

# Folic Acid Reverses the Effects of Cannabis on the Brain of New Born Wistar Rats

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## Abstract

There are conflicting reports on the effect of Cannabis on brain cells, some reports support damage to brain cells while others do not. This work was carried out at the neuroscience unit of the Department of Veterinary Anatomy, University of Ibadan Oyo State, Nigeria. It investigated the neurodegenerative effect of extract of *Cannabis sativa* plant on brain regions in newborn Wistar rats exposed to Cannabis *in utero* and also combining the antioxidant and cell proliferative properties of folic acid to reverse these changes particularly in the Purkinje layer of the cerebellum in the group that received folic acid and cannabis. Four groups of pregnant Wistar rats were treated from Day 5 to 20 of pregnancy as follows: group A received 2ml of normal saline solution per os, group B received 2.0 mg /kg body weight of ethanolic extract of *Cannabis sativa* plant dissolved in normal saline per os, group C received 2.0 mg /kg body weight of *Cannabis sativa* extract and 40 mg/kg body weight of folic acid tablets (Emzor®) and group D received 2 mg/kg body weight of folic acid per os. The rat pups from group B showed deficit in locomotor function with spongiosis and astrogliosis in the corpus callosum, cerebellum and hippocampal regions of the brain. While pups from group A showed normal locomotor activities with no visible lesions in regions of the brain such as the cerebrum, cerebellum and the hippocampus. Group C pups were presented with normal locomotor activities with congestion of vessels in cerebrum and meninges and scattered areas of cell loss in the cerebrum, cerebellum hippocampus and corpus callosum and group D pups showed normal locomotor function, with vascular congestion in the meninges and cerebrum. We, therefore, concluded that folic acid which is a crucial factor in cell division, neurotransmitter production and an antioxidant in the nervous system could have a reversal of behavioural and locomotor deficits on nervous tissues exposed to *Cannabis sativa* extract by reducing the rate of cell death and increasing cellular stability and integrity in brain.

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## Keywords

Ethanollic Extract of *Cannabis sativa*, Folic Acid, Transplacental Administration, Locomotor Deficit

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## 1. Introduction

Cannabis plant originated from Northwest Himalayas. It is an annual deciduous plant with leaves palmately compound or digitate and possess serrated leaflets. Three varieties of cannabis plant known are *Cannabis sativa*, *Cannabis indica* and *Cannabis ruderalis* [1] [2]. Marijuana is a derivative prepared from the *Cannabis sativa* plant. It possesses cannabinoids, which produce mental and physical effects when consumed and is the most widely used illicit drug [3] [4] [5].

The active ingredient in cannabinoid is tetrahydrocannabinol (THC) and it gives three-phase effect in rats. A dose of 0.2 mg/kg decreased locomotor activity, while 1 - 2 mg/kg stimulated movements and at 2.5 mg/kg there is catalepsy [6]. When taken orally, the psychoactive effects take longer to manifest and generally last longer, typically lasting for 4 - 10 hours after consumption [7].

Works on Marijuana have shown significant performance impairment, 1 - 2 hours after use and residual effects up to 24 hours. There are evidences of cannabinoid receptors in rat brains during prenatal development and transfer of cannabinoids through placental blood during the gestation both in humans and in rodents [8] [9].

However, animal studies have yielded conflicting results. Treatment of rats with high doses of THC given orally for 3 months [10] or subcutaneously for 8 months [11] was reported to lead to neural damage in the hippocampal CA3 zone, with shrunken neurons, reduced synaptic density and loss of cells. However, in another study the potent synthetic cannabinoid WIN55, 2122 was administered twice daily (2 mg/kg) to rats and led to an apparent increase in hippocampal granule cell density, and increased dendritic length in the CA3 zone. In perhaps the most severe test of all, rats and mice were treated with THC 5 days each week for 2 years and no histopathological changes were observed in brain, even after 50 mg/kg/day (rats) or 250 mg/kg/day (mice) [12].

Folic acid/vitamin B9 is biologically active as tetrahydrofolate [13] [14]. It aids rapid cell division and growth, such as in infancy and pregnancy [15]. It improves memory status by reducing oxidative stress and maintaining the integrity of neurons during aging [16] and offer a protective role in neural cells in pre-eclampsia model rats [17].

Cannabis has been documented to cause damage to nerve cells through apoptotic mechanism [18] and available data suggest recovery of nerve cells from apoptotic damage by administration of Folic acid [19]. However, there are no available literatures on attempt to reverse the damage to nerve cells by Cannabis

using an anti-apoptotic factor.

The study, therefore, will investigate the effect of high dose folic acid on cannabis-induced neurotoxicity using changes in body and brain weights, behavioral tests, histology and immunohistochemistry.

## 2. Materials and Method

Four groups of Wistar rats with three matured females and one male per group were used in this research.

This work was carried out at the neuroscience unit of the Department of Veterinary Anatomy, University of Ibadan Oyo State, Nigeria from 24-5-2012 to 29-6-2012.

They were housed in a well-ventilated room in plastic rat cages, given water and feed ad libitum and allowed to mate. The animals received humane care according to criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science.

Ethic regulations were followed in accordance with National and Institutional Guidelines for the Protection of Animal Welfare during the experiments.

Mating success was confirmed on the second day of introduction of the male rats by vulva examination for white plug. The females successfully whelped 21 days after mating and were allowed to suckle their pups for 15 days before sacrifice was done.

### 2.1. Preparation of Extract of *Cannabis sativa* Plant

Extract of *Cannabis sativa* plant was prepared by ethanol method [20]. 89.9 g of the leaves and seeds were soaked in 600 ml of ethanol 70% for 24 hrs and evaporated to dryness at 40°C to yield 11.889 g of the extract (delta 9 tetrahydrocannabinol) in syrup form.

2.054 g of extract was dissolved in normal saline (500 ml), and given at dose rate of 2 mg/kg/os using stomach tubes.

### 2.2. Folic Acid Tablets

Folic acid tablet (5 mg, Emzor®), was dissolved in normal saline and given at the dose rate of 40 mg/kg and 2 mg/kg/os using stomach tubes.

### 2.3. The Treatment Groups

**Group A**—Members received 0.2 ml of normal saline *per os* from Day 5 to 20 of pregnancy.

**Group B**—Members were given 2 mg per kg body weight of *Cannabis sativa* extract *per os* from Day 5 to 20 of pregnancy.

**Group C**—Members received 2 mg per kg body weight of *Cannabis sativa* extract *per os* and 40 mg per kg body weight of folic acid *per os* from Day 5 to 20 of pregnancy.

**Group D**—Members received 2 mg per body weight of folic acid *per os* from

Day 5 to 20 of pregnancy.

#### **2.4. Sample Collection**

The body weights of pups were taken on Days 6 & 15 (four pups/group) using a bench top balance and subjected to behavioral test on Day 15 (eight pups/group).

Sacrifice was done by administering ketamine 100 mg/kg Bwt and xylazine 10 mg/kg Bwt im.

The cranium was dissected to remove the brain, weighed and processed for Immunohistochemistry [21].

#### **2.5. Immunohistochemistry**

Procedure for anti-glial fibrillary acid protein:

Fixation of brain tissue is done in 4% phosphate buffered formalin for 72 hrs. Tissue is then processed for routine paraffin wax embedment and section cut at 5  $\mu$ m for immunohistochemistry

Slides containing cut sections are heated in 10 mM citrate buffer for 25 minutes in microwave to retrieve the antigen. This is followed by peroxidase quenching in 3% hydrogen peroxide/methanol.

All sections were blocked in 2% skimmed milk and probed with anti-GFAP mouse monoclonal antibody 1:200 (sigma) for 16 hrs at room temperature.

Detection of bound antibody was done using appropriate HRP-conjugated secondary antibody in VECTASTAIN kit (Vector Lab USA).

Reaction product was enhanced with DAB for 6 - 10 minutes, with subsequent dehydration in ethanol and mounting on slides.

Immuno-reactive cells were viewed and pictures obtained using TSVIEW® software on a mounted microscope.

#### **2.6. Histology**

Fixed brain tissues were processed for histological examination using the routine paraffin-wax embedding method.

Sections, 5  $\mu$ m thick, were stained with Haematoxylin and Eosin and observed under the light microscope.

Pictures of slides were obtained using TSVIEW® software on a mounted microscope.

#### **2.7. Behavioural Studies**

The pups were tested on: the open field (test for fear, hind limb strength and locomotor activity), hanging wire (test for fore limb strength) and for negative geotaxis (test for vestibular activity) [22] [23].

#### **2.8. Statistical Analysis**

The data obtained were presented as mean  $\pm$  SEM, subjected to one way Anova test using graph pad prism 4 system to show differences between the mean values of all groups. A value of  $p < 0.05$  was interpreted as statistically significant.

### 3. Results

#### 3.1. Body Weight Gain

There was an observed increase in body weights in the treated groups B, C and D at Day 6 while this trend was abrupted at Day 15 in group C ( $20.02 \pm 0.71$ ) compared to group A ( $24.67 \pm 0.75$ ) (Table 1 and Figure 1).

#### 3.2. Brain Weight

At Day 6, the brain weights in groups B ( $0.50 \pm 0.02$ ) and D ( $0.50 \pm 0.01$ ) were reduced compared to group A ( $0.55 \pm 0.02$ ) while group C had increased brain weight. At Day 15, groups C ( $1.11 \pm 0.04$ ) and D ( $1.08 \pm 0.02$ ) had reduced brain weight compared to group A ( $1.17 \pm 0.02$ ) while group B had an increased value (Table 2 and Figure 2).

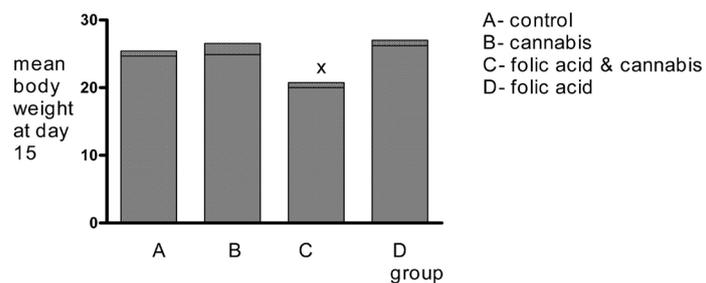


Figure 1. Graph showing the mean values of body weight of rat pups at Day 15.

Table 1. Mean values of body weight gain in grams in 6- and 15-day-old rats.

	A	B	C	D
Day 6	$8.40 \pm 0.29$	$8.47 \pm 0.39$	$8.72 \pm 0.22$	$10.01 \pm 0.78$
Day 15	$24.67 \pm 0.75$	$24.88 \pm 1.66$	$20.02 \pm 0.71$	$26.20 \pm 0.80$

Body weight data are presented in mean  $\pm$  SD. \*Indicates a statistically significant difference in the folic acid and cannabis group compared to the control.

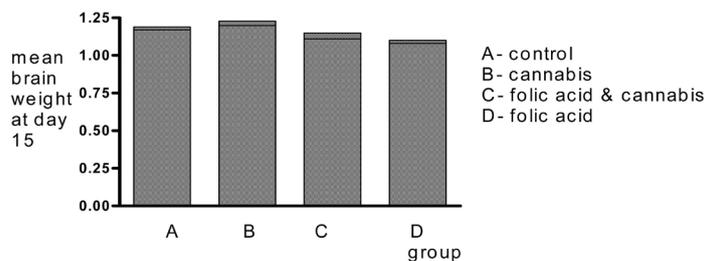


Figure 2. Graph showing the mean values of brain weight at Day 15.

Table 2. Mean values of brain weight in grams in 6- and 15-day-old rats.

	A	B	C	D
Day 6	$0.55 \pm 0.02$	$0.50 \pm 0.02$	$0.57 \pm 0.25$	$0.50 \pm 0.01$
Day 15	$1.17 \pm 0.02$	$1.198 \pm 0.03$	$1.11 \pm 0.04$	$1.08 \pm 0.02$

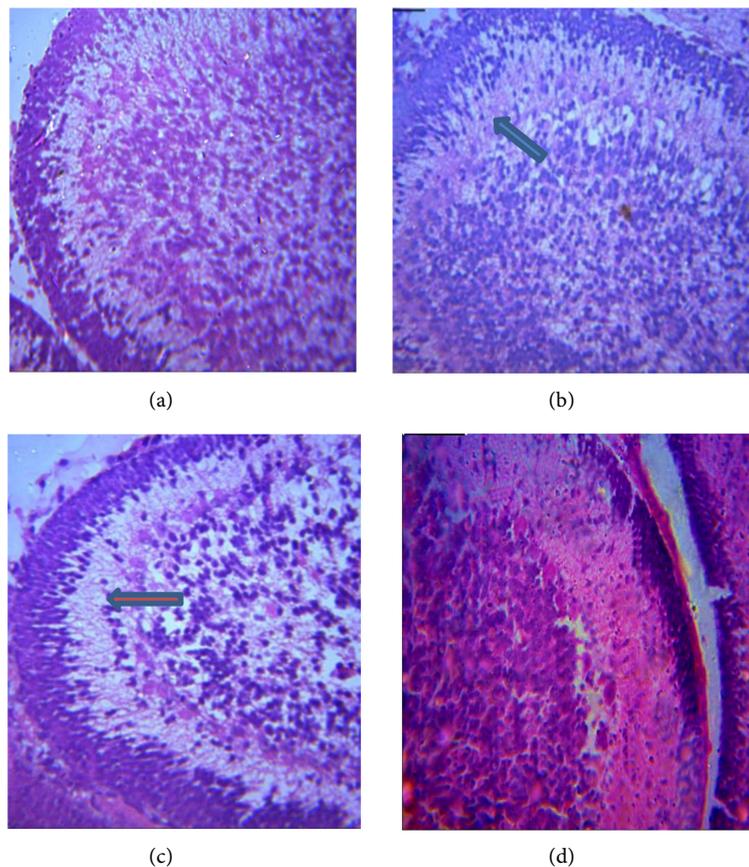
Brain weight data are presented as mean  $\pm$  SEM.

### 3.3. Behavioural Tests

There is a reduction in locomotor activity (line crossing value of  $7.00 \pm 5.66$ ), strong forelimb support (hanging wire value  $14.00 \pm 7.28$ ) and slower vestibular response (negative geotaxis value  $5.19 \pm 2.75$ ) in the cannabis exposed rats (group B) compared to the control group A, group C values showed a recovery of these deficits, except for the fore limb support ( $27.81 \pm 19.13$ ) where they showed greater strength (**Table 3**).

### 3.4. Histology

The histology of brain sections revealed diffuse spongiosis in some regions of the brain especially, the hippocampus, corpus callosum and alteration of cellular integrity in the Purkinje cells of the cerebellum in the cannabis groups. Severe capillary congestion at the cortex and sub-meninges were also seen. However, these lesions were not seen in the control and folic acid groups. The folic acid/cannabis group appeared to have a reduction in the severity of lesions, as indicated by the mild loss of eosinophilia in Purkinje cells in the cerebellum (**Figures 3(a)-(d)**).



**Figure 3.** (a)-(d) Photomicrograph of cerebellum (6 days old pups) H and E  $\times 400$  (a) (Control), (b) (Cannabis treated); (c) (Cannabis and Folic acid treated) and (d) (Folic acid treated). Blue arrow indicates area of loss of eosinophilia, Red arrow indicates area of return of eosinophilia.

**Table 3.** Comparison of mean values for behavioural test on 15-day-old rats.

	Group A	Group B	Group C	Group D
	Control	Cannabis only	Cannabis + folic acid	Folic acid
Negative geotaxis (secs.)	3.35 ± 1.28	5.19 ± 2.75	3.15 ± 2.22	4.28 ± 1.30
Hanging wire test (sec)	13.35 ± 14.25	14.00 ± 7.28	27.81 ± 19.13	50.43 ± 31.59
Line crossing	10.00 ± 4.51	7.00 ± 5.66	24.00 ± 10.90	7.00 ± 5.31
Centre time (secs.)	9.00 ± 7.91	20.00 ± 14.25	8.75 ± 3.88	7.87 ± 13.32
Rearing	2.00 ± 2.23	1.00 ± 0.88	2.00 ± 1.35	2.00 ± 1.80
Stretching (fear)	4.87 ± 3.13	5.12 ± 2.29	5.87 ± 2.16	2.75 ± 2.71 ✕
Grooming	1.00 ± 1.18	2.00 ± 1.64	1.00 ± 0.96	2.00 ± 1.51

✕ indicates a statistically significant difference ( $p < 0.05$ ) in the group that receive cannabis only (5.12 ± 2.29) compared to control group A (4.87 ± 3.13) and folic acid group D (2.75 ± 2.71).

### 3.5. Immunohistochemistry

The Anti-Glia Fibrillary Acid Protein immunohistochemistry revealed reactive astrogliosis in most regions of the brain especially, the hippocampus and corpus callosum in the cannabis treated groups compared to the control and folic acid only groups which had normal astrocytes in these regions. Astrocytes in the folic acid/Cannabis group were reactive but the intensity of reaction was less compared to the cannabis group (**Figures 4(a)-(d)**).

## 4. Discussion

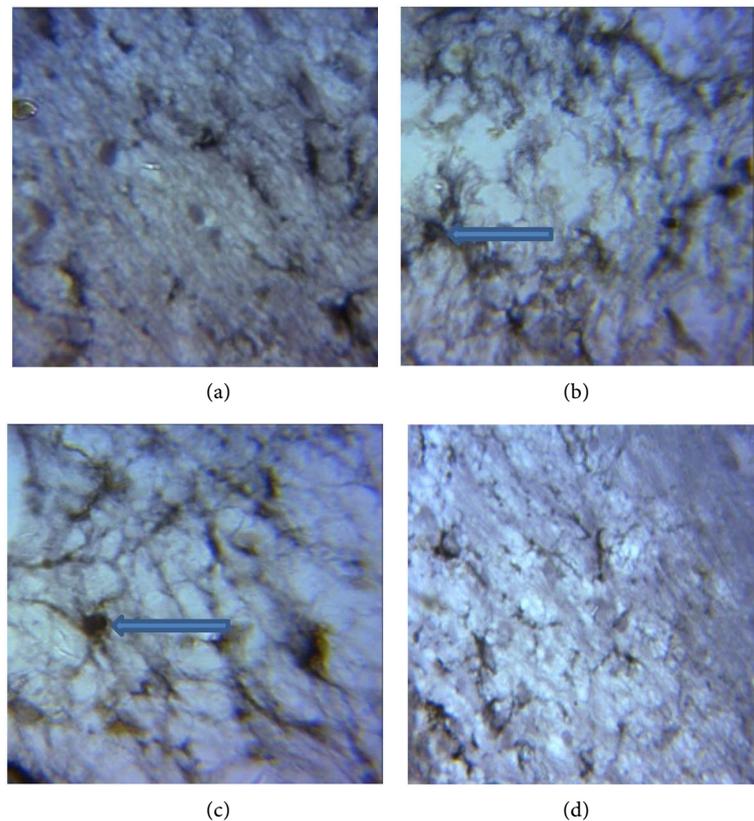
In this study, body weight measurements at Day 6 showed that both cannabis and folic acid caused an increase in body weight, but at Day 15, this increase was abrupted in the cannabis/folic acid group and this was statistically significant. This is possible because the groups that received cannabis had vascular congestion in most of the body organs (especially in the liver), which will gradually lead to cell loss and decrease weight. This is comparable to the studies by [24] and [25] which says that Cannabis possesses anti-proliferative property.

Cannabis, as used in this study, caused swelling and congestion of blood vessels to the brain which later result in loss of cells, particularly in the Purkinje layer of the cerebellum, thereby, causing a decrease in brain weight. This is comparable to findings by [26] which showed that cannabis causes vasodilation and congestion in the CNS, leading to structural damage to cells or total loss of cell.

In this study folic acid caused decrease brain weight. It disagrees with findings of [27] which states that folic acid causes an increase in brain mass.

Behavioural test results showed that cannabis causes locomotor deficit, panic increase in muscle strength, isolation, fear, incoordination and anxiety. These findings are comparable to those of [28] [29] [30]. However, the deficit in locomotor function and incoordination were reversed by folic acid but folic acid did not improve confidence status of the pups.

Histology revealed, in the cannabis group, structural damage to cells and areas of complete cell loss in corpus callosum, hippocampus and cerebellum. In the



**Figure 4.** (a) - (d) Photomicrograph of Corpus Callosum (6 days old pups) GFAP  $\times 1000$  (a) (Control); (b) (Cannabis treated); (c) (Cannabis and folic acid treated) and (d) (Folic acid treated). Arrows indicate reactive astroglia.

cerebellum, the cells in the Purkinje layer had pyknotic nuclei with loss of cytoplasmic eosinophilia. However, in the folic acid/ cannabis group, the cytoplasmic eosinophilia returns, though areas of complete cell loss persist. These lesions were not seen in the folic acid and control groups confirming the fact that cannabis is capable of causing neurodegenerative changes in the CNS through an apoptotic mechanism [11] [17] [31].

Immunohistochemistry revealed that the intensity of astrocytic response was reduced when folic acid was used as an antidote to nerve cells exposed to cannabis intoxication. This is comparable to the study carried out on mice by [31] which showed that it was possible to slow down astrocytic response by administration of some factors especially anti-stroke factors.

In conclusion, this study has been able to show that deficit in locomotor function can occur in pups whose dams received cannabis in normal saline by oral route during gestation due to loss of cell integrity in the Purkinje cell layer of the cerebellum. When these cells are exposed to Cannabis, they build up excitatory impulses which can start off programmed cell death. Folic acid removes the apoptotic factor homocysteine from nerve cells, thereby stopping the process of cell death. These nerve cells will then be available for subsequent impulse transduction, creating a situation that resembles the reversal of deficit in locomotor

function caused by cannabis. However, the work was not able to quantify the extent of damage to nerve cells by cannabis and the stage at which recovery sets in. It is, therefore, necessary that further studies be carried out at ultrastructural level, to quantify and describe the damages observed and extend the number of days that the pups were observed before sacrifice.

### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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