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Neurobehavioral and Immunohistochemical Assessment of the Cerebellum in Adult Male Albino Wistar Rats Following *Cannabis Sativa* Administration

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Abstract
Reports abound on the use and effects of *Cannabis sativa*. High consumption may result in schizophrenia. It put some consumers in a state of coma, while some become unconscious after its consumption. Besides, it restores emotional stability in others and fills some consumers with a sense of wellbeing. Epidemiologic data suggest that *Cannabis sativa* use is a serious public health problem because it is highly addictive and is associated with a variety of neurological complications. The cerebellum is implicated in locomotor activity and is richly endowed with cannabinoid-1, CB1 receptors; there was the need to assess the microstructure of the cerebellum and to evaluate locomotor activity on an animal model following consumption of *Cannabis sativa* via various routes of administration. Graded doses of various preparations of *Cannabis sativa* were administered daily for 28 days as follows: Group B: 0.41 mg/kg, 0.2 mg/kg, and 0.13 mg/kg body weight of soxtlet extract of *Cannabis sativa* via oral ingestion. Group C: 4grams, 2grams and 1grams of grounded dried leaves of *Cannabis sativa* via inhalation. Group D: A mixture of 8grams, 4grams and 2 grams of grounded dried leaves of *Cannabis sativa* and 90grams of animal feed in each case. Group A served as the control while rats in Group E were given 1gm of 10% Tween 80 via oral ingestion. Before and after 28 days of administration of *Cannabis sativa*, the open field maze and Morris water maze experiments on neurobehavioral were carried out. Data were expressed as means ± standard error of the mean (M±SEM) and subjected to one way analysis of variance (ANOVA) using PRIMER, EXE, version 3.01. Significant difference between means was assessed by Student-Newman-Keuls post hoc test. 95% level of significance (P = .05) was used for the statistical analysis; while Microsoft excel 2010 package was used for graphs and error bars. The findings of this study thus suggest that the extract of *Cannabis sativa* obtained via soxtlet extraction technique has a mild and lesser psychoactive effect compared to other preparations of *Cannabis sativa*, thus presents a better method to be adopted in preparation of medical *Cannabis*.
Keywords: *Cannabis sativa*. Cerebellum. Albino *Wistar* rats

1. INTRODUCTION

Cannabis sativa is the taller variety of *Cannabis* plant, characterized by narrow serrated leaves and loose spear-like flower clusters that can be extremely resinous. (www.CannabisDispensary.ca; http://thegreencross.org/why-cannabis; www.kanducannameds.com). *Cannabis sativa*, also known as marijuana, pot, reefer, buds, grass, weed, ganja, boom, gangster, Mary Jane, sinsemilla, joint, hash oil, and Thai stick, just to mention but a few, refers to preparations of the Cannabis plant intended for use as a psychoactive drug and as medicine (Houghton, 2007 & Fusar-Poli, et al., 2009).

Some people consume *Cannabis sativa* for recreational use which is characterized by mild euphoria, relaxation, and intensification of ordinary sensory experiences and as a cure to some disease conditions such as chronic pain, low blood pressure and alleviation of some undesirable side effects arising from chemotherapy treatment of cancer. There is also a wide spread impression that consumption of *Cannabis sativa* is harmful. Cannabis sativa produces a constellation of effects in humans, including alterations in perception and mood, intoxication, increased heart rate, physical dependence upon chronic use, and cognitive impairment (Pacher, et al., 2006).

Cannabis has psychoactive and physiological effects when consumed. The minimum amount of THC required to have a perceptible psychoactive effect is about 10 micrograms per kilogram of body weight. Aside from a subjective change in perception and, most notably, mood, the most common short-term physical and neurological effects include increased heart rate, lowered blood pressure, impairment of short-term and working memory, psychomotor coordination, and concentration. (Riedel, and Davies, 2005),

In 2004, the United Nations estimated that global consumption of Cannabis sativa indicated that approximately 4.0 percent of the adult world population (162 million people) used *Cannabis sativa* annually, and that approximately 0.6 percent (22.5 million) of people used *Cannabis sativa* daily. (United Nations Office on Drugs and Crime, 2006).

Since the early 20th century *Cannabis sativa* has been subject to legal restrictions with the possession, use, and sale of *Cannabis sativa* preparations. The United Nations has found that *Cannabis sativa* is the most

used illicit drug in the world. (World Drug Report 2010; "*Cannabis*: Legal Status", 2011; Erowid.org.)

Chemically, the major psychoactive compound in *Cannabis sativa* is delta-9-tetrahydrocannabinol (Δ 9-THC); it is one of 400 compounds in the plant, including other cannabinoids, such as cannabidiol (CBD), cannabinol (CBN), and tetrahydrocannabivarin (THCV), which can produce sensory effects unlike the psychoactive effects of delta-9-tetrahydrocannabinol (Fusar-Poli, *et al*. 2009).

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Δ 9-THC is metabolized mainly to 11-OH-THC (11-hydroxy-THC) by the human body. This metabolite is still psychoactive and is further oxidized to 11-Nor-9-carboxy-THC (THC-COOH). In humans and animals, more than 100 metabolites could be identified, but 11-OH-THC and THC-COOH are the dominating metabolites. Metabolism occurs mainly in the liver by cytochrome P450 enzymes CYP2C9, CYP2C19, and CYP3A4. (Geibpraserta, *et al*, 2009).

Pathomechanism include excitotoxicity, which may lead to an acute or sub-acute leukoencephalopathy, and vascular complications, including vasoconstriction, vasculitis, or hypertension, which may lead to intracranial hemorrhage or ischemia. (Geibpraserta, *et al*, 2009).

Receptors for THC and other cannabinoid compounds (termed CB1 receptors) and endogenous CB1 receptor ligands (termed endocannabinoids) are present in the brain, especially in the frontal cortex, basal ganglia, cerebellum, and limbic regions. Cannabinoid actions in the basal ganglia and the cerebellum probably account for effects on psychomotor control. In humans, *Cannabis sativa* impairs balance and may induce prolonged immobility. (John, 2003).

Cerebellar damage produces disorders in fine movement, equilibrium, posture and motor learning (Fine, Ionita and Lohr, 2002).

1.2. SPECIFIC OBJECTIVES OF THE STUDY

a. Conduction of neurobehavioral studies for locomotion and exploratory activities on adult albino male wistar rats using open field maze.
b. Demonstration of the expression of neuron specific enolase (NSE), in the cerebellum of adult albino male *Wistar* rats using immunohistochemical staining method.

2. MATERIALS AND METHODS

Cannabis sativa was legally obtained from the National Drug Law Enforcement Agency (NDLEA), Akwa Ibom State Command, and identified as such by the same agency with a reference code (an equivalent of voucher specimen number) NDLEA/AKSC/59/VOL.1/57.

The dried leaves of *Cannabis sativa* were grounded and weighed. The extract was obtained via soxlet extraction technique and was screened for the presence of alkaloids, saponins, tannins, and cardiac glycoside using standard methods (Odebiyi and Sofowora, 1978; Trease and Evans, 1989).

Determination of LD_{50} using Lorke's method. The aim of LD_{50} study was to ascertain the highest dose of *Cannabis sativa* at which the mice/rat will survive and the lowest dose at which the mice/rat will die; hence, the dosage the mice/rat will tolerate.

Mice were used for this particular study. The mice were sourced from the animal house of the Department of pharmacology, Faculty of pharmacy, University of Uyo. Based on the outcome of this study, the maximum dosage of the active ingredients of *Cannabis sativa* that produced 100% mortality was 700mg/kg, while the minimum dosage that produced 0% mortality was 600mg/kg.

$LD_{50} = \sqrt{AB}$

Where:

A = the maximum dosage that produced 100% mortality

B = the minimum dosage that produced 0% mortality

$LD_{50} = \sqrt{AB}$

$= \sqrt{600 \times 700}$

$= 648.07$mg/kg

For the administration of Cannabis sativa to mice, 10%, 20% and 30% of $\sqrt{600 \times 700}$ (648.07mg/kg) was used.

Where:

The value of 10% of 648.07mg/kg = low dose = 64.81mg/kg

The value 0f 20% of 648.07mg/kg = middle dose = 129.61mg/kg

The value of 30% of 648.07mg/kg = high dose = 194.42mg/kg

10% Tween 80 was the vehicle used to dissolve Cannabis sativa in water.

10% Tween 80 was prepared by adding 10ml of Tween 80 to 90ml of water.

The rats for this research were sourced from the animal house of the Faculty of Basic Medical Sciences, University of Uyo. Body weight ranging from 200g to 220g was one of the main criteria used for selection. Two weeks was allowed for the rats to adapt to their new surroundings. The rats were housed in clean transparent cages in a standard animal room. They were fed pelletized rodent diet ad libitum (Vital feeds PLC), and had free access to good drinking water.

A total of fifty (55) adult male albino *Wistar* rats were used for the research. The rats were divided into five groups: A, B, C, D, and E.

Group A, the control, was made up of five male rats and were given normal clean distilled water.

Group B, was given the extract of *Cannabis sativa* via oral ingestion. Group B, comprised of three subgroups, namely, B1, B2, and B3. B1 comprising of 5 male rats was given high dose (0.41 mg/kg body weight) of *Cannabis sativa* via oral ingestion. B2 comprising of 5 male rats was given middle dose (0.2 mg/kg body weight) of *Cannabis sativa* via oral ingestion. B3 comprising of 5 male rats was given low dose (0.13 mg/kg body weight) of *Cannabis sativa* via oral ingestion.

Group C, was exposed to smoke from ground dried leaves of *Cannabis Sativa*. Group C comprised of three subgroups, namely, C1, C2, and C3. This was done by placing red-hot charcoal at the bottom level of a desiccator. The required number of grams of dried grounded *Cannabis sativa* leaves was on each occasion spread on top of the red-hot charcoal. Galvanized wire gauze was immediately placed above this compartment to separate it from the compartment above it containing the rat and to serve as a platform for the rat. The desiccator was covered with its lid once the rat was placed inside, and timing started. The rats were allowed to inhale the smoke for three minutes. After every three minutes, the lid was opened for ten seconds to alternate the flow of smoke and fresh air to the animals to mimic puffing. C1 comprised of 5 male rats. All the rats in this group were made to inhale (were exposed to) smoke from 4grams of grounded dried leaves of *Cannabis sativa* for three minutes; with 10 seconds interval between each minute to enable them breath in fresh air. C2 comprised of 5 male rats. All the rats in this group were made to inhale (were exposed to) smoke from 2grams of ground dried leaves of *Cannabis sativa* for three minutes; with 10 seconds interval between each minute to enable them breath in fresh air. C3 comprised of 5 male rats. All the rats in this group were made to inhale (were exposed to) smoke from 1gram of ground dried leaves of *Cannabis sativa* for three minutes; with 10 seconds interval between each minute to enable them breath in fresh air.

Group D, was given a mixture of grounded dried leaves of *Cannabis sativa* and animal feed. Group D, comprised of three subgroups, namely, D1, D2, and D3. D1 comprising of 5 male rats were given a mixture 8grams of grounded dried leaves of Cannabis sativa and 90grams of animal feed. D2 comprising of 5 male rats were given a mixture 4grams of grounded dried leaves of *Cannabis sativa* and 90grams of animal feed. D3 comprising of 5 male rats were given a mixture 2grams of grounded dried leaves of Cannabis sativa and 90grams of animal feed.

Group E, has only one group, E. It comprised of 5 male rats. They were given 1gm of 10% Tween 80 via oral ingestion.

3.STATISTICAL ANALYSIS

Data were expressed as means ± standard error of the mean (M±SEM) and subjected to one way analysis of variance (ANOVA) using PRIMER, EXE, version 3.01. (MacGraw-Hill, 1992). Significant difference between means was assessed by Student- Newman-Keuls post hoc test. 95% level of significance (P = .05) was used for the statistical analysis; while Microsoft excel 2010 package was used for graphs and error bars.

4. RESULTS.

4.1. The comparison of frequency of line crossing

The comparison of frequency of line crossing in open field maze was significantly lowest ($P<0.001$) for the rats in group C1, that were made to inhale smoke from 4grams of ground dried *Cannabis sativa* leaves compared to the control group A. The frequency of line crossing in C1 was significantly lower compared to C2, C3 that inhaled middle and low doses of *Cannabis sativa*. The frequency of line crossing in C1 was also lower than other experimental groups, namely: B1, B2, B3, and D1, D2, D3, that were given high, middle and low doses of the extract of *Cannabis sativa* via ingestion, and a mixture of feed and grounded dried leaves of Cannabis sativa, respectively. The same thing was observed in rats of group E, that was given 10% tween 80. The frequency of line crossing was significantly low (P = .05) in B1, B2, B3, C2, C3, D1, D2, and D3 compared to the control. The figures are shown in table 1 below.

4.1.2. Comparison of the frequency of rearing

It was observed that the experimental groups especially those with high and middle doses, namely: B1, B2, C1, C2, D1, D2, in addition to B3, C3, D3 were significantly lower ($P<0.001$) compared to control group A and

experimental group E. The result is shown in table 2 below.

4.1.3. Comparison of the frequency of center square duration

In the open field, there was no significant difference ($P \leq 0.01$) between the experimental groups with respect to center square duration. However, the high dose, middle dose and low dose treated subgroups in each group were significantly lower ($P < 0.05$) compared to the control group A and experimental group E that were given 5ml of 10% Tween 80.

4.1.4. Comparison of the frequency of stretched attend posture (SAP)

The frequency of stretched attend posture (SAP) was higher in all the experimental groups compared to the control group A and experimental group E. There was no significant difference ($P \leq 0.01$) between the control A and E.

Table 1. Comparison of the frequency of line crossing in the open field test following administration of *Cannabis sativa*(*C.S.*)

Group	Subgroup	Frequency of line crossing
A	A	117.80±1.02
B	B1	79.80±0.86*
	B2	83.60±1.03*
	B3	101.20±1.24*^{ab}
C	C1	53.50±1.03*^{ab}
	C2	59.40±0.51*^{ab}
	C3	59.80±0.58*^{ab}
D	D1	72.60±1.12*^{ab}
	D2	73.60±1.12*^{ab}
	D3	78.40±0.81*^{b}
E	E	100.40±3.44*^{ab}

KEY:

Values are expressed as mean±standard error of mean (S±SEM). NS = not significant compared to control at P=.05. * = significant compared to control at P=.05. a = significant compared group 2 at p=0.05. b = significant compared group 3 at p=.05

A = control group
B1 = rats given 0.41mg/kg body weight of soxtlet extract of C.s. via oral ingestion
B2 = rats given 0.20mg/kg body weight of soxtlet extract of C.s. via oral ingestion
B3 = rats given 0.13mg/kg body weight of soxtlet extract of C.s. via oral ingestion
C1 = rats exposed to smoke from 4g of grounded dried leaves of C.s.
C2 = rats exposed to smoke from 2g of grounded dried leaves of C.s.
C3 = rats exposed to smoke from 1g of grounded dried leaves of C.s.
D1 = rats given a mixture of 8g grounded dried leaves of C.s.and 90g of animal feed.
D2 = rats given a mixture of 4g grounded dried leaves of C.s.and 90g of animal feed.
D3 = rats given a mixture of 2g grounded dried leaves of C.s.and 90g of animal feed.
E = rats given 1gm of 10% Tween 80 via oral ingestion.

Table 2. Comparison of the frequency of rearing in the open field test following administration of *Cannabis sativa.* (C.s.)

Group	Subgroup	Frequency of rearing
A	A	11.87±0.63
B	B1	10.19±0.01*a
	B2	10.37±0.08*
	B3	11.91±0.29^{NSab}
C	C1	6.22±0.20*^{abc}
	C2	6.05±0.14*^{abc}
	C3	7.00±0.11*^{abc}
D	D1	7.89±0.13*^{b}
	D2	9.84±0.13*
	D3	10.38±0.10*
E	E	12.78±0.34*^{ab}

Values are expressed as mean±standard error of mean (S±SEM). NS = not significant compared to control at P=.05. * = significant compared to control at P=.05. a = significant compared group 2 at p=.05. b = significant compared group 3 at p=0.05.

Table 3. Comparison of the frequency of center square duration in the open field test following a dministration of *Cannabis sativa*(*C.s*.)

Group	Subgroup	Frequency of center square duration
A	A	6.60±0.24
B	B1	4.60±0.24*
	B2	5.40±0.24*^{a}
	B3	5.80±0.20^{NSabc}
C	C1	2.20±0.20*^{abc}
	C2	2.40±0.24*^{abc}
	C3	2.80±0.37*^{abc}
D	D1	4.00±0.32*
	D2	4.60±0.24*
	D3	4.80±0.20*^{a}
E	E	6.40±0.24^{NSabc}

Values are expressed as mean±standard error of mean (S±SEM). NS = not significant compared to control at P=.05. * = significant compared to control at P=.05. a = significant compared group 2 at p=0.05. b = significant compared group 3 at p=0.05.

Table 4 Comparison of the frequency of stretched attend posture (SAP) in the open field test following administration of *Cannabis sativa*(C.s.)

Group	Sub group	Stretched attend position
A	A	6.00±0.06
B	B1	$6.40\pm0.11^{*ac}$
	B2	$6.50\pm0.08^{*a}$
	B3	6.88±0.03*
C	C1	$8.90\pm0.05^{*ab}$
	C2	$7.30\pm0.08^{*abc}$
	C3	$8.84\pm0.17^{*abc}$
D	D1	$6.40\pm0.06^{*bc}$
	D2	$6.70\pm0.05^{*bc}$
	D3	$6.70\pm0.05^{*bc}$
E	E	6.08 ± 0.07^{NSab}

Values are expressed as mean±standard error of mean (S±SEM). NS = not significant compared to control at P=.05. * = significant compared to control at P=.05. a = significant compared to group B1 at p=0.05. b = significant compared to group B2 at p=.05. c = significant compared to group B3 at p=.05

NSE IMMUNOSTAINING METHOD FOR DEMONSTRATION OF NEURONS IN THE CEREBELLUM

(Immunostaining method for neuron specific enolase (NSE). X400).

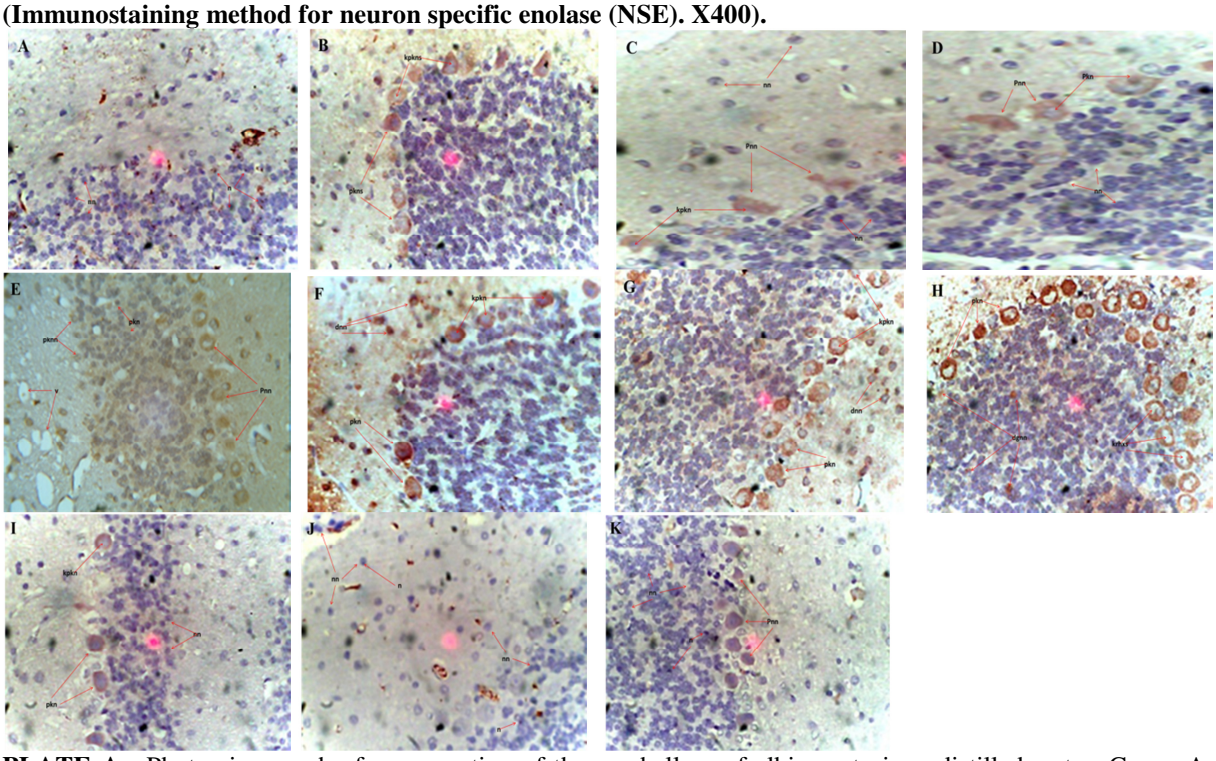

PLATE A . Photomicrograph of cross section of the cerebellum of albino rat given distilled water. Group A. Section is characterized by monomorphic population of small-sized purple-colored interneurons (granule cells),(nn), and dark stained nuclei(n). (red arrows). Inference: not affected (negatively stained).

PLATE B. Photomicrograph of cross section of the cerebellum of albino rat given 0.41mg/kg of soxtlet extract of *Cannabis sativa* via oral ingestion. Group B1. Section revealed pyknosis(pkns) of purkinje neurons with concurrent swelling and degeneration of the nuclei suggesting karyopyknosis(kpkns). Inference: moderately affected (moderately positive).

PLATE C. Photomicrograph of cross section of the cerebellum of albino rat given 0.20mg/kg of soxtlet extract

of *Cannabis sativa* via oral ingestion. Group B2. Section is characterized by monomorphic population of small-sized purple-colored interneurons(nn). The section revealed a single layer of degenerating brown colored projection neurons (Purkinje neurons) (Pnn) with karyopyknotic nuclei (kpkn). (red arrows). Inference: slightly affected (slightly positive). .

PLATE D. Photomicrograph of cross section of the cerebellum of albino rat given 0.13mg/kg of soxtlet extract of *Cannabis sativa* via oral ingestion. Group B3. Section is characterized by monomorphic population of small-sized purple-colored interneurons(nn). The section revealed a single layer of degenerating light-brown colored projection neurons (Purkinje neurons) (Pnn) with karyopyknotic nuclei (kpn). (red arrows). Inference: slightly affected (slightly positive).

PLATE E. Photomicrograph of cross section of the cerebellum of albino rat exposed to smoke from 4g of grounded dried leaves of *Cannabis sativa*. Group C1. Section is characterized by classic appearance of complete degenerating Purkinje neurons(Pnn) with pyknotic granule cell neurons(pknn) and nuclei(pkn). In addition, there is vacuolation(v) within the underlying granule cell layer. Inference: severely affected (strongly positive).

PLATE F. Photomicrograph of cross section of the cerebellum of albino rat exposed to smoke from 2g of grounded dried leaves of *Cannabis sativa*. Group C2. Section revealed pyknotic Purkinje neurons(pkn) and karyopyknotic nuclei(kpkn) in addition to degenerated neurons(dnn) in the overlying molecular layer. Inference: severely affected (strongly positive).

PLATE G. Photomicrograph of cross section of the cerebellum of albino rat exposed to smoke from 1g of grounded dried leaves of *Cannabis sativa*. Group C3. Section revealed pyknotic Purkinje neurons(pkn) and karyopyknotic nuclei(kpkn) in addition to degenerated neurons(dnn) in the overlying molecular layer. Inference: severely affected (strongly positive).

PLATE H. Photomicrograph of cross section of the cerebellum of albino rat given a mixture 8g of grounded dried leaves of *Cannabis sativa* and 90g of animal feed. Group D1. Section revealed pyknotic Purkinje neurons(pkn) and karyorrhexis(krhxs) in addition to degenerated neurons(dnn) in the overlying molecular layer. Degeneration occurred in a few granule cell layer neurons(dgnn). Inference: severely affected (strongly positive).

PLATE I. Photomicrograph of cross section of the cerebellum of albino rat given a mixture 4g of grounded dried leaves of *Cannabis sativa* and 90g of animal feed. Group D2. Section is characterized by monomorphic population of small-sized purple-colored interneurons (granule cells),(nn) with pyknotic Purkinje neurons(pkn) with concurrent swelling and degeneration of the nuclei suggesting karyopyknosis(kpkns). Inference: slightly affected (slightly positive).

PLATE J. Photomicrograph of cross section of the cerebellum of albino rat given a mixture 2g of grounded dried leaves of *Cannabis sativa* and 90g of animal feed. Group D3. Section is characterized by monomorphic population of small-sized purple-colored interneurons (granule cells),(nn) with dark stained nuclei(n). (red arrows). Inference: not affected (negatively stained).

PLATE K. Photomicrograph of cross section of the cerebellum of albino rat given 1gm of 10% tween 80. Group E. Section is characterized by monomorphic population of small-sized purple-colored interneurons(nn) (granule cells), and a single layer of large-sized projection neurons (Purkinje neurons) (Pnn); and dark stained nuclei(n) (red arrows). Inference: not affected (negatively stained).

5. DISCUSSION AND CONCLUSION

In normal adolescents the volume of cerebellar gray matter starts to decrease around puberty and continues until early adulthood (Diamond, 2000; Ostby et al, 2009). This increase in gray matter volume in the cerebellum replicates results in adults (Cousijn et al, 2012) and in adolescents (Medina et al, 2010). Alterations of this phenomenon have been observed in various psychiatric conditions (Mackie et al, 2007) (Jarvis et al, 2008) (Castellanos et al, 2002) (Pujol et al, 2004) (Hill et al, 2003) and in adolescents with familiar history of severe alcohol abuse (Hill et al, 2007).

It has been hypothesized that this normal reduction in gray matter volume in the cerebellum is due to the pruning of the synaptic connections (Cohen-Cory, 2002). One possible reason for abnormal pruning could be the toxic effect of THC at a critical period of brain maturation. Endogenous cannabinoids have an important role in synaptic pruning due to their interaction with GB1 receptors controlling the release of glutamate and GABA (Bossong and Niesink, 2010). Exogenous cannabinoids might disturb this system by competing for the receptors, thus inhibiting the pruning particularly in receptor-rich areas like the cerebellum (Casu et al, 2005) or the prefrontal cortex (Bossong and Niesink, 2010).

Those opposed to *Cannabis sativa* use substantiated their stand by citing evidence of the personal and social harms of *Cannabis* use. It is unjustifiable to deny the clinical potential of *Cannabis sativa.* The present findings have shown that the Soxhlet extract of *Cannabis sativa*, compared to other preparations of *Cannabis sativa*, is not likely to produce severe toxicological effects on the central nervous system (cerebellum, with respect to this study). Based on these findings, this mode of preparation can be used in the development of therapeutic strategies in view of the very low toxicity and the generally benign side effects of this extract.

RECOMMENDATION

This could be regarded as preliminary probes; further research aimed at understanding the real ingredients or part(s) of the contents of *Cannabis sativa* that was either eliminated or destroyed during the Soxhlet extraction process may help in the development of therapeutic strategies to prevent or ameliorate *Cannabis sativa*-induced impairments and its disruptive effect on performance in a wide range of animal models of learning and memory.

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