eISSN 2255-0569

ORIGINAL

The effect of *Cannabis sativa* on memory, apoptotic genes and inflammatory cytokines in rat

El efecto del Cannabis sativa sobre la memoria, los genes apoptóticos y las citoquinas inflamatorias en la rata

Farshid Parsa[1], Seyed Ebrahim Hoseini[2], Davood Mehrabani[3], Seyedeh. Sara Hashemi[4]

1. Department of Biology, Shiraz Branch, Islamic Azad University, Shiraz, Iran. 2. Department of Biology, Faculty of Science, Zand Institute of Higher Education, Shiraz, Iran. 3. Stem Cell Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. Burn and Wound Healing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran; Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, Li Ka Shing Center for Health Research and Innovation, University of Alberta, Edmonton, AB, Canada. 4. Burn and Wound Healing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

Corresponding author
Seyed Ebrahim Hoseini
Department of Biology, Faculty of Science,
Zand Institute of Higher Education, Shiraz, Iran
E-mail: ebrahim.hossini@yahoo.com

Received: 27 - VII - 2021
Accepted: 2 - VIII - 2021

doi: 10.3306/AJHS.2021.36.04.96

Abstract

Objectives: *Cannabis sativa* L. has important ingredients of delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD). CBD is non-psychotropic, but THC is psychotropic responsible for making people feel "high". This study investigated changes in memory, apoptosis and inflammatory cytokines following *C. sativa* use in experimental rats.

Methods: Forty-five Wistar rats were randomly divided to 3 equal groups of experimental receiving cannabis subcutaneously (2 mg/kg in 0.6 mL volume) for 3 weeks, sham receiving ethanol identically (0.6 mL), and control receiving normal saline similarly (0.6 mL). The animals' spatial memory was assessed for 3 weeks using mean percentage of alternation and number of entries in a Y-maze to confirm cannabis effect on the brain. Real-time PCR was conducted for expression analysis of primer pairs for Bax and Bcl-2 genes in response to cannabis. Changes in inflammatory cytokines of IL-1, IL-6, IL-10, TNFα, INFγ, superoxidase dismutase (SOD) and malondialdehyde (MDA) were assessed following cannabis use.

Results: Significant reduction in memory and expression of Bcl-2 genes and increase in expression of Bax and inflammatory cytokines of IL-1, IL-6, IL-10, TNFα, INFγ and SOD were noted following cannabis use.

Conclusions: Based on our findings and reduction in memory and expression of Bcl-2 gene, and increase in expression of Bax gene and inflammatory cytokines following cannabis use, the paramount public health importance of cannabis use, when targeted for medical purposes should come into consideration.

Keywords: *Cannabis sativa*, Memory, Apoptosis, Inflammation.

Resumen

Objetivos: El *Cannabis sativa L.* tiene importantes ingredientes de delta-9-tetrahidrocannabinol (THC) y cannabidiol (CBD). El CBD no es psicotrópico, pero el THC es psicotrópico, responsable de hacer que las personas se sientan "colocadas". Este estudio investigó los cambios en la memoria, la apoptosis y las citoquinas inflamatorias tras el uso de *C. sativa* en ratas experimentales.

Métodos: Cuarenta y cinco ratas Wistar fueron divididas aleatoriamente en 3 grupos iguales de experimentación que recibieron cannabis por vía subcutánea (2 mg/kg en 0,6 mL de volumen) durante 3 semanas, de simulación que recibieron etanol de forma idéntica (0,6 mL) y de control que recibieron solución salina normal de forma similar (0,6 mL). Se evaluó la memoria espacial de los animales durante 3 semanas utilizando el porcentaje medio de alternancia y el número de entradas en un laberinto en Y para confirmar el efecto del cannabis en el cerebro. Se realizó una PCR en tiempo real para el análisis de la expresión de los pares de cebadores para los genes Bax y Bcl-2 en respuesta al cannabis. Se evaluaron los cambios en las citoquinas inflamatorias de IL-1, IL-6, IL-10, TNFα, INFγ, superoxidasa dismutasa (SOD) y malondialdehído (MDA) tras el consumo de cannabis.

Resultados: Se observó una reducción significativa de la memoria y la expresión de los genes Bcl-2 y un aumento de la expresión de Bax y de las citoquinas inflamatorias de IL-1, IL-6, IL-10, TNFα, INFγ y SOD tras el consumo de cannabis.

Conclusiones: Basándonos en nuestros hallazgos y en la reducción de la memoria y la expresión del gen Bcl-2, y el aumento de la expresión del gen Bax y de las citoquinas inflamatorias tras el consumo de cannabis, debe tenerse en cuenta la importancia primordial del consumo de cannabis para la salud pública, cuando se destina a fines médicos.

Palabras clave: *Cannabis sativa*, Memoria, Apoptosis, Inflamación.

Introduction

Cannabis plant with scientific name of *Cannabis sativa L*. has important ingredients of delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), while CBD is non-psychotropic part and THC a psychotropic chemical responsible for making people feel "high". Cannabinoids are endogenous lipid-based retrograde neurotransmitters that produce endocannabinoids in the body bound to cannabinoid receptors [G-protein coupled receptors of cannabinoid receptor type 1 (CB1R) and cannabinoid receptor type 2 (CB2R)] expressed throughout central and peripheral nervous system. CB1R receptors are noted in excitatory glutamatergic neurons and inhibitory GABA-ergic neurons mostly prevalent in hippocampus, basal ganglia, frontal cortex, cerebellum, hypothalamus, spinal cord and peripheral nervous system. CB2R receptors are mostly visible on hematopoietic, immune and glial cells expressed in the periphery under normal healthy condition, but in conditions of disease or injury, an upregulation can happen within the brain in unhealthy conditions. So THC and CBD are mostly responsible for the therapeutic potentials of cannabis and cannabinoids, even, THC in cannabis and cannabinoid makes the consumers to feel "high" that can limit their clinical use[1,2].

Smoking was reported as the most common route of cannabis administration over the past few decades that can cause an increase in THC and a decrease in cannabidiol leading to an increase in the persistence of neuroanatomic changes in the brain during adolescence[3]. There are conflicting results for dopamine release after cannabis use. Acute THC administration was demonstrated to elicit striatal dopamine release in animals[4] and humans[5], but others reported no evidence for THC-induced dopamine release[6], because THC induces quantitatively less dopamine release in comparison to other psychostimulants such as amphetamine or methylphenidate[7].

Cannabis was illustrated to have benefit in various neuropsychiatric disorders including anxiety, psychosis, autism spectrum disorder, neuropathic pain, cancer pain, multiple sclerosis, migraine, Parkinson disease, Alzheimer disease, Huntington disease, epilepsy, hypoxic-ischemic injuries, and HIV, as well as chronic cardiovascular and respiratory effects. Also, adverse events such as diarrhea and somnolence may occur following cannabis use[1,2,7].

Erectile dysfunction is the most common male sexual disorder after cannabis use[8]. Regular marijuana use can have neurotoxic effects leading to disruptions in the brain development and significantly change neuro-developmental trajectories, neurochemical communication and genetic expression of neural development9. Chronic cannabis use can increase the risk of developing substance use disorders, and influence motivation, cognitive and executive function and emotion processing[7,10,11].

Cannabis is the most commonly used substance of abuse in the United States after alcohol and tobacco and its use has a global increasing trend along with the progressive legalization of both recreational and medical cannabis, despite the various health effects of this substance[2,12]. Neuropsychological studies revealed considerable understanding of the effects of cannabis on the brain due to advances in neuroimaging providing the chance to track neuronal activation, neuroanatomic changes, and metabolic and neurotransmitter activity during brain development[13].

With worldwide increases in cannabis use and decreases in perceived risk, it is necessary to reevaluate its adverse effects especially on nervous system[14] and the brain as the mostly target organ after administration of cannabis. A suppressive effect of THC on secretion of different cytokines by cultured murine spleen cells was previously reported[15]. THC at doses of 5-100 mg/kg was demonstrated to decrease the plasma concentration of interferon-alpha and –beta[16], while the immunomodulatory concentrations of used THC were more than physiological range of THC (1-100 ng/ml) observed in plasma of human marijuana smokers[17].

Watzl et al.[18] showed that CBD suppressed the secretion of IL-1, TNF and IFN, and THC the release of IFN. So this study was carried out to assess changes in memory, apoptotic genes and inflammatory indicators of malondialdehyde (MDA), superoxidase dismutase (SOD), interleukins of IL-1, IL-6, IL-10, tumor necrosing factor alpha (TNFα), and interferon-gamma (INFγ) following *C. sativa* use in experimental rats.

Methods

Preparation of *C. sativa*

C. sativa L. was donated by Shiraz Islamic Azad University herbarium in Shiraz, Iran. The whole plant was left at room temperature in shadow to be dried and then changed to powder (particles not greater than 1.8 inch). The maceration method was applied for extraction. In brief, the powder was soaked in a closed container containing 70% ethanol for 2-4 days, was later shaken twice per day, was filtered and was separated by a rotary evaporator. The extract was put in oven at 40°C to be dried. To reach the requested concentration for the research, the dried extract was finally dissolved in distilled water.

Animals and grouping

Forty five male 8 weeks old Wistar rats (200-220 g) were purchased from Shiraz University of Medical Sciences, Shiraz, Iran and kept at 22±1°C with 12 h light/dark cycle, and had free access to water and food and were allowed to accommodate to their condition before the experiments. The study was approved in Ethics Committee of Shiraz Islamic Azad University (7-E-IR-

MIAU.REC.80-B-1397), and all experiments were carried out based on guidelines of Iran Veterinary Organization.

Rats were randomly divided to three equal groups. The experimental group received daily subcutaneous administration of 2 mg/kg of the cannabis for three weeks in 0.6 mL volume. The sham group was injected identically with equal volume of 70% ethanol and the control group received distilled water similarly. All experiments were undertaken between 08:00 and 12:00 AM.

The memory Y-maze test

The Y-maze was used to assess animals' spatial memory as described before[19]. Briefly, Y-maze possess three similar 16 arms, while the maze floor involves soiled animal bed. Animals were investigated in dark phase of their cycle. First test included blockage of one arm with black Plexiglas considered as the 'Novel' arm in the second test. The other remaining arm was the 'Start' arm and the last arm was the 'Other', and all arms were randomized between animals. The rats were allowed to explore the start and other arms for 15 minutes as acquisition trial. Quantification of the entries into an arm was recorded when the rat entered 10 cm of a given arm. After the first assessment, the rats returned to their cage. After 4 hours, the animals were transferred in the same start arm as first testing was undertaken.

The arm that was previously blocked was available in the second test and the animals were allowed to explore all arms for 5 minutes. In the second trial, first arm entrance between the novel and other arm was designated as the First Choice denoting to the percentage of rats with recognition of the novel arm-arm discrimination memory. The time spent in each arm for each minute was quantified and recorded (Dwell Measure) to indicate the inspective exploratory behaviors. The number of entries made into each arm for each minute was quantified and recorded (Entry Data) to depict the inquisitive behavior of responses to novelty. The interaction on Dwell and/or Entry data between arm choice, exposure and period time of injection showed the spatial recognition memory of the previously unvisited arm. Arm entry was completed after location of the entire tail was within the arm. Alternation was defined as successive entries into the three arms on overlapping triple sets.

Quantitaive real time PCR

To quantify expression of Beta-2 microglobulin (B2m), Bax, and Bcl-2 genes, brain tissue was harvested for total cellular ribonucleic acid (RNA) extraction 1 to 3 weeks after treatment of the cells to cannabis using an RNA extraction kit (Cinna Gen Inc., Tehran, Iran). The ratio of optical density (A260/A280 and A260/A230) was assessed using a Nanodrop™ spectrophotometer (Nanodrop; Thermo Fisher Scientific Inc., USA), the quantity and quality of obtained RNA were determined. The cDNA was synthesized using 1000 ng total RNA in a first strand complementary DNA (cDNA) synthesis reaction applying the Revert Aid™ first strand cDNA synthesis kit (Thermo Fisher Scientific Inc., USA). The Bax and Bcl-2 genes were target and B2m was the housekeeping gene and an endogenous control. The sequences of the genes were determined using NCBI database and primer sets were designed by primer3 software (**Table I**).

Table I: Primers used in the present study.

Genes	Primer Sequences	Sizes (bp)
Bax	Forward: 5'-CTGCAGAGGATGATTGCTGA-3' Reverse: 5'-GATCAGCTCGGGCACTTTAG-3'	174
Bcl-2	Forward: 5'-ATCGCTCTGTGGATGACTGAGTAC-3' Reverse: 5'-AGAGACAGCCAGGAGAAATCAAAC-3'	134
B2m	Forward: 5'-CGTGCTTGCCATTCAGAAA-3' Reverse: 5'-ATATACATCGGTCTCGGTGG-3'	244

Abbreviation: bp, base pair.

Real time-polymerase chain reaction (PCR) was carried out using SYBR Green I as reporter dye and Step One Real time-PCR reactions (Applied Biosystems, USA). In each reaction, 200 nM of each primer was used for targeting the specific sequence. The PCR conditions were set as 10 min at 94°C followed by 40 cycles of 15 s at 94°C, 60 s at 60°C, and melting curve analysis ramping of 65-95°C. The amplification signals of different samples were normalized to B2m cycle threshold (Ct), and then the 2-delta delta cycle threshold (2-DDCt) method was applied to compare mRNA levels of various groups, which represented a fold-change in data analysis[20].

Assessment of inflammatory cytokines

A blood sample was provided in each group on days 7, 14 and 21 after interventions by cardiac puncture in tubes with heparin sodium for hematological tests and in serum-separated tubes for biochemical parameters. The coagulated blood was left to clot at room temperature for 30 min, and was latter centrifuged at 3600× g for 15 min. The following parameters were determined by commercial kits (Merck, Germany): IL-1, IL-6, IL-10, TNFα, INFγ, superoxidase dismutase and MDA.

Statistical analysis

Data were shown as mean±SEM (tandard error of the mean) and analyzed using statistical package for the social sciences software (SPSS, Version 20, Chicago, IL, USA). One-way Analysis of variance (ANOVA) and Post-hoc Tukey's were used for comparison. P values less than 0.05 were considered statistically significant.

Results

The memory Y-maze test

Regarding behavioral alteration when the groups were compared after 1, 2 and 3 weeks of cannabis use; a significant difference was noted between the groups ($P \leq 0.01$, $P \leq 0.01$, $P \leq 0.001$, respectively) revealing spatial

memory impairment following cannabis administration (**Figure 1**). Regarding the number of entries when comparison between the groups was conducted after 1, 2 and 3 weeks of cannabis use, a significant statistical difference was visible after one ($P \leq 0.01$), two ($P \leq 0.05$) and 3 weeks ($P \leq 0.01$) denoting to an impairment in spatial memory following cannabis use (**Figure 2**).

Figure 1: Comparison of the effect of cannabis on mean percentage of behavioral alterations between the control, sham and the experimental groups; 1, 2 and 3 weeks after interventions (P = 0.01, P = 0.01, P = 0.001, respectively).

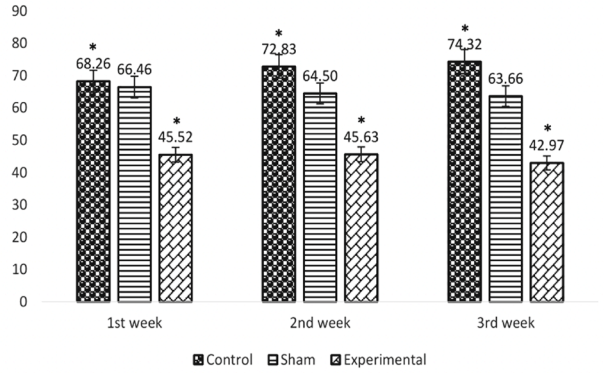


Figure 2: Comparison of the effect of cannabis on mean number of entries by rats between the control, sham and the experimental groups; 1, 2 and 3 weeks after interventions (P = 0.01, P = 0.05, P = 0.01, respectively).

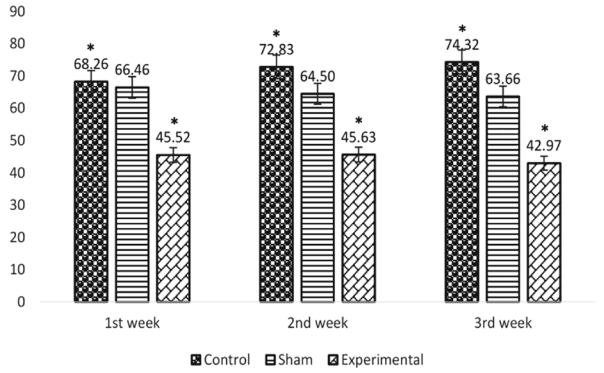
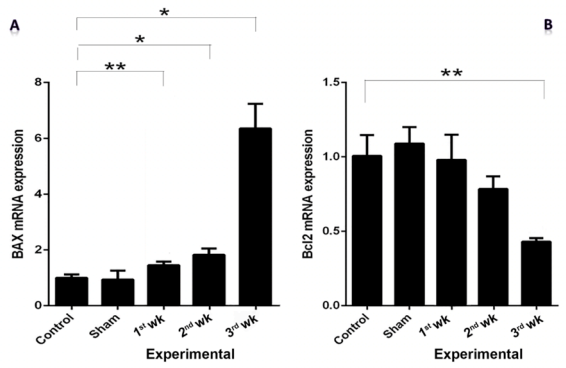


Figure 3: Using real-time quantitative PCR and assessment of the effect of Cannabis on various genes: (A). Bax (*P = 0.0003) (**P = 0.01) vs. control, (B). Bcl-2 (**P = 0.003) vs. control.



Quantitaive real time PCR

Regarding the expression of apoptotic genes, cannabis resulted to an increase in Bax expression (P = 0.0003, P = 0.01), and a decrease in Bcl-2 expression (P = 0.003) (**Figure 3**).

Assessment of inflammatory cytokines

Regarding MDA level, no significant changes were noted following cannabis use ($P > 0.05$). For SOD level, there were significant modification between sham and control group (P = 0.02) and between control and cannabis group after two (P = 0.01) and 3 (P = 0.03) weeks illustrating an increasing trend. Regarding IL-1, IL-6, and IL-10, there were significant modification between sham and control group (P = 0.0001) and between control and cannabis group after one (P = 0.0001), two (P = 0.0001) and 3 (P = 0.0001) weeks revealing a rising trend. The changes between sham and control group and between control and cannabis group after one, two and 3 weeks were also statistically significant ($P < 0.05$) demonstrating an increase. Totally, an increase was noted in IL-1, IL-6, IL-10, TNFα, INFγ and SOD following cannabis use, but MDA illustrated no significant change following cannabis use (**Table II**).

Discussion

C. sativa plant with the major psychoactive constituent of Δ-9 tetrahydrocannabinol (THC) is the most widely cultivated, trafficked and consumed substance among approximately 147 million people annually, consisting 2.5% of the world population[21]. Although cannabis is used for therapeutic purposes and has wide-ranging potential uses in medicine, with increasing legalization, still the adverse events in central nervous system and other health outcomes limit its adoption. In this study we showed an increase in IL-1, IL-6, IL-10, TNFα, INFγ and SOD following cannabis use, but MDA did not show any significant changes following cannabis use.

Similar to our findings, a suppressive effect of THC on secretion of different cytokines by cultured murine spleen cells was previously reported[15]. THC at doses of 5-100 mg/kg was demonstrated to decrease the plasma concentration of interferon-alpha and –beta[16], while the immunomodulatory concentrations of used THC were more than physiological range of THC (1-100 ng/mL) observed in plasma of human marijuana smokers[17]. It was shown that CBD suppressed the secretion of IL-1, TNF and IFN, and THC the release of IFN[18].

We showed that tumor necrosis factor secretion was affected by cannabis. It was already reported that murine macrophage phagocytosis was impaired by THC concentrations above 5 pg/mL[15]. Animal and human studies revealed that THC at physiological levels of 2.5 ~g/mL had no effect on NK cell activity *in vivo* and *in*

Table II: Changes in hematological variables following cannabis use.

Variable \ Group	Control (Mean ± SD)	Sham (Mean ± SD)	Cannabis 1 week (Mean±SD)	Cannabis 2 weeks (Mean ± SD)	Cannabis 3 weeks (Mean ± SD)
MDA **P value**	31.50 ± 2.12 N/A	19.66 ± 6.02 0.12	20.66 ± 3.05 0.18	18.75 ± 4.57 0.07	21.50 ± 5.80 0.193
Super Oxidase Dismutase **P value**	325.00 ± 35.35 N/A	409.66 ± 51.43 0.02	427.00 ± 8.71 0.01	426.50 ± 11.03 0.01	409.00 ± 19.18 0.029
IL-10 **P value**	325.00 ± 35.35 N/A	493.33 ± 4.93 0.0001	502.66 ± 5.50 0.0001	476.50 ± 18.06 0.0001	480.75 ± 9.53 0.0001
INFγ **P value**	136.00 ± 15.55 N/A	447.66 ± 25.10 0.0001	204.66 ± 8.32 0.03	406.75 ± 16.56 0.0001	575.75 ± 27.98 0.0001
TNF **P value**	647.50 ± 67.17 N/A	1291.66 ± 175.61 0.0001	948.00 ± 22.51 0.05	1058.50 ± 119.32 0.006	1321.50 ± 58.54 0.0001
IL-6 **P value**	11.00 ± 1.41 N/A	61.33 ± 10.69 0.0001	43.66 ± 1.52 0.0001	49.50 ± 1.73 0.0001	59.25 ± 3.30 0.0001
IL-1 **P value**	337 ± 11.31 N/A	944.66 ± 93.24 0.0001	744.00 ± 29.51 0.0001	814.50 ± 35.45 0.0001	930.75 ± 45.38 0.0001

vitro[22,23], while in vitro concentrations of THC above 5 pg/mL could decrease the NK cell activity[24,25], which was a dose-dependent modulation for IFN in another study[18]. No effect was noted no effect on IFN release compared with the control[26], but at concentrations more than 5 pg/mL THC, the release of interferon-alpha (IFN-α) and interferon-beta (IFN- β) was suppressed[15,18,26].

Cannabis use was demonstrated to be associated with a potentially beneficial decrease in systemic inflammation and immune activation such as IL-6 and TNFα in the context of antiretroviral-treated HIV infection[27]. Several studies illustrated the anti-inflammatory activity of CBD in cell lines and animal models of skin inflammation[28-30]. Sangiovanni et al.[29] showed that cannabis can inhibit the release of mediators of inflammation involved in wound healing via impairment of the NF-κB pathway and inhibiting the TNFα-induced NF-κB-driven transcription and inhibition of the release of IL-8 and MMP-9 in HDF and HaCaT cell lines. It was noted that cannabinoid compounds mitigated inflammation associated with alcohol use including circulating levels of the pro-inflammatory cytokines interleukin 6 (IL-6), IL-8, and IL-1β in the blood[31]. Our findings showed a decrease in expression of Bcl-2 and an increase in expression of Bax genes which were also confirmed by other researchers too[32-35].

Conclusions

In summary, based on our findings revealing a reduction in memory and expression of Bcl-2, an increase in expression of Bax and inflammatory cytokines of IL-1, IL-6, IL-10, TNFα, INFγ and superoxidase dismutase, we can conclude that changes in memory, apoptotic genes and inflammatory indicators following cannabis use emphasize paramount importance of cannabis when targeted for medical use. The results can be added to the literature regarding its public health issues.

Acknowledgements

The authors thank Islamic Azad University for financial support of this study, grant number 7-E-IR-MIAU. REC.80-B-1397, for funding this research. The authors would like to thank the efforts and assistance of the distinguished experts of the Stem Cell Research Center and the Comparative and Experimental Medical Research Center of the Shiraz University of Medical Sciences who collaborated in implementation of this project.

Conflict of interests

The authors have no conflict of interest.

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