time (sec) between the days 1 and 4 in Morris' water maze test. Values are expressed as mean ± SEM (n=8). Two-way ANOVA and Tukey's post hoc tests were used for statistical analysis. ***P<0.001 compared to Control group, #P<0.05, ##P<0.01, and ###P<0.001 compared to Alzheimer group. *Abbreviations:* A = Alzheimer; C = Control; SEM = standard error of the mean; ANOVA = analysis of variance; CBD = cannabidiol; CBG = cannabigerol.

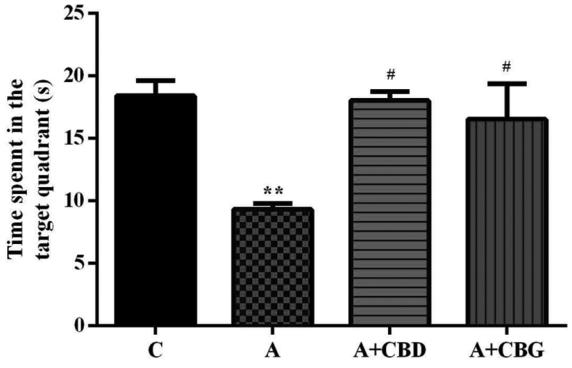

Fig. 4 — Determination of time spent in the target quadrant (sec) utilizing Morris' water maze test. Values are expressed as mean ± SEM (n=8). One-way ANOVA and Tukey's post hoc tests were used for statistical analysis. ** p<0.01 compared to Control group, # p<0.05 compared to Alzheimer group. *Abbreviations:* A = Alzheimer; C = Control; SEM = standard error of the mean; ANOVA = analysis of variance; CBD = cannabidiol; CBG = cannabigerol.

determined utilizing ELISA kits. In the hippocampus specimens, TNF-α levels were found to be increased in the Alzheimer group as compared to the Control group (P<0.01), however the CBD and CBG treatments managed to reverse this elevation (P<0.01) (Fig. 6A). When the cerebral cortex specimens were examined, an increase in the TNF-α level was observed in the Alzheimer group as compared to the Control group (P<0.05), whereas the Alzheimer+CBD and Alzheimer+CBG treatment groups showed the reduced levels of TNF-α (P<0.001) (Fig. 6B).

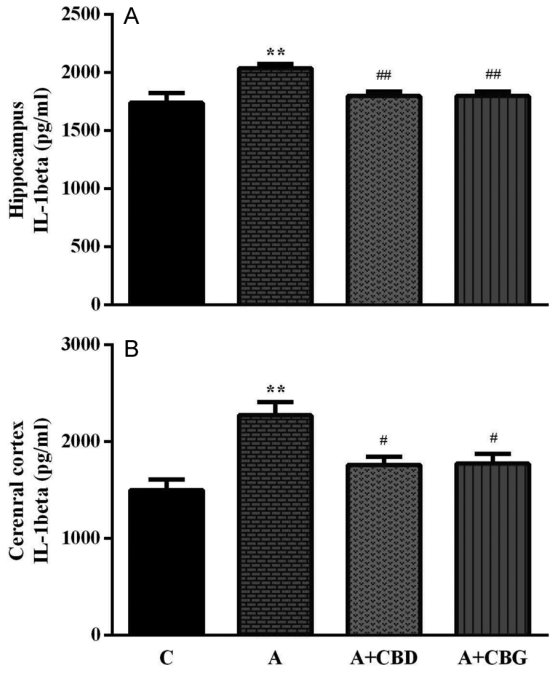

Fig. 5 — (A) Determination of IL-1ß levels (pg/mL) in hippocampus, and (B) in cerebral cortex specimens. Values are expressed as mean ± SEM (n=8). One-way ANOVA and Tukey's post hoc tests were used for statistical analysis. **P<0.01 compared to Control group, $^{\#\#}P$<0.01 compared to Alzheimer group, $^{\#}P$<0.05 compared to Alzheimer group. *Abbreviations:* A = Alzheimer; C = Control; SEM = standard error of the mean; ANOVA = analysis of variance; CBD = cannabidiol; CBG = cannabigerol; IL-1β = interleukin-1beta.

Immunohistochemical analysis

The expression of $A\beta_{1\text{-}42}$ in cerebral cortex and hippocampus tissues was analyzed immunohistochemically. $A\beta_{1\text{-}42}$ immunoexpression was not observed in the cortex and hippocampus tissues of the Control group (Fig. 7A & Fig. 8A). Strong $A\beta_{1\text{-}42}$ immunoexpression was seen in both the cortex and hippocampus regions of the Alzheimer group as compared to the Control group (Fig. 7B & Fig. 8B). Weak immunoexpression was observed in

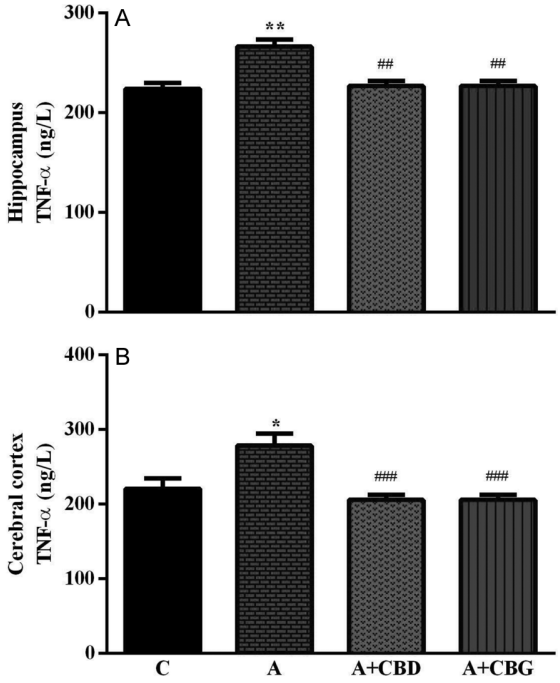

Fig. 6 — (A) Determination of TNF-α levels (ng/L) in hippocampus, and (B) cerebral cortex specimens. Values are expressed as mean ± SEM (n=8). One-way ANOVA and Tukey's post hoc tests were used for statistical analysis. *P<0.05 compared to Control group, **P<0.01 compared to Control group, $^{\#\#}P$<0.01 compared to Alzheimer group, $^{\#\#\#}P$<0.001 compared to Alzheimer group. *Abbreviations:* A = Alzheimer; C = Control; SEM = standard error of the mean; ANOVA = analysis of variance; CBD = cannabidiol; CBG = cannabigerol; TNF-α: tumor necrosis factor-alpha.

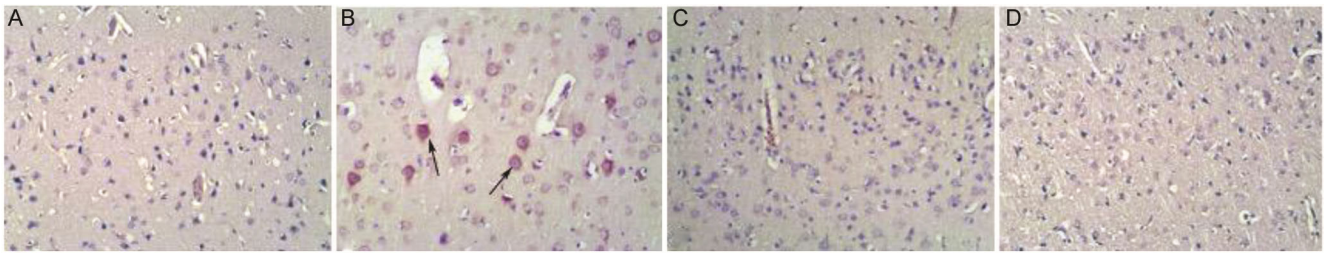

Fig. 7 — Evaluation of amyloid beta 1-42 ($A\beta_{1\text{-}42}$) expression in cerebral cortex specimen. (A) $A\beta_{1\text{-}42}$ immunoexpression is not observed in the cortex tissue of Control group. (B) Strong $A\beta_{1\text{-}42}$ immunoexpression is seen in neuron cell cytoplasm in the Alzheimer group cortex tissue (arrow). (C) Weak $A\beta_{1\text{-}42}$ immunoexpression is seen in the Cannabidiol group. (D) Weak $A\beta_{1\text{-}42}$ immunoexpression is seen in the Cannabigerol group. (10x, HRP streptavidin-biotin).

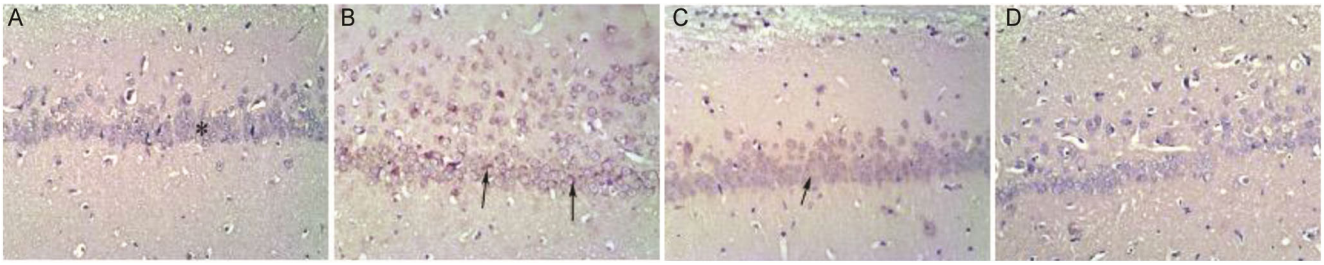

Fig. 8 — Evaluation of amyloid beta 1-42 ($A\beta_{1-42}$) expression in hippocampus specimen. (A) $A\beta_{1-42}$ immunoexpression is not observed in the cortex tissue of Control group (*). (B) Strong $A\beta_{1-42}$ immunoexpression is seen in the Alzheimer group cortex tissue (arrow). (C) Weak $A\beta_{1-42}$ immunoexpression is seen in the Cannabidiol group. (D) $A\beta_{1-42}$ immunoexpression is not observed in the cortex tissue of the Cannabigerol group. (10x, HRP streptavidin-biotin).

the CBD group sections from both regions (Fig. 7C and Fig. 8C). Also, in the CBG group sections, weak $A\beta_{1-42}$ immunoexpression was observed in the cortex region, but no expression was observed in the hippocampus region (Fig. 7D and Fig. 8D).

Discussion

Alzheimer's disease is a neurodegenerative disease characterized by progressive dementia that occurs in the middle- and late-age population. Pathological factors associated with AD include aggregation of extracellular Aβ plaques, accumulation of neurofibriller tangles, oxidative stress, and inflammation. Aβ plaques consists of Aβ peptides in fibrillar form[35]. It is well known that Aβ peptides cause neurodegenerative changes by interacting directly with functional protein complexes on the cell surface or indirectly disrupting normal physiological processes through astrocyte and microglia activation. Different doses of synthetic or naturally secreted Aβ peptides (e.g., $A\beta_{1-40}$, $A\beta_{1-42}$, and $A\beta_{25-35}$) are administered to the brain by intracerebroventricular or intrahippocampal injection to induce experimental AD in rats. Experimental studies have shown that $A\beta_{1-42}$ is more toxic among these peptides[38]. Studies have reported that $A\beta_{1-42}$ peptide triggers lipid peroxidation by triggering intracellular signalling pathways through various membrane receptors. 4-Hydroxynonenal metabolites enhanced by $A\beta_{1-42}$-induced lipid peroxidation have been found to impair the functions of important membrane proteins and Ca^{2+} homeostasis, resulting in increased free oxygen radicals and neuronal damage[39]. In addition, oxidative processes convert non-aggregated $A\beta_{1-42}$ to aggregated $A\beta_{1-42}$, while $A\beta_{1-42}$ itself increases free radical production, thus resulting in a pathological cycle[40]. For these reasons, we chose $A\beta_{1-42}$ to induce AD model in our study and based on our behavioural test results coupled with biochemical and histological analyses, the AD model was successfully induced.

The open field test was performed to evaluate locomotor activity before other behavioural experiments. There was no significant difference between the groups on the number of squares crossed and rearing. It is known that tetrahydrocannabinol (THC), the psychoactive component of *C. sativa*, has a stimulating effect and increases locomotor activity in rats in the open field test[41]. The fact that non-psychoactive components (cannabidiol and cannabigerol used in our study) did not cause any changes in the open field test indicates that they do not have stimulant or sedative effects, andit is important that other behavioural parameters were not affected.

The passive avoidance test was used to assess the emotional learning and memory function[42]. The shortening of the latency time of the rat placed in the light compartment to the dark compartment where electricity is applied indicates that learning and memory are impaired[43]. In this study, it was observed that the administration of $A\beta_{1-42}$ reduced the latency time in the subjects demonstrating emotional learning and memory impairment. On the other hand, we observed that cognitive functions improved when the AD-induced rats were given CBD, while CBG treatment was ineffective. In previous studies, it has been shown that CBD corrects impaired cognition in the passive avoidance test[44]. The effectiveness of CBG in passive avoidance test was shown for the first time in our study and it was not effective in restoring impaired cognition. It has been suggested that stimulation of the cannabinoid receptor type 1 (CB1) receptor impairs learning-memory functions, while CB1 antagonists improve this function[45]. It has also been shown that CBD, which we used in our study, acts as an allosteric modulator of CB1, binding to sites other than the ligand-binding site and inhibiting

its activity, while CBG binds to this receptor as a weak agonist[42]. Since CBG is a weak agonist, it may be ineffective in the AD induced cognitive disfunctions in the passive avoidance test.

In our experimental studies, Morris' water maze test was utilized to investigate 3-dimensional (3D) learning and memory functions in rats. In this test, the rat's ability to find the platform hidden in the quarter of the tank was investigated with the help of clues in the environment[46]. The latency time (i.e., the time to reach the platform) for 4 days and the time spent in the target quadrant on the probe test day were used as learning-memory evaluation parameters. It was expected that a healthy rat would reach the platform in a relatively shorter time on the next test days, which would indicate that the learning and memory functions are enhanced. In our study, an increase in the time to reach the platform was observed in the Alzheimer group as compared to the Control group, especially on the 3rd and 4th days, which indicates that the learning-memory was impaired in the Alzheimer group. In our study, both CBD and CBG treatments shortened the latency time as compared to the Alzheimer group and re-organised the deterioration in the learning memory observed in the Alzheimer group.

It is reported that when the platform is removed in the prob test, the increase in the time spent in the target quarter where the platform is located indicates that the rat had gained learning skills[47]. In our study, the time spent in the target quadrant of the Alzheimer group decreased as compared to the Control group, proving that learning memory was impaired in the AD-induced rats. The CBD and CBG treatments rescued $A\beta_{1-42}$ induced special memory deficits.

It has been reported that pro-inflammatory cytokines including TNF-α and IL-1 (especially IL-1β) are increased in the hippocampus and cerebral cortex, thus cause inflammation in AD[48]. TNF-α levels in these regions have been reported to cause amyloid plaque formation, which mediates impaired learning and memory function[49]. Prevention of this inflammatory response is one of the important goals in the treatment of AD. In our study, we measured TNF-α and IL-1β levels in hippocampus and cerebral cortex specimens utilizing ELISA to evaluate their induced inflammation in AD. Indeed, we observed that inflammation increased in Alzheimer induced rats, which was modulated/reduced by the CBD and CBG treatments equally. It is well-known that marked inflammation occurs in AD that is induced by $A\beta_{1-42}$. In addition, it was shown that cannabinoids effectively prevent the $A\beta_{1-42}$ mediated increase in the pro-inflammatory cytokines and the neurodegeneration that occurs after its *in vitro* and *in vivo* administration[50]. Therefore, such drugs with neuroprotective and anti-inflammatory effects, especially devoid of psychoactive effects[13], may be of interest in the prevention of AD inflammation[51].

As with our immunohistochemical analyses, an increase in $A\beta_{1-42}$ expression was observed in both the cerebral cortex and hippocampus tissues of the AD group rats as compared to the Control group. This predictable increase[52] in the icv $A\beta_{1-42}$-induced AD model indicates that the model was successfully established. Notably, the increase in the AD group was reduced by CBD and CBG treatments in the cerebral cortex. Considering the hippocampus results, while relatively low $A\beta_{1-42}$ expression was observed in the CBD group, $A\beta_{1-42}$ expression was not observed in the CBG group. Based on our IHC analysis, CBG treatment showed profound decreased in the amyloid plaque formation as compared to CBD treatment.

Cannabidiol has low affinity for CB1 and CB2, and studies show that mechanisms unrelated to these receptors are responsible for their pharmacological effects. These non-CB-receptor-mediated actions would explain their effects on several neurotransmitter systems, such as modulating glutamatergic activity. CBD reduced glutamate-mediated neurotoxicity observed in the hippocampus and prefrontal cortex by the modulation of glutamate released by cannabinoids[53]. Consistent with these data, in our study, CBD administration ameliorated $A\beta_{1-42}$ induced memory impairment presumably through interactions between the CBD and the glutamatergic system. Also, previous studies have shown that glutamate toxicity can be prevented by the antioxidant properties of CBD. CBD interrupts free radical chain reactions by capturing free radicals or converting them into less active forms. The direct antioxidant effects of CBD are related to pro-oxidant enzyme activity, chelation of transition metal ions, interruptions of free radical chain reactions, antioxidant enzyme activity, non-enzymatic antioxidant levels, oxidative modifications of lipid protein, and the effect on DNA. CBD has also been found to exert partial agonist activity on the serotonin (5-HT) receptor type 5-HT1, inhibit 5-HT reuptake, and reduce overall 5-HT neurotransmission[54]. CBD induces a variety of pharmacological effects, possibly by increasing 5-HT

neurotransmission, followed by activation of post-synaptic 5-HT1A receptors. However, the activated 5-HT1A receptor can act as a membrane antioxidant by trapping ROS[55]. Thus, CBD's affinity to 5-HT receptors appears to be responsible for, but not limited to, its neuroprotective effects[56].

There are limited studies in the peer-reviewed iterature that discusses the effects of *C. sativa* (and some of its related effective compounds) on AD and are not sufficient to elucidate their mechanism(s) of action. Nonetheless, there are studies in the peer-reviewed literature that show CBD and CBG effectiveness in various neurodegenerative diseases. These studies are rather incomplete in terms of elucidating the effects of CBD on AD, and there is no available study showing the effects of CBG on AD in the literature. Based on the neuroprotective and neuromodulatory effects of CBD and CBG in other neurodegenerative diseases, it is believed that it might be effective in AD. As expected, in our study of behavioural experiments in rats and biochemical and histological analyses, CBD and CBG treatments improved the defects of AD in the $A\beta_{1-42}$ induced AD model. Thus, our study observations could advocate that *C. sativa* (a natural product of herbal origin and its components) can be useful in the development of effective treatment for AD.

Conclusion

Since the commonly used products (e.g., cosmetics, energy bars, and beverages) contain substantial amounts of non-psychoactive cannabis ingredients, more people are consuming these products in their daily lives. However, studies have shown that non-psychoactive cannabis components are effective in various diseases. Our study highlights the importance of studies with cannabis derivatives, as we have elucidated the efficacy of cannabis derivatives (*viz*., CBD and CBG) on Passive avoidance test, Morris's water maze test, inflammatory cytokines' levels, and $A\beta_{1-42}$ expression in cerebral cortex and hippocampus specimens of $A\beta_{1-42}$ induced AD rats. A mechanistic study with the standard drug may be required in further preclinical studies before concluding/suggesting the use in a clinical setting.

Conflict of Interest

The authors declare no conflict of interest.

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