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Evaluation of Sub-chronic Toxicity Effect of n-Hexane fraction of Cannabis sativa Leaves in Mice

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ABSTRACT

Background: *Cannabis sativa* (Cannabaceae) has been cultivated for centuries in different parts of the world for its psychoactive property. However, despite the pharmacological potential and economic prospects, there is a dearth of information on the toxicity profile of Nigerian Cannabis. This study aims at evaluating the sub-chronic toxicity of the n-hexane fraction of *C. sativa* leaves (nHCS) in mice.
Method: The n-hexane fraction was obtained from the methanol extract of *C. sativa*. Oral median lethal dose (LD_{50}) of nHCS was determined in mice following the OECD 423 method. Forty mice were divided into four groups (n=10); Group 1 (Vehicle-treated group), received 10 ml/kg while groups 2 – 4 received nHCS (200, 400 and 800 mg/kg respectively) orally for 28 days. Five mice in each group (n=5 per group) were selected for recovery, and the remaining were sacrificed for collection of blood, brain, liver, kidney and oviduct. After 21-days of recovery, the remaining animals were subjected to the same procedure. Haematological parameters were determined using a standard procedure while the biochemical assays were carried out on the liver enzymes (aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and kidney functional parameters (urea and creatinine). Histopathology assessment was done on essential organs such as liver, kidney, brain and fallopian tube using H & E. Data obtained were analyzed using descriptive statistics and ANOVA at $\alpha_{0.05}$.
Results: The LD_{50} of nHCS was found to be above 2000 mg/kg per oral. The 28-day repeated dose administration results showed that there was no significant alteration in most of the haematological parameters. However, both platelet and lymphocyte counts were significantly increased at the dose of 200 mg/kg while the white blood cell count was significantly increased at the doses of 400 and 800 mg/kg (8.80±1.50, 9.40 ± 1.20 vs 4.40 ± 0.70 $x10^3/\mu L$), when compared with control. The biochemical results revealed that only urea level was significantly increased at dose of 200 and 400 mg/kg and this effect was reversed during the recovery period. Histopathological results showed that there was a dose-dependent deleterious effect on the liver, kidney, brain cortex and fallopian tube that was reversed back to normal during the recovery period.
Conclusion: Our findings suggest that the LD_{50} is greater than 2,000 mg/kg and *Cannabis sativa* n-hexane extract exhibited no toxicity at low dose level (200 mg/kg, p.o.), but there was evidence of neuronal, hepatic, renal and reproductive toxicities at higher doses (400 and 800 mg/kg, p.o) suggesting the need to exercise caution when used subchronically.

INTRODUCTION

Plant source plays an important role in medicine in world health. Plant sources have an essential role in medicine and world health[1,2]. Therapeutic or curative aids have been found to be derived from medicinal herbs or plants. Medicinal plant use is now widely accepted as a major component of global health systems[2,3]. This involves using medicinal plants as a possible resource for preserving health as well as for the treatment of illnesses. Approximately two-thirds of the global population, or several countries, rely on herbal medicine for their main healthcare needs[2,3].

Records show that some of the commonly used traditional medications contain bioactive elements, or active chemicals, derived from plants[3]. Plant-derived medications have been found through recent studies that looked into traditional, therapeutic, and curative therapies[3].

World Health Organisation estimates state that around 80% of individuals in less developed nations get their primary care solely from traditional medicine practitioners[4,5]. Therefore, it is important to note that not less than 3.3 billion people consistently use medicinal plants in less developed countries because they are the "mainstay" of traditional medicine[6,7]. Even though synthetic drugs have advanced, medicinal plants have given humanity access to a wide range of potent medications that can treat or completely eradicate infections and illnesses. As a result, the use of plant-based medications is growing worldwide. Despite the recent developments in contemporary (synthetic) medicine, many illnesses and infections remain untreated, and there are many diseases for which appropriate treatments are still lacking. This has made the development of safer medications for the treatment of illnesses urgently necessary, for both humans and the environment. It has been noted that a large number of people, particularly in less developed nations, use medicinal plants to treat and prevent illnesses[8]. Additionally, it has been noted that these medicinal herbs[8] have strong therapeutic effects when taken raw or in the form of crude extracts; yet, they can also have poisonous effects when taken in excess or for an extended length of time. Chronic users of high quantities of crude therapeutic plant extracts have reported hazardous side effects including kidney problems issues[4].

C. sativa is a unique and complex plant in terms of its ingredients and physiological features, some of which have opposing effects. Humans have consumed C. *sativa* since ancient times for both therapeutic and recreational purposes[9]. The toxicity profile of Nigerian cannabis has not been examined, despite its pharmacological and economic potentials. In addition, there is a growing demand in several countries, including Brazil, Thailand, Australia and Zambia, for it legalisation for medicinal use which is the impetus behind this research. Therefore, taking into consideration the increase rate of *cannabis* legalization for medical use in world in countries such as Brazil, Australia, Czech, Canada and Zambia among others, it is critical to assess the possible toxicity of Nigerian cannabis to ascertain its safety. Hence, the need for the evaluation of the toxic effects of *C. sativa* on the essential organs such as liver, kidney, brain and fallopian tube in mice for 28 days.

MATERIALS AND METHODS

Extracts Preparation

The dried *C. sativa* leaves were milled and thereafter soaked in 70% methanol for 72 hours. A rotary evaporator operating at a lower pressure and around 40^0C was used to filter the extract and remove the solvent. Methanol extract of the plant was dissolved in a mixture of distilled methanol and water and the fractions were then extracted using n-hexane. The extracts obtained were then stored in the refrigerator at 4 $^{\circ}$C until needed for the experiment.

Animals

Healthy adult female mice (20-25 g) were used for all the experiments. The animals were fed with standard laboratory pellets (Grand Cereals, United African Company Plc., Nigeria) and water was provided *ad libitum* except on the day prior to the start of experiment[10]. The experiment's animal handling followed the ethical guidelines for using animals[11]. For the sighting test, three (3) mice per dose level were used; however, animals were grouped into four (4) groups consisting of 10 female mice per group for the repeated dose toxicity study.

Ethical consideration

The Animal Care and Use Research Committee, University of Ibadan, Ibadan granted ethical permission for the study (File number Fid. No.: 22/009).

METHODOLOGY

LD_{50} Determination

This was done in compliance with OECD recommendation 423[12] that is a stepwise procedure, starting with the best estimate of the LD_{50} and the subsequent doses are either increased or reduced depending on the outcome for the previous dose, usually by a factor 3. 2. Therefore, the highest dose (2000 mg/kg, per oral) was administered to commence the LD_{50} determination and thereafter followed the steps accordingly as stated in the guidelines 423.

Repeated dose (sub-chronic) toxicity / recovery tests

OECD Guideline 407 for a 28-day repeated dosage study was followed in conducting the repeated dose toxicity test [13, 14,15] Forty (40) female mice were divided into four groups (n=10). Group 1 served as control (vehicle) and mice in this group were dosed daily with olive oil (10 µl/g body weight, p.o.). Groups 2 – 4 were daily administered 200, 400 and 800 mg/kg body weight of *C. sativa* n-hexane leaves extract, respectively, the highest dose being one-fifth (1/5) of the LD_{50}. Following the 28 days repeated toxicity study (i.e. after the toxicity phase), each

group of mice were split into two equal groups at random. One subgroup of mice was referred to as the "toxicity set," and the other as the "recovery set." The recovery sets were furthermore allowed a non-dosing recovery period of 21 days. All the animals during the 28-day repeated dosing and 21-day recovery period were sacrificed by cervical dislocation. Samples were collected for haematological, biochemical and histological assays.

Evaluation of heamatological parameters

Blood collected was analyzed using Mindray BC 2800 Haematology Auto-Analyzer (Mindray, India). The whole blood was presented to the sample probe of the auto-analyzer, and the probe automatically aspirated 13 µl sample. When aspiration was done, the sample was removed and the probe rose up. The analyzer analyzed the sampleand displayed the results on the screen and thereafter printed out. White blood cell (WBC), haematocrite (HCT), mean corpuscular volume (MCV), haemoglobin (HGB), red blood cell (RBC), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution unit (RDW-CV, RDW-SD), platelet distribution width (PDW), platelet count (PLT), procalcitonin (PCT) and mean platelet volume (MPV) results were obtained.

Biochemical Parameters

The sections of liver and kidney frozen were homogenized in extraction medium using an electrical homogenizer (Model WT-130, Success Technologies, Malaysia). Supernatant from homogenates of liver and kidney were used to assess the liver function tests (aspartate aminotransferase (AST), alanine aminotransferase (ALT), Alkaline phosphatase (ALP) and renal biomarkers (urea, creatinine). Assayed for biochemical indices of liver and kidney functions were carried out from the samples. Quantitative assessment of urea, creatinine, AST, ALT and, ALP was performed using commercial biochemical assay kits in accordance with manufacturer's instructions.

Histological Examination

The brain, liver, kidney and fallopian tube were removed and preserved in 10% formalin for histological assessment. Gross and microscopic examinations were carried out on repeated dose toxicity studies[10,12]. Following fixation, the tissues were immersed in progressively increasing grades of alcohol for dehydration at room temperature: 0% alcohol, 80% alcohol, 90% alcohol, absolute alcohol I, and absolute alcohol II. Dehydrated tissues were cleaned at room temperature twice with xylene to replace the alcohol. Melted paraffin wax was then inserted twice into the tissues. To give rigid support for the microtomy, paraffin wax was applied to the infiltrated tissues. Using a Leica rotary microtome (Bright B5143 Huntington, England), the tissue was divided into slices that were 5 µm thick. To help the folded portions spread, they were floated in a 45 °C water bath before being mounted on fresh, spotless glass slides. To improve adherence to the slide, the sections were then dried at 40°C on a slide drier. After that, they were de-waxed in xylene and treated with progressively higher grades of alcohol, which were 100%, 90%, 80%, 70%, and 50% alcohol. The stain employed was hematoxylin. The extra stains were washed off with a water rinse. After removing extra dye from the tissues by differentiating the sections in a solution of 70% alcohol and 1% hydrochloric acid, the sections were rinsed until the nuclei turned blue. After counterstaining with eosin, they were washed with water. After a brief washing, which the pieces were quickly dried off and dehydrated in the following alcohol concentrations: 50%, 70%, 80%, 90%, and pure alcohol. According to the description of Onaolapo et al.[16], after being cleared in xylene and mounted in DPX (Distrene Plasticizer and xylene) with a cover slip, they were examined under a microscope using a photomicrograph acquired with a Leica DM750 Camera Microscope (x 400).

Statistical analysis

Data analyses were performed using Graphpad Prism software (Graphpad Software, Inc.). The results were expressed as mean and standard error of mean. One-way ANOVA was used for the analysis followed by Tukey's and Dunnet's *Post hoc* multiple comparison test.

RESULTS

Determination of LD_{50} and Sighting Study

Following a preliminary assessment of the effects of the extract on mice, a starting dose of 2000 mg/kg was selected for LD_{50} determination and sighting studies. LD_{50} of *Cannabis sativa* n-hexane leaves extract was established to be greater than 2000 mg/kg, p.o.

Haematological Results

The haematological parameters after 28-day of repeated administration are presented in **Table 1**. There were no significant changes in the haematological parameters by oral administration of *C. sativa* n-hexane leaves extract at the administered dose except WBC at 400 and 800 mg/kg that showed significant increase.

Table 1: Effect of *C. sativa* n-hexane leaves extract on Haematological parameters in mice given 28-day repeated oral administration

Haematological Parameters	Treatment Groups (nHCS, mg/kg, p.o)			
	Vehicle	200	400	800
WBC ($x10^3$)/µL	4.40 ± 0.70	8.30 ± 1.10	8.80 ± 1.50*	9.40 ± 1.20*
RBC ($x10^6$)/µL	8.02 ± 0.39	9.09 ± 0.29	8.50 ± 0.40	8.36 ± 0.86
HGB (g/dL)	11.30 ± 0.40	12.60 ± 0.40	11.40 ± 0.60	12.50 ± 0.30
HCT (%)	45.50 ± 2.70	47.90 ± 1.20	47.50 ± 1.60	46.40 ± 4.50
MCV (fL)	56.60 ± 0.90	53.10 ± 040*	56.30 ± 1.20	55.60 ± 0.40
MCH (pg)	13.50 ± 0.30	14.00 ± 0.20	13.00 ± 0.20	15.70 ± 1.80
MCHC (g/dL)	25.20 ± 0.80	26.50 ± 0.10	23.60 ± 0.40	28.20 ± 3.00
PLT /µL	694.80 ± 65.05	1014.80 ± 100.32*	746.00 ± 83.50	815.60 ± 32.42
LYM%	72.30 ± 3.20	79.70 ± 4.40	86.90 ± 1.70*	81.80 ± 1.80
NEUT%	27.70 ± 3.20	20.30 ± 4.40	13.10 ± 1.70*	18.20 ± 1.80
LYM# ($x10^3$)/µL	3.50 ± 0.60	9.40 ± 0.90*	6.30 ± 0.50*	6.00 ± 0.80
RDW-SD fl	37.00 ± 1.20	29.70 ± 0.30*	34.40 ± 0.90	36.00 ± 3.00
RDW-CV %	20.30 ± 1.10	15.60 ± 0.60*	17.30 ± 0.80	18.20 ± 1.30

Values are presented as mean ± SEM (n=5). Vehicle: (Olive oil, 10 µl/g, p.o); *p<0.05 vs vehicle treated group.

Biochemical Results Following Repeated Dose Oral Administration

Urea level was significantly ($p<0.05$) increased at the dose of 200 mg/kg and 400 mg/kg when compared with the vehicle-treated control group, however, creatinine level was not significantly affected (**Table 2**). Liver enzymes assay revealed that ALP was only significantly decreased at 200 mg/kg while ALT was significantly increased at 800 mg/kg (Table 3).

Table 2: Effect of *C. sativa* n-hexane leaves extract on kidney function in mice given 28-day repeated oral administration

	Kidney Function Parameters	
Treatment (nHCS, mg/kg)	**Creatinine (µmol/L)**	**Urea (mmol/L)**
Vehicle	29.23 ± 6.12	27.50 ± 3.87
200	18.49 ± 3.04	72.25 ± 2.17*
400	27.73 ± 5.20	45.55 ± 5.29*
800	17.30 ± 1.61	36.99 ± 3.00

Values are presented as mean ± SEM (n=5). Vehicle: (Olive oil, 10 µl/g, p.o); **p<0.05 vs vehicle treated group.* nHCS: *C. sativa* n-hexane leaves extract.

Table 3: Effect of *C. sativa* n-hexane leaves extract on liver function in mice given 28-day repeated oral administration.

	Liver function Parameters		
Toxicity (nHCS, mg/kg)	**ALP (U/L)**	**ALT (U/L)**	**AST(U/L)**
Veh	74.96 ± 9.46	54.19 ± 6.26	84. 04 ± 7.34
200	52.61 ± 3.48*	63.25 ± 5.82	77.74 ± 4.12
400	67.23 ± 5.38	38.07 ± 2.55	80.11 12.72
800	92.36 ± 3.50	92.08 ± 0.91*	90.25 ±9.86

Values are presented as mean ± SEM (n=5). Vehicle: (Olive oil); **p<0.05 vs vehicle treated group.* nHCS: *C. sativa* n-hexane leaves extract.

Histopathology Results

C. sativa n-hexane leaves extract repeated-dose 28-days toxicity study photomicrographs of the histopathological analysis are as shown in Plates 1, 2, 3 and 4.

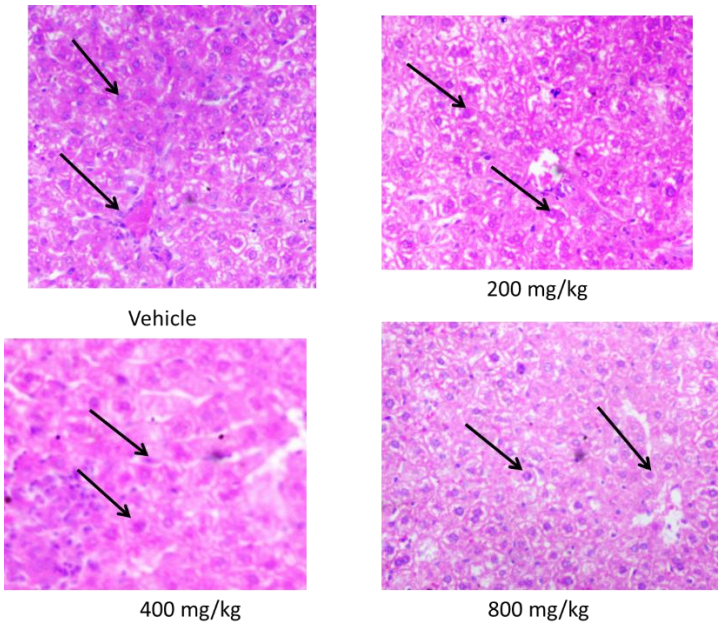

Plate 1 **X400**

Vehicle-Treated Liver: An examination of the liver revealed sheets of radially organized hepatocytes encircling a terminal hepatic venule. There were sinusoidal gaps between hepatocyte cords. Hepatocytes displayed well-staining nuclei, which are consistent with normal histology.

Liver Treated groups (200-800 mg/kg): With increasing doses, there was a progressive loss of normal liver architecture, including enlarged hepatocytes with pale-staining nuclei, loss of intervening sinusoids, and the presence of many vacuoles, all of which are consistent with liver injury.

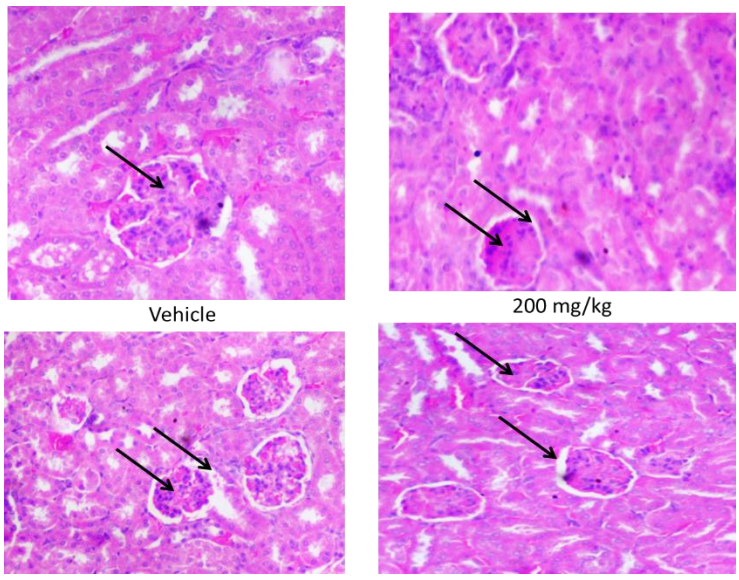

Plate 2 **x400**

Vehicle-Treated Kidney: The kidney control slide revealed a well-defined cortex, medulla, glomeruli, Bowman's capsule, Bowman's space, proximal and distal renal tubules, and blood vessels, as well as deeply stained nuclei of the glomeruli and tubular epithelium, all of which are consistent with normal histology.

Kidney Treated groups (200-800 mg/kg): With increasing concentrations, there was a graded loss of normal kidney architecture, as demonstrated by crumpling of the glomeruli, the presence of pale staining nuclei of the glomeruli and tubular epithelium that were consistent with renal injury.

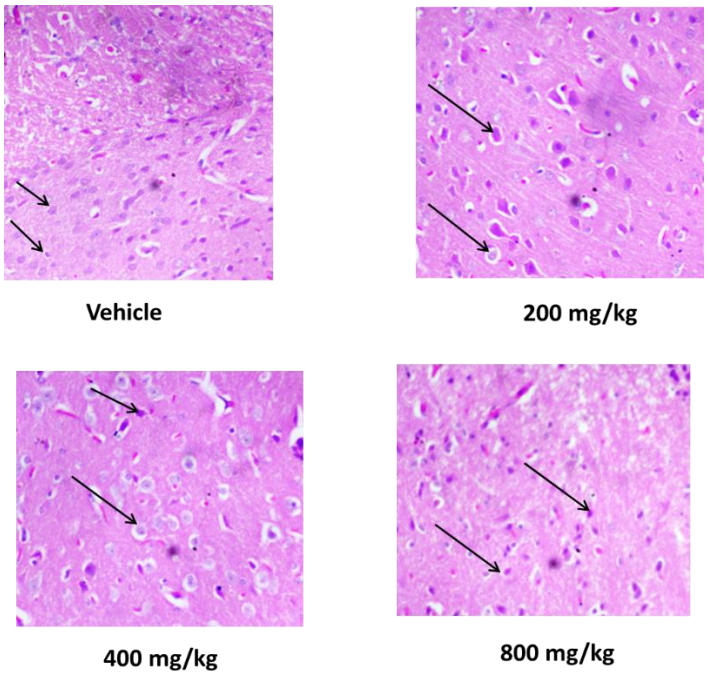

Plate 3 **X400**

Control cerebral cortex: Examination of the brain slide in the control slide revealed features in keeping with cerebral cortex architecture. This is composed of multipolar- shaped pyramidal cells with rounded vesicular nuclei, small round-vesicular glial neurons scattered within a pink-staining neuropil and granule cells that are visible as circular-shaped neurons with large open-face nuclei, prominent nucleoli, and scant cytoplasm. These characteristics are consistent with normal cerebral cortex histology.

Cerebral cortex groups (200-800 mg/kg): There was evidence of shrinking and pale staining nuclei in degenerating pyramidal cells with pale margins as the concentrations increased. Degenerating granule cells with pale-staining pyknotic nuclei were also evident.

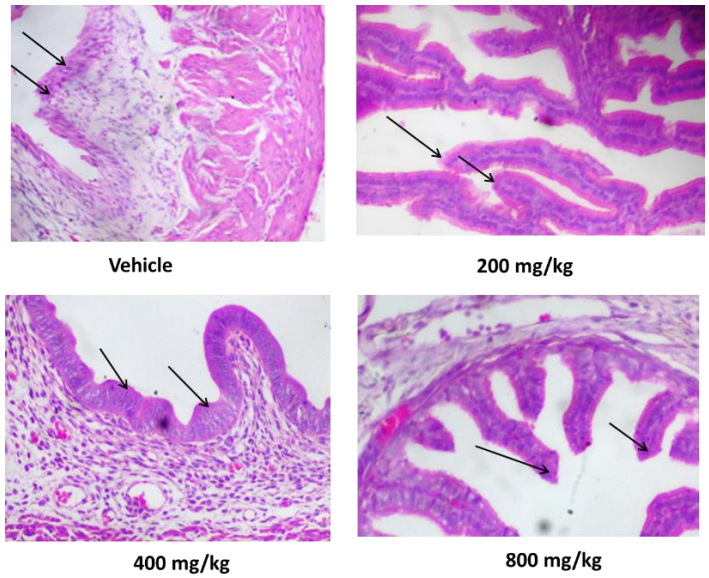

Plate 4 **X400**

Control Fallopian tubes: Analyzing the control group's fallopian tube slide showed normal fallopian tube lining the branching finger-like projections of lamina propria with its connective tissue stroma, composed of ciliated columnar cells and peg cells with bulbous apical projections.

Fallopian tube treated groups (200-800 mg/kg): With increasing concentrations there was graded loss of normal architecture with loss of ciliated columnar epithelium and peg cells.

Recovery Phase Results

The Haematological Parameters after the 21–Day Recovery Period

In the haematological investigation, there was a significant increase in neutrophils while lymphocytes (%) were significantly ($p<.0.05$) decreased at all dose levels. However, there was a significant decrease in MCHC at 200 mg/kg. It was also observed that there was a decrease in haemoglobin and haematocrit at 400 mg/kg when compared with the vehicle–treated mice during the recovery period (**Table 4)**.

Table 4. Effect of *C. sativa* n-hexane leaves extract on haematological parameters in mice after 21 days recovery period, immediately after the 28-day repeated oral administration

Haematological Parameters	Treatment Groups (nHCS, mg/ kg, p.o)			
	Vehicle	200	400	800
WBC ($x10^3$)/µL	7.00 ± 1.40	11.10 ± 2.10	10.40 ± 1.80	13.50 ± 0.44
RBC ($x10^6$)/µL	8.47 ± 0.35	8.04 ± 0.64	6.82 ± 0.66	8.77 ± 019
HGB (g/dL)	12.30 ± 0.50	11.20 ± 0.80	9.50 ± 1.10*	12.40 ± 0.10
HCT (%)	42.30 ± 2.30	41.40 ± 3.10	32.10 ± 3.40*	43.70 ± 0.80
MCV (fL)	49.90 ± 0.80	51.60 ± 0.50	50.30 ± 0.70	49.80 ± 0.20
MCH (pg)	14.50 ± 0.10	14.00 ± 0.40	14.80 ± 0.30	14.20 ± 0.20
MCHC (g/dL)	29.00 ± 0.40	27.10 ± 0.60*	29.40 ± 0.70	28.50 ± 0.30
PLT /µL	797.40 ± 82.00	978.60 ± 107.89*	969.00 ± 45.00	655.00 ± 24.00
LYM%	93.40 ± 1.40	78.00 ± 4.90*	73.00 ± 5.10*	73.00 ± 2.80*
NEUT%	6.80 ± 1.10	22.00 ± 4.90*	27.00 ± 5.10*	27.00 ± 2.80*
LYM# ($x10^3$)/µL	6.50 ± 1.30	9.10 ± 0.90	6.10 ± 1.70	9.80 ± 0.20
RDW-SD fl	29.70 ± 0.60	31.50 ± 1.10	33.40 ± 1.30	32.70 ± 1.90
RDW-CV %	16.30 ± 0.90	20.10 ± 2.60	18.10 ± 1.10	18.90 ± 1.20

Values are presented as mean ± SEM (n=5). Vehicle: (Olive oil, 10 µl/g, p.o); *p<0.05 vs vehicle treated group.

Biochemical Results after the 21–Day Recovery Period

The results showed no significant effects on the kidney function (**Table 5**), however, ALT and AST were significantly decreased (**Table 6**).

Table 5. Effect of *C. sativa* n-hexane leaves extract on biochemical parameters of mice after 21 days recovery period, immediately after the 28-day repeated oral administration

Kidney Function Parameters		
Recovery (nHCS, mg/kg)	**Creatinine (µmol/L)**	**Urea (mmol/L)**
Vehicle	34.30 ± 3.00	10.52 ± 0.67
200	38.20 ± 3.50	9.97 ± 0.49
400	43.90 ± 3.80	10.15 ± 0.27
800	39.00 ± 5.30	12.46 ± 1.76

Values are presented as mean ± SEM (n=5). Vehicle: (Olive oil, 10 µl/g, p.o); nHCS: *C. sativa* n-hexane leaves extract.

Table 6. Effect of *C. sativa* n-hexane leaves extract on liver function in mice after 21 day recovery period, immediately after the 28-day repeated oral administration.

Liver Function Parameters			
Recovery (nHCS, mg/kg)	**ALP (U/L)**	**ALT (U/L)**	**AST(U/L)**
Vehicle	37.73 ± 6.47	92.65 ± 8.28	259.61 ± 18.57
200	47.09 ± 5.70	75.02 ± 8.08	279.05 ± 47.56
400	46.84 ± 3.59	34.70 ± 4.64*	121.86 ± 12.65*
800	49.68 ± 11.94	23.02 ± 0.81*	121.19 ± 12.67*

Values are presented as mean ± SEM (n=5). Vehicle: (Olive oil, 10 µl/g, p.o); **p<0.05 vs vehicle treated group.* nHCS: *C. sativa* n-hexane leaves extract.

Histology Results (Recovery)

The vehicle-treated recovery liver, kidney, cerebral cortex and fallopian tube showed normal features, while 200, 400 and 800 mg/kg nHCS treated mice showed reversal of all organ injury.

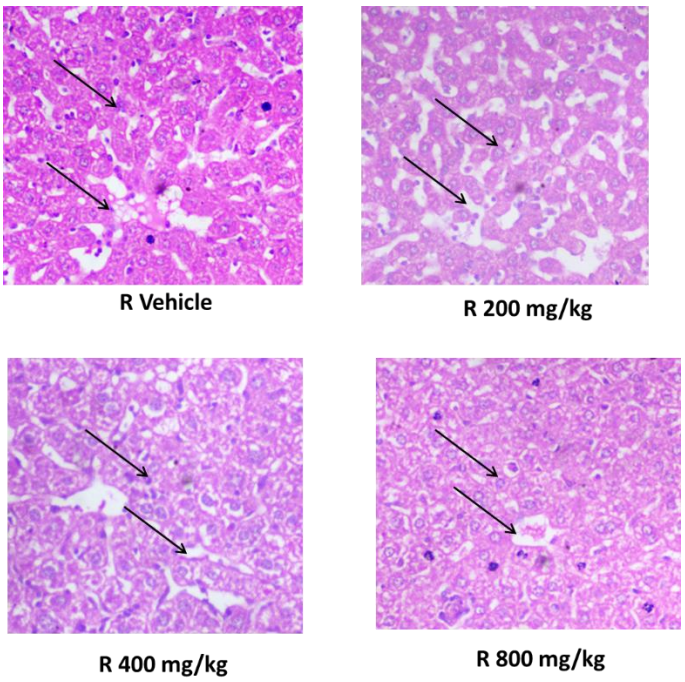

Plate 5 **X400**

The liver of the olive oil-treated mice showed normal features while the recovery nHCS -treated mice showed reversal of organ injury**.**

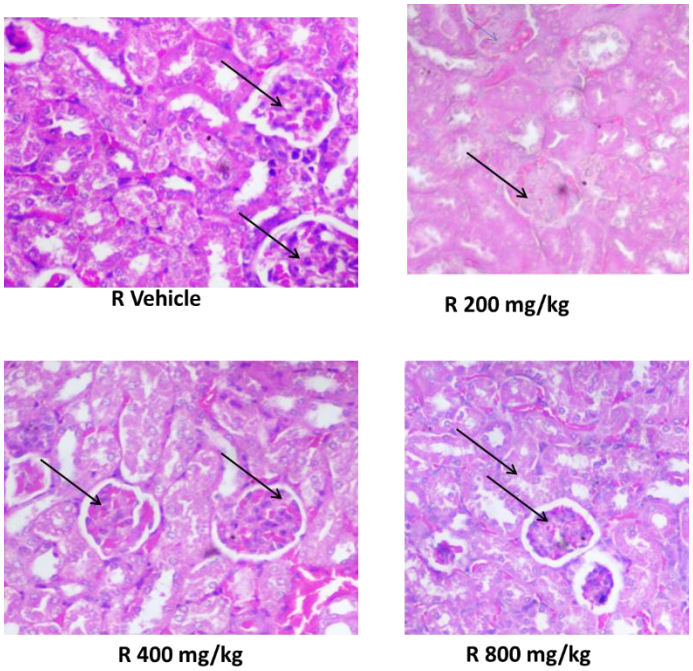

Plate 6 **x400**

The kidney of the olive oil-treated mice showed normal features while the recovery nHCS -treated mice showed reversal of organ injury**.**

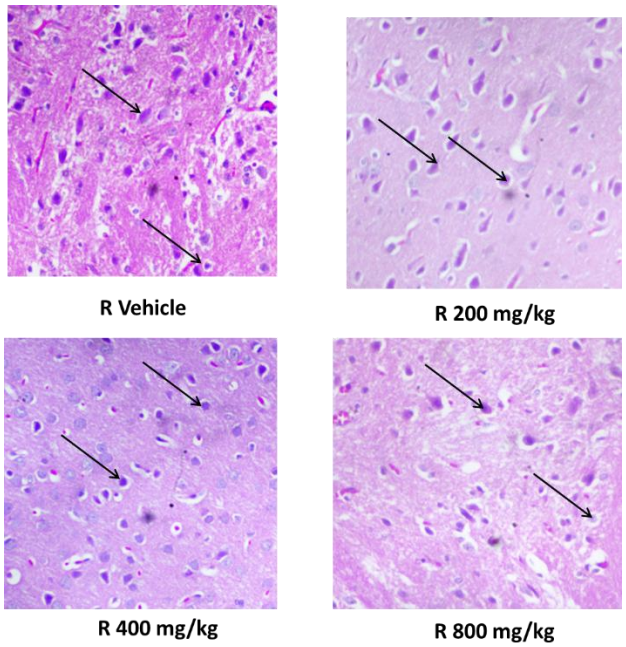

Plate 7 **x400**

The cerebral cortex of olive oil-treated mice showed normal features while the recovery nHCS -treated mice showed reversal of organ injury**.**

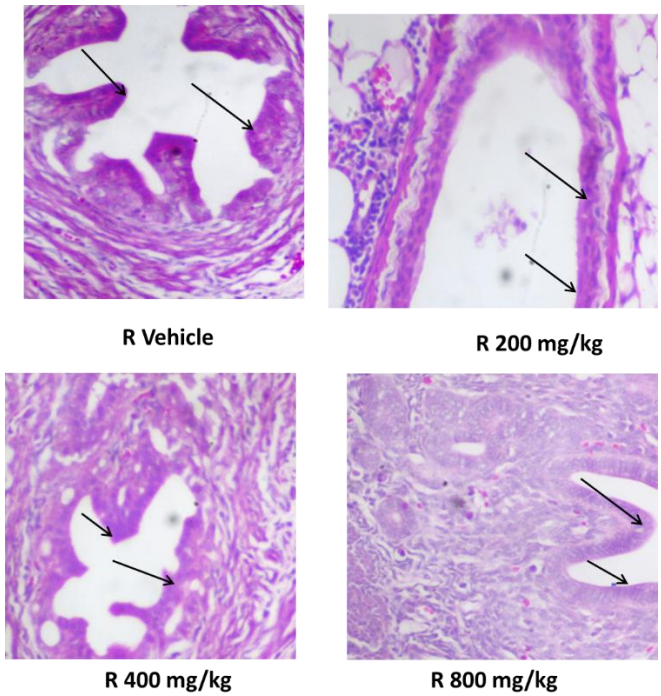

Plate 8 X400

The fallopian tube of the olive oil-treated mice showed normal features while the recovery nHCS -treated mice showed reversal of organ injury**.**

DISCUSSION

The LD_{50} of the n-hexane cannabis extract was determined in accordance with OECD guidelines 423 following a stepwise procedure[12]. The LD_{50} of *C. sativa* n-hexane leaves extract was observed to be greater than 2000 mg/kg similar to the results earlier reported by Ewing et al. [17] 2019 and Balafrej et al.[18]. It was observed that acute oral administration of nHCS did not lead to deaths.

In the 28 days repeated dose sub-chronic study, the hematological parameters investigations in mice showed that n-hexane-extract of *C. sativa* caused no significant effects in parameters such as neutrophils, hemoglobin, white blood cells, monocytes, eosinophil, and basophils among others when compared with vehicle-treated mice. Most of these parameters results are within the standard ranges[19]. However, there was an increase in platelet and

lymphocyte counts at 200 mg/kg suggesting a possible high risk of inducing a systemic inflammatory process as earlier reported that an increase in platelet (thrombocytosis) and lymphocyte (lymphocytosis) counts can suggest a risk of systemic inflammation because both cell types play key roles in the body's inflammatory response[20,21]. In this study, it was observed that this increase in both platelet and lymphocyte counts was reversed to normal during the recovery period.

The biochemical indices result showed a significant increase in urea level at 200 and 400 mg/kg even though the values were within the standard range in mice. It was also observed that this increase was reversed back to normal during the recovery period. It is well recognised that creatinine is a more reliable measure of renal function. It was observed in this study that nHCS (200- 800 mg/kg, p.o.) following the 28-day repeated administration did not cause any significant changes on creatinine level when compared with vehicle-treated mice. However, there was a significant increase in urea level at the doses of 200 and 400 mg/kg. Our study showed that there were no significant changes in both ALT and AST in the 28-day repeated administration at all doses used when compared with the vehicle- treated group. The liver transaminases, mainly ALT alanine transaminase (ALT) and aspartate aminotransferase (AST) are known to be localized within the cells of liver, heart, gill, muscles, kidney and some organs[22] and therefore, they can be useful to determine the deleterious effect of substance on these essential organs. These enzymes are important in assessing and monitoring liver cytolysis.

In our study in female mice, an increase in doses from 200 mg/kg to 800 mg/kg administered orally resulted in a progressive deterioration of normal liver architecture, including enlarged hepatocytes with pale-staining nuclei, loss of intervening sinusoids, and the presence of many vacuoles, all of which are consistent with liver injury that was reversed during the 21-day recovery period. This is similar to the previous report by Ewing et al.[17] where it was observed that hexane-derived cannabis extract containing 57.9% cannabidiol (CBD) administered to mice caused hepatotoxicity. It was established that CBD exhibited clear signs of hepatotoxicity, possibly of a cholestatic nature that may involve numerous pathways associated with lipid and xenobiotic metabolism[17].

The histopathology results of the 28-day repeated oral administration showed that there was a loss of normal architecture with swollen hepatocytes with pale-staining nuclei, loss of intervening sinusoids, and the presence of numerous vacuoles dose-dependently suggesting possible liver injury. This is similar to the previous report by Ewing et al.[17] where it was observed that hexane-derived cannabis extract containing 57.9% cannabidiol administered to mice caused hepatotoxicity. Furthermore, it was also observed in the kidney that there was a graded loss of normal kidney architecture, as demonstrated by crumpling of the glomeruli, the presence of pale staining nuclei of the glomeruli and tubular epithelium that were consistent with renal injury. In the ceerebra cortex, there was evidence of shrinking and pale staining nuclei in degenerating pyramidal cells with pale margins as the doses increased. However, all these deleterious observations in the histopathology of these essential organs were reversed during the recovery period because recovery nHCS-treated mice showed reversal of organ injury, thus suggesting that the changes observed during the 28-day repeated treatment seemed to be reversible**.**

CONCLUSION

In this study, it was demonstrated that *Cannabis sativa* extract may be regarded as safe at low doses but not safe at high doses if administered for a long period. The study concludes that caution is necessary when using high doses of *Cannabis sativa* over extended periods.

Declaration of Conflict of Interest

The authors declare that there are no conflicts of interest with respect to the research.

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