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TOXICOLOGICAL EVALUATION OF AQUEOUS AND METHANOLIC LEAF EXTRACT OF CANNABIS SATIVA IN LIVER AND BRAIN OF MALE RATS

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ABSTRACT
The toxicological evaluation of aqueous and methanolic leaf extract of Cannabis sativa (marijuana) was investigated in liver and brain of male rats. Twenty seven (27) male albino rats were divided into three groups (A, B and C) comprising nine rats each. Animals in group A (115.43±2.45g), B (135.56±3.27g) and C (145.92±2.13g) were administered 0.5 ml of distilled water, 700 mg/kg body weight aqueous and methanolic extract of C. sativa leaves respectively. Treatment was done once daily during which 3 rats from each group were sacrificed at interval of 7 days until the 21st day. Phytoconstituents present in the methanolic extract are alkaloids, steroids, phenolics, terpenoids, saponins, flavonoids, carbohydrates, tannins, cardiac glycosides, starch and reducing sugars while the aqueous extract contains all except phenolics, tannins and starch. From week1, week2 and week3, while treatment with both aqueous and methanolic extract significantly ($p< 0.05$) decreased WBC, MCV, monocyte, lymphocytes, total and direct bilirubin as well as the activities of ALP, AST, ALT in the aqueous and methanol group when compared with the control group, it increased platelet count. However, treatment with both extract from week1 to week3 did not significantly ($p> 0.05$) affect the levels of PCV and hemoglobin when compared with the control group. Histological section of the liver and brain were normal for the control group from week1 to week3. However, it showed acute hepatocellular necrosis and central vein inflammation for both aqueous and methanol treated groups from week1, week2 and week3. In the brain, it showed neurosis, neuronal degeneration and perivascular congestion in both groups throughout the exposure period. The effect of methanolic extract was greater than that of the aqueous extract both in liver and brain. Overall, the various alterations in biomolecules which do not compare favourably with the control values as evidenced with degeneration in histology suggest that the plant extract has both functional and structural toxicity. This study also supports the speculation that consumption of Cannabis sativa may contribute to increasing incidence of liver failure and brain damage.

KEYWORDS: Cannabis sativa, toxicological evaluation, neurosis, phytoconstituents, hepatocellular necrosis.

INTRODUCTION
It has been confirmed by WHO that traditional (herbal) medicines serve the health needs of about 80 percent of the world's population; especially for millions of people in the vast rural areas of developing countries (WHO, 1993; WHO, 1999). The use of traditional medicine (TM) and complementary and alternative medicine (CAM) has increased significantly over the past few years. This is because the use of plants in traditional medical practice for treatment of various ailments is usually regarded as harmless and safe in humans because they are derived from natural sources (Newman and Cragg, 2007). One other main reason for the increasing use of traditional medicine is a growing trend for patients to take a more proactive approach to their own health and to seek out different forms of self-care. In the process, many consumers have turned to natural traditional medicinal products and practices, under the assumption that "natural means safe" (WHO, 2004). However, this is not necessarily the case. A number of reports have revealed examples of incorrect use of traditional medicines by consumers, including incidents of overdose, unknowing use of suspect or counterfeit herbal medicines, and unintentional injuries caused by unqualified practitioners (WHO, 2004). Many studies have given reports on various toxic effects of herbal medicines, such as hepatotoxicity (Nwachukwu and Iweala, 2009) and nephrotoxicity (Colson and De-Broe, 2005; Asif, 2012).

In Nigeria, the use of medicinal plant in traditional medicine has remained as the most affordable and easily accessible source of treatment in the primary health care system (Hosseinzadeh et al., 2015). However, the use of

right dosage for these medicinal plants is not currently regulated, and thus there is toxicity risk of inappropriate use, incorrect dosage and consequent deleterious effects on tissues and organs of the biological system.

Cannabis sativa, an annual herbaceous plant, is an angiosperm belonging to the Cannabaceae family (Burkill, 1985; John, 2010), is called Indian hemp or Ganja in English. Cannabis sativa preparation is known by various names worldwide. It is called Marijuana in America; Bhang, Ganja and Charas in India; Kif in North Africa; Dogga in South Africa; Krori in Tunisia, Habak in Turkey; Hashish in Middle East; Djomba or Liamba in Central Africa and Brazil; Sodom, Tampl, Gum, Gauge and stuff in Kinshasa; Swala and Whiskt in Ghana; Grifa in Mexico and Ma-cohna in some parts of South America (Sachindra and Pradhan, 1977). In the native Nigerian languages, it is called igbò in Yoruba; Nwonkaka in Igbo and Ikya in Tiv (Saalu, 2016).

It is an annual, greenish or brownish herb attaining as much as 5 m tall, native of temperate central western Asia, and of very ancient cultivation in Asia and the Mediterranean region (Ontario Hemp Alliance, 2010). It is now dispersed by man to vey many countries, and cultivated, often illegally, including occasionally in territories of West African region. It contains over 300 compounds with at least 66 of them being cannabinoids (Burns and Ineck, 2006; Downer and Campbell, 2010). Novak et al (2001) reported some of the important chemicals found in Cannabis sativa plant to include Δ-9-tetrahydrocannabinol (THC), α-pinene, myrcene, trans-β-ocimene, α-terpinolene, trans-caryophyllene and α-humulene.

Cannabis sativa leaves are bitter, astringent, tonic, aphrodisiac, alterative, intoxicating, stomachic, analgesic and abortifacient. C. sativa is grown and processed for many uses; they are used in convulsions, otalgia, abdominal disorders, malarial fever, dysentery, diarrhoea, skin diseases, hysteria, insomnia, gonorrhoea, colic, tetanus and hydrophobia (Brady et al., 2009). Its excessive use causes dyspepsia, cough, impotence, melancholy, dropsy, restlessness and insanity (Merzouki et al., 2000). The bark is tonic, and is useful in inflammations, haemorrhoids and hydrocele. The inflorescence of female plant is intoxicating, stomachic, soporific, abortifacient and useful in convulsions (Nath et al., 1997). Seeds are carminative, astringent, aphrodisiac, antiemetic and anti-inflammatory. The resin is smoked to allay hiccough and bronchitis. It is useful in insomnia, sick headaches, neuralgia, rnigrain, mania, whooping cough, asthma, dysuria and in relieving pain in dysmenorrhoea and menorrhagia. In northeastern India, some of the plants species including Cannabis sativa have been used for treatment of specific human ailments such as allergies, burns, cuts and wounds, inflammation, leprosy, leucoderma, scabies, smallpox and sexually transmitted diseases (Dilara and Nath, 2000). The different preparation of Cannabis sativa has been used in Asian traditional medicine for treatment of variety of diseases including: inflammation, nausea, headache, hematochesia, diarrhea, and alopecia (McPartland, 2004). Generally speaking, the plant has three major economic uses: for fibre, for seed-oil, and for its narcotic resin (Grotenhermen and Russo, 2002; Aryana and Williams, 2007).

Almost no plant has been studied as much as the Cannabis plant (C. sativa); more than 10 papers have been published describing various aspects of cannabis as a biologically active plant (Hazekamp, 2009). For example, Musa et al 2011 and Musa et al 2012 reported the anti-inflammatory activity of the petroleum ether extract of C. sativa (L) in rats as well as hepatoprotective and toxicity assessment of the seed oil of the plant respectively. Tijani and Adekomi (2011) as well as Tijani et al 2014 reported the neurotoxic effects of aqueous leaf extract of C. sativa on the visual cortex of adult Wistar rats and histological changes in the vital organs of male rats following short term exposure to smoke extract of C. sativa respectively. Okon et al (2014 a and b) examined the effect of long term administration of C. sativa on body weight feed and water intake as well as those of locomotion and exploratory behavior in mice respectively. Aizpurua-Ppolaizola (2014) worked on identification and quantification of cannabinoids in Cannabis sativa by HPLC-MS while Odokuma and Ogbor-Omorie (2015) saw the histomorphologic effects of C. sativa on the brains of adult Wistar rats. Obembe et al (2013) and Obembe et al (2014) worked on the effect of chronic consumption of C. sativa on bleeding time, prothrombin time and platelet count and haematological parameters following orogastric feeding in rats respectively. Mukhtar and Elbagir (2011) evaluate the effect of C. sativa on hematological indices in rats and men while VarshaZade et al (2013) reported the antifertility effect of C. sativa leaves on female albino rats.

The consumption of Cannabis sativa could have deleterious effect which may lead to insult on organs of the biological system. Such insults usually manifest as alteration in the levels of hormones, enzymes and other marker biomolecules. The toxicity which could as well result in cell, tissue or organ damage, commonly affect some vital body organs. The aim of this research work was therefore to study the toxicological changes in the liver and brain of male rats following oral administration of aqueous and methanolic extract of Cannabis sativa leaves.

MATERIALS AND METHODS

Materials

Plant Materials and Authentication

Cannabis sativa leaves which were purchased from Pyata village, Bosso Local Government Area, Niger State, Nigeria were authenticated at National Tropical Botanical Garden, French Polynesia, France with Voucher Specimen Number (PTBG0000040397).

Experimental Animals

Male Wistar rats (Rattus norvegicus) weighing 115.43±2.45g, 135.56±3.27g, 145.92±2.13g were obtained from the Animal House of Bingham University, Karu, Nasarawa State, Nigeria.

Assay Kits and Chemicals

The assay kits for the determination of total and direct bilirubin, alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase were products of Randox Laboratory Ltd, Co-Atrium, Uk. Sodium hydroxide which was used for the preparation of 0.4N sodium hydroxide employed as one of the reagents for the determination of aspartate and alanine aminotransferase respectively.

Other Reagents

All other chemicals and reagents used which were of analytical grade were products of Sigma Aldrich Ltd., Buchs, Canada and are prepared in volumetric flask using glass wares with distilled water. The reagents were stored in neat, air-tight reagent bottles except for the Biuret reagent which was stored in plastic containers.

Methods

Preparation of Aqueous Extract of Cannabis sativa Leaves

Dried leaves of Cannabis sativa were pulverized in a blender (PHILIPS, Model HR-1724, Brazil) to obtain smooth powder. A known weight (100 g) of the powdered sample was extracted in 500 ml of distilled water for 72 hours at room temperature. The mixture was filtered with Whatman No. 1 filter paper (Maidstone, UK) and the resulting filtrate concentrated in a water bath (Model: NL-420S, NEWLIFE® Medical Instrument, England) to give a brownish-black residue (extract). The extract was then reconstituted in distilled water to give the required dose of 700 mg/kg body weight used throughout the experimental period.

Preparation of Methanolic Extract of Cannabis sativa Leaves

Dried leaves of Cannabis sativa were pulverized in a blender (PHILIPS, Model HR-1724, Brazil) to obtain smooth powder. A known weight (100 g) of the powdered sample was extracted in 1000 ml of methanol in a Soxhlet extractor. The resulting mixture was concentrated in a Rotary Evaporator (MODEL: RE-52A, Shanghai Ya Rong Biochemistry Instrument Factory, China) to obtain an extract. The extract was transferred into water bath (Model: NL-420S, NEWLIFE® Medical Instrument, England) where it was further evaporated to give the required brownish-black residue. This was then reconstituted in distilled water to give the required dose of 700 mg/kg body weight used throughout the experimental period.

Screening of Secondary Metabolites

Screening of secondary metabolites to detect the presence of alkaloids, saponins, tannins, flavonoids, cardiac glycosides, carbohydrates, reducing sugar, starch, steroids terpenoids and phenolics were carried out by adopting the procedures described by (Harborne, 1973; Odebiyi and Sofowora, 1978; Trease and Evans, 1989; Sofowora, 1993; El-Olemy et al., 1994; Walls et al., 1996; Awe and Sodipo, 2001; Edeoga et al., 2005; Ganesan and Bhatt, 2008).

Animal Grouping and Extract Administration

The animals which were housed in aluminium cages placed in well ventilated standard housing conditions (temperature: 28-31 ^{0}C; photoperiod: 12 hours; humidity: 50-55%) were allowed free access to rat pellets (Vital Feed®, Grand Cereals Ltd, Jos, Plateau State, Nigeria) and tap water. The cages were also cleaned on daily basis. The animals were acclimatized for two weeks before the commencement of the experiment. Twenty seven (27) male albino rats were divided into three groups (A, B and C) comprising nine rats each. Animals in group A (115.43±2.45g), B (135.56±3.27g) and C (145.92±2.13g) were administered 0.5 ml of distilled water, 700 mg/kg body weight aqueous and methanolic extracts respectively. Treatment was done once daily during which 3 rats from each group were sacrificed at interval of 7 days until the 21st day. This experimental investigation was done in accordance with the standard humane animal care as outlined in the "Guide for the Care and Use of Animals in Research and Teaching", as approved by the Institute of Laboratory Animal Resource, National Research Council, DHHS, Pub. No NIH 86-23 (National Institute of Health, 1985).

Preparation of Plasma, Serum and Tissue Supernatant

The rats were anaesthesized in a glass jar containing cotton wool soaked in diethyl ether. Thereafter, the neck area of the rats was quickly shaved to expose the jugular veins. The veins after being slightly displaced (to prevent blood contamination by interstitial fluid) were sharply cut with sterile scapel blade and an aliquot (2 ml) of the blood was collected into EDTA sample bottles to give plasma for the haematological analysis. Blood (5 ml) collected in plain bottles was centrifuged (using High Speed Centrifugal Machine, Model: YXJ-2, Essex, England) at 2000 g for 10 minutes and serum used for the biochemical analyses. Animals were dissected during which liver and brain were removed, weighed, homogenized, centrifuged (3000 g at 15 minutes) and the resulting supernatant kept frozen for 24 hours before being used for the determination of selected biochemical parameters.

Determination of Haematological Parameters

Haematological Autoanalyzer (Beckman Coulter, Inc. Fullerton, CA, USA) was used for the determination of haematological parameters: Packed cell volume (PCV), hemoglobin (Hb) concentration, white blood cell (WBC) count, platelet count, mean cell volume (MCV), monocyte and lymphocyte by adopting the procedure

described by Dacie and Lewis (1995) and Lewis et al (1995).

Histological Examination

The tissues of animals (liver and brain) was fixed in 10% (v/v) formaldehyde, dehydrated through ascending grades of ethanol (70%, 90% and 95% v/v), cleaned in xylene and embedded in paraffin wax (melting point 56 ^{0}C) (Krause, 2001; Avwioro, 2010). Tissue sections were then prepared according to the procedure described by Disbrey and Rack (1970) and Drury and Wallington (1973) and stained with haematoxylin/eosin (H&E). The histology slides was read with a binocular light research microscope (OLYMPUS, Model: XSZ-209BN, New York Microscope Company Inc., New York). Cross section of the liver was captured at x400 while that of the brain was captured at x100 with Kodak Digital Camera package (Model: Powershot M4505, Germany).

Statistical Analysis

Results were expressed as the mean ± SD of nine determinations. Means were analyzed using Duncan's Multiple Range Test and complemented with Student's t-test. The differences were considered statistically significant at $p<0.05$. All these analyses were done using SPSS 20.0 Software (Statistical Package for Social Sciences, Inc., Chicago, IL, USA).

RESULTS

Phytoconstituents present in the methanolic extract of Cannabis sativa leaves are alkaloids, steroids, phenolics, terpenoids, flavonoids, saponins, carbohydrates, tannins, cardiac glycosides, starch and reducing sugars while the aqueous extract contains all except phenolics, tannins and starch. This is shown in Table 1.

Table 1: Secondary metabolite content of aqueous and methanolic extract of Cannabis sativa leaves.

Class of compound	Methanolic extract	Aqueous extract
Alkaloids	+++	+
Steroids	++	+
Phenolics	++	_
Terpenoid	+	+
Flavonoids	++	+
Tannins	+	_
Starch	+	_
Cardiac glycosides	++	+
Reducing sugars	++	+
Saponins	+++	+
Carbohydrates	+	+

(+) = Present in minute amount, **(++)** = Present in moderate amount, **(+++)** = Present in appreciable amount, **(-)** Not present.

Treatment of animals with both aqueous and methanolic extract of C. sativa leaf at 700 mg/kg body weight significantly ($p< 0.05$) increased the platelet count in the aqueous and methanol groups from week1, week2 and week3 when compared with the control group (Table 2).

Throughout the experimental period of 3 weeks, treatment of animals with both aqueous and methanolic extract at 700 mg/kg body weight did not significantly ($p< 0.05$) alter the levels of hemoglobin (Hb) and packed cell volume (PCV) in the aqueous and methanol groups when compared with the control group (Table 2).

From week1, week2 and week3, treatment of animals with both aqueous and methanolic extract leaf at 700 mg/kg body weight significantly ($p< 0.05$) decreased the levels of white blood cell (WBC), mean corpuscular volume (MCV), monocyte and lymphocyte in the aqueous and methanol groups when compared with the control group (Table 2).

Table 2: Haematological parameters of male rats following oral administration of aqueous and methanolic extract of Cannabis sativa leaves.

Duration	Parameters	Control Group (Mean±SD)	Aqueous Group (Mean±SD)	Methanol Group (Mean ± SD)
Week1	PCV (%)	43.43 ± 0.21^a	43.43 ± 0.21^a	43.43 ± 0.21^a
Week2		43.43 ± 0.21^a	43.43 ± 0.21^a	43.43 ± 0.21^a
Week3		43.42 ± 0.21^a	43.63 ± 0.21^a	43.63 ± 0.21^a
Week1	PLT (10^3/ML)	281.43 ± 1.86^a	471.17 ± 1.55^b	513.67 ± 3.27^c
Week2		280.43 ± 2.57^a	488.93 ± 9.90^b	530.43 ± 1.15^c
Week3		280.17 ± 1.90^a	504.27 ± 3.55^b	540.80 ± 0.95^c
Week1	Lymphocytes (%)	93.61 ± 1.91^a	80.26 ± 5.23^b	75.756 ± 3.11^c
Week2		93.13 ± 0.78^a	73.37 ± 6.75^b	67.69 ± 5.17^c
Week3		93.21 ± 0.26^a	73.36 ± 5.82^b	68.42 ± 7.32^c
Week1	WBC (10^6/µL)	7.20 ± 0.30^a	4.13 ± 0.15^b	3.30 ± 0.27^c
Week2		7.20 ± 0.30^a	2.07 ± 0.85^b	1.70 ± 0.45^c
Week3		7.20 ± 0.61^a	2.13 ± 0.15^b	1.17 ± 0.21^c
Week1	MCV (fl)	56.97 ± 3.02^a	43.34 ± 6.09^b	37.36 ± 9.18^c
Week2		56.33 ± 3.79^a	36.51 ± 196^b	31.98 ± 6.61^c
Week3		56.46 ± 0.91^a	24.32 ± 4.07^b	22.50 ± 2.90^c

Week1	Monocytes (%)	4.13±0.25[a]	2.17±0.67[b]	1.80±0.10[c]
Week2		4.20±0.66[a]	1.53±0.55[b]	1.03±0.15[c]
Week3		4.20±0.36[a]	1.20±0.36[b]	0.60±0.10[c]
Week1	Hemoglobin (G/DL)	14.48±0.07[a]	14.48±0.07[a]	14.46±0.07[a]
Week2		14.47±0.07[a]	14.49±0.07[a]	14.49±0.07[a]
Week3		14.48±0.07[a]	14.55±0.07[a]	14.47±0.07[a]

Data are mean ± SD of nine determinations. Test values with superscript different from their respective control across the row are significantly different ($p<0.05$).
PCV= Packed cell volume; PLT= Platelet; WBC= White Blood Cell; MCV= Mean Corpuscular Volume

Treatment of animals with both aqueous and methanolic extract of C. sativa leaf at 700 mg/kg body weight significantly ($p< 0.05$) decreased the concentration of serum total and direct bilirubin as well as the activities of alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the aqueous and methanol groups from week1, week2 and week3 when compared with the control group (Table 3).

Table 3: Effect of aqueous and methanolic extract of Cannabis sativa leaves on selected liver biomarkers of male rats.

Duration	Parameters	Control Group (Mean±SD)	Aqueous Group (Mean ±SD)	Methanol Group (Mean ± SD)
Week1	ALP (U/L)	128.44±3.73[a]	155.49±11.88[b]	170.95±8.02[c]
Week2		128.52±3.42[a]	181.41±14.03[b]	213.88±3.85[c]
Week3		128.66±1.13[a]	199.22±5.85[b]	225.65±7.01[c]
Week1	AST (U/L)	74.40±1.03[a]	83.65±18.61[b]	98.30±4.88[c]
Week2		74.72±0.10[a]	114.52±10.25[b]	128.45±7.86[c]
Week3		73.41±2.68[a]	124.57±14.13[b]	129.25±8.35[c]
Week1	ALT (U/L)	35.10±2.55[a]	46.50±4.87[b]	55.30±2.87[c]
Week2		35.33±0.83[a]	63.62±6.55[b]	75.31±3.15[c]
Week3		35.80±2.50[a]	67.32±14.05[b]	75.63±5.41[c]
Week1	Direct Bilirubin (µmol/L)	0.04±0.01[a]	0.14±0.044[b]	0.21±0.01[c]
Week2		0.03±0.01[a]	0.24±0.03[b]	0.35±0.03[c]
Week3		0.04±0.01[a]	0.24±0.03[b]	0.35±0.02[c]
Week1	Total Bilirubin (µmol/L)	0.11±0.02[a]	0.35±0.04[b]	0.54±0.05[c]
Week2		0.12±0.02[a]	0.64±0.03[b]	0.93±0.05[c]
Week3		0.13±0.07[a]	0.64±0.02[b]	0.95±0.04[c]

Data are mean ± SD of nine determinations. Test values with superscript different from their respective control across the row are significantly different ($p<0.05$)
ALP = Alkaline Phosphatase; **AST=** Aspartate Aminotransferase; **ALT=** Alanine Aminotransferase

Histological section of the liver showed normal portal vein (PV), central vein (CV) and sinusoid (S) from week1 to week3 (Plate 1a). The brain showed normal pyramidal cell (PY), perivascular space (PS), vacuole (V) and neuronal cells (NC) for the control group from week1 to week3 (Plate 1b).

At week1, the cross section of liver and brain of aqueous group revealed tissue degeneration (TD), hepatocellular degeneration (HD) and acute hepatic necrosis (AH) as well as necrosis (N), neuronal degeneration (ND) respectively (Plate 2a and c). A cross section of liver and brain of methanol group revealed tissue degeneration (TD), kupffer (KC) and acute hepatic necrosis as well as lymphocytic cuffing (LC), and neuronal degeneration (ND) respectively (Plate 2b and d).

At week2, the cross section of liver and brain of aqueous group revealed hepatocellular degeneration (HD) and acute hepatic necrosis (AH) as well as neurosis (N), neuronal degeneration (ND) respectively (Plate 3a and c). The cross section of liver and brain of methanol group revealed acute hepatic necrosis (AH) and central vein (CV) as well as congestion of vascular channel (VC), and pyramidal cell (PY) respectively (Plate 3b and d).

At week3, the cross section of liver and brain of aqueous group revealed hepatocellular necrosis (HN) and acute hepatic necrosis (AHN), central vein inflammation (CVI) and (CIR) as well as perivascular congestion (PC), and necrosis (N) respectively (Plate 4a and c). A cross section of liver and brain of methanol group revealed acute hepatic necrosis (AHN), tissue degeneration (TD), central vein inflammation (CVI) as well as multi-focal congestion of vascular channel (VC) respectively (Plate 4b and d).

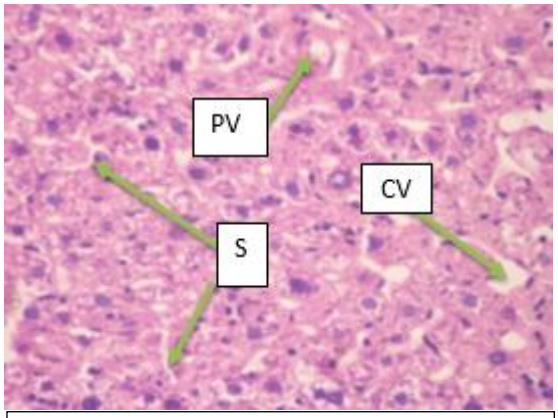

Plate 1a: Cross section of liver of rat (control group) administered distilled water indicating: normal portal vein (PV), central vein (CV) and sinusoid (S) (x400; H&E).

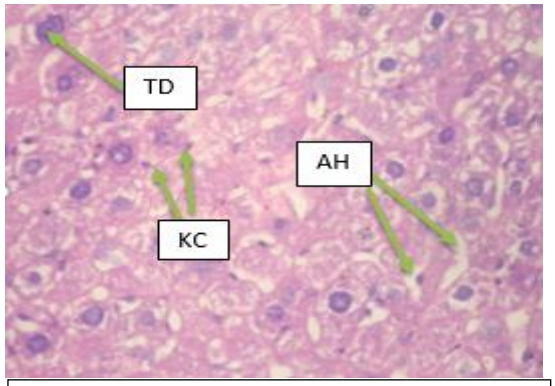

Plate 2b: Cross section of liver of rat (methanol group) administered 700mg/kg body weight of methanolic extract of *C. sativa* leaf for Week1 showing: tissue degeneration (TD), kupffer (KC) and acute hepatic necrosis (AH) (x400; H&E).

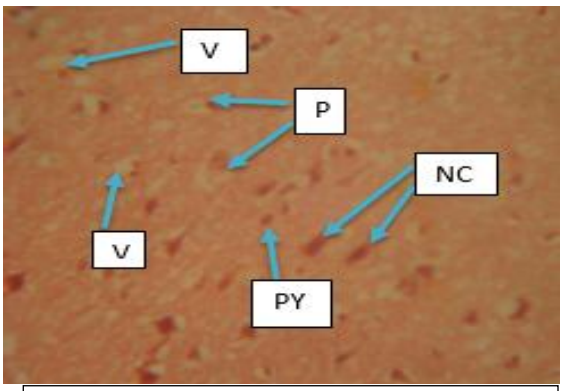

Plate 1b: Cross section of brain of rat (control group) administered distilled water showing normal pyramidal cell (PY), perivascular space (PS), vacuole (V) and neuronal cells (NC) (x100; H&E).

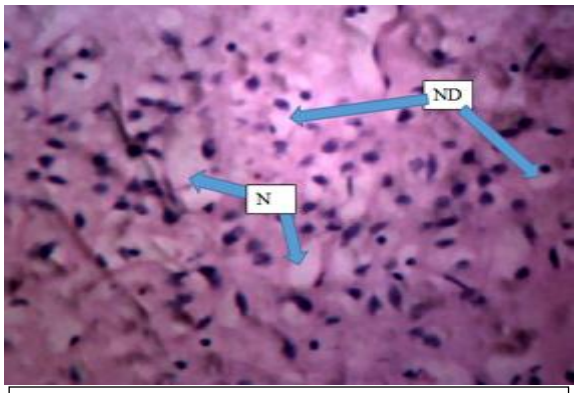

Plate 2c: Cross section of brain of rat (aqueous group) administered 700mg/kg body weight of aqueous extract of *C. sativa* leaf for Week1 showing: Necrosis (N), Neuronal degeneration (ND) (x100; H&E).

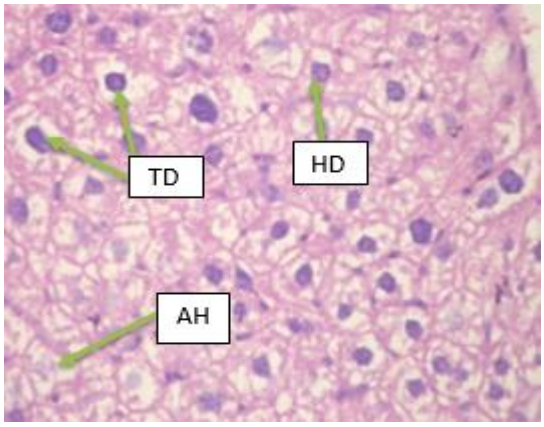

Plate 2a: Cross section of liver of rat (aqueous group) administered 700mg/kg body weight of aqueous extract of *C. sativa* leaf for Week1 indicating: tissue degeneration (TD), hepatocellular degeneration (HD) and acute hepatic necrosis (AH) (x400; H&E).

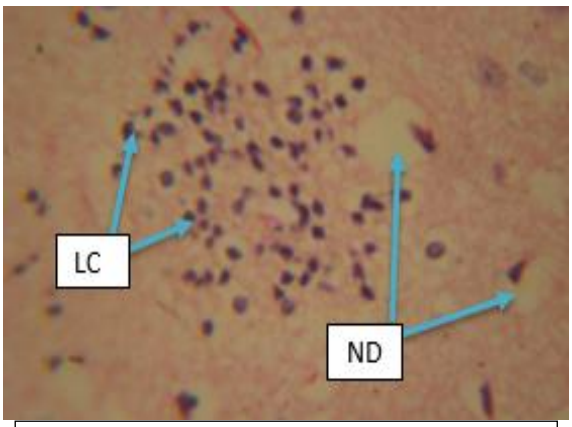

Plate 2d: Cross section of brain of rat (methanol group) administered 700mg/kg body weight of methanolic extract of *C. sativa* leaf for Week1 indicating: Lymphocytic cuffing (LC), and neuronal degeneration (ND) (x100; H&E).

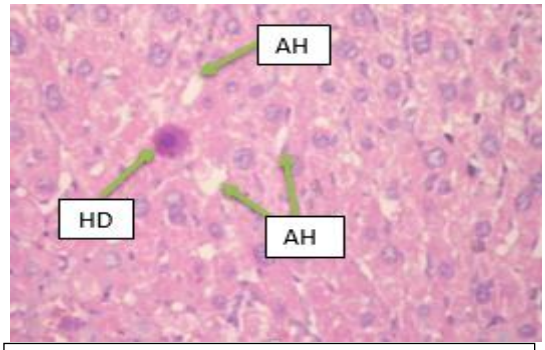

Plate 3a: Cross section of liver of rat (aqueous group) administered 700 mg/kg body weight of aqueous extract of *C. sativa* leaf for Week2 indicating: hepatocellular degeneration (HD) and acute hepatic necrosis (AH) (x400; H&E).

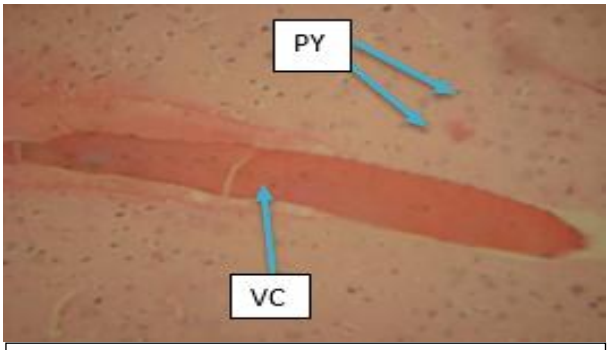

Plate 3d: Cross section of brain of rat (methanol group) administered 700 mg/kg body weight of methanolic extract of *C. sativa* leaf for Week2 showing: congestion of vascular channel (VC), and pyramidal cell (PY) (x100; H&E).

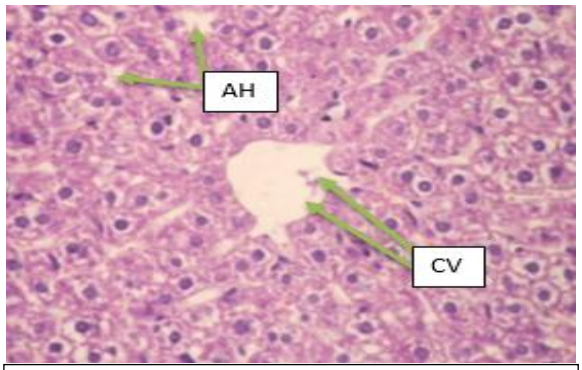

Plate 3b: Cross section of liver of rat (methanol group) administered 700 mg/kg body weight of methanolic extract of *C. sativa* leaf for Week2 showing: acute hepatic necrosis (AH) and central vein (CV) (x400; H&E).

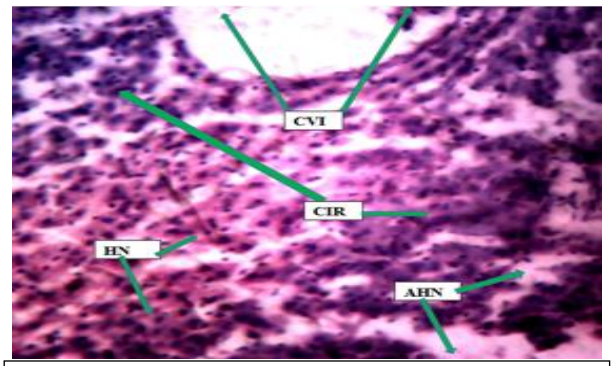

Plate 4a: Cross section of liver of rat (aqueous group) administered 700 mg/kg body weight of aqueous extract of *C. sativa* leaf for Week3 showing: hepatocellular necrosis (HN) and acute hepatic necrosis (AHN), central vein inflammation {CVI) and (CIR) (x400; H&E).

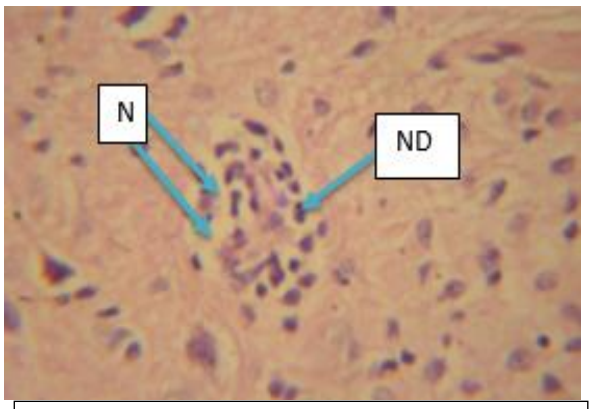

Plate 3c: Cross section of brain of rat (aqueous group) administered 700 mg/kg body weight of aqueous extract of *C. sativa* leaf for Week2 indicating: Neurosis (N), Neuronal degeneration (ND) (x100; H&E).

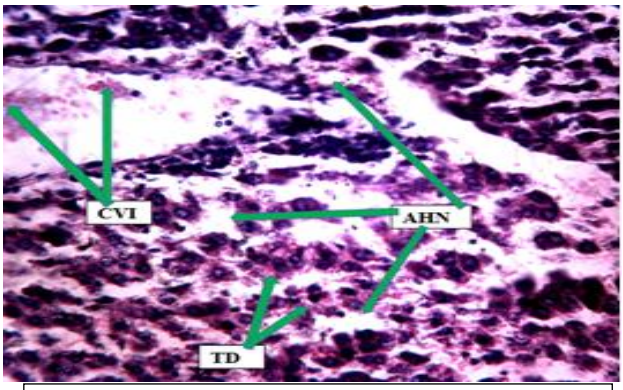

Plate 4b: Cross section of liver of rat (methanol group) administered 700 mg/kg body weight of methanolic extract of *C. sativa* leaf for Week3 indicating: acute hepatic necrosis (AHN), tissue degeneration (TD), central vein inflammation (CVI) (x400; H&E).

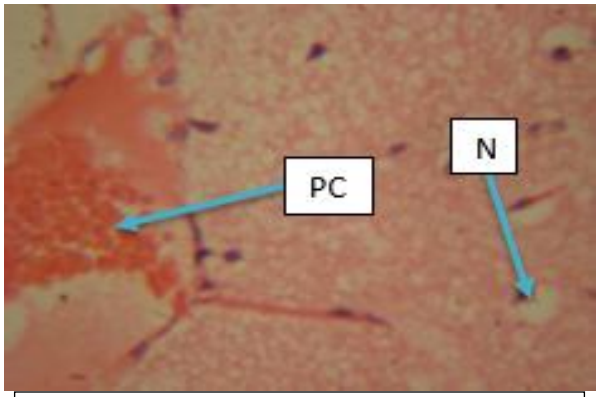

Plate 4c: Cross section of brain of rat (aqueous group) administered 700mg/kg body weight of aqueous extract of *C. sativa* leaf for Week3 showing: perivascular congestion (PC), and necrosis (N) (x100; H&E).

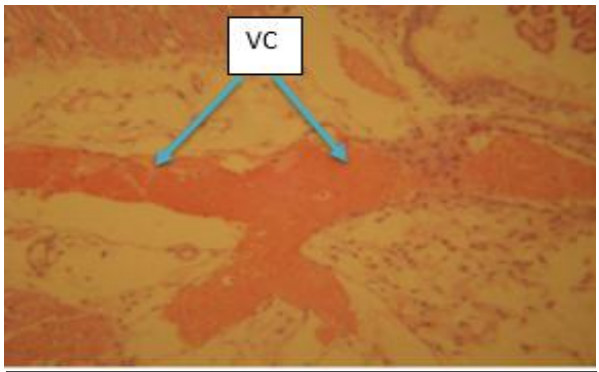

Plate 4d: Cross section of brain of rat (methanol group) administered 700 mg/kg body weight of methanolic extract of *C. sativa* leaf for Week3 showing: multi-focal congestion of vascular channel (VC) (x100; H&E).

DISCUSSION

Screening of secondary metabolite of aqueous and methanolic extract of C. sativa leaves which revealed the presence of significant amount of secondary metabolite contents might be responsible for some of the various pharmacological and toxicological effects of the plant. The number of secondary metabolite obtained from methanolic extract of C. sativa leaves was greater than the aqueous extract suggesting that methanol is a better solvent for extraction of C. sativa leaves than water.

The analysis of blood indices has proven to be a valuable approach for analyzing the health status of animal models as these indices provide reliable information on metabolic disorders, deficiencies, chronic stress status and blood relating functions before they are present in a clinical setting (Bahmani et al., 2001). Alterations in blood parameters may be due to changes in cellular integrity, membrane permeability of cells or even due to exposure to toxic chemicals (Hoffbrand, 1997; Edet et al., 2013). The non-significant effect in packed cell volume (PCV) by both extracts at 700 mg/kg body weight suggests that the extracts may not affect the rate of production of RBCs (erythropoiesis). The non-significant effect in haemoglobin (Hb) by both extracts at 700 mg/kg body weight suggests that the extracts may not affect the oxygen-carrying capacity of the blood and the amount of oxygen delivered to the tissues (Seigler, 2003). The non-significant effect in haemoglobin (Hb) and packed cell volume (PCV) by both extracts at 700 mg/kg body weight in the rats suggest that the extract could possess erythropoetic activity which will enhance the PCV and Hb level and thereby correct anaemia. The significantly high level of platelet count in both the aqueous and methanol groups at 700 mg/kg body weight compared with control group may imply better blood clotting ability. It may also be due to stimulatory effect on thrombopoietin (Kaushansky, 1995; Li et al., 1999).

The increase in white blood cell (WBC) count in the aqueous and methanol group at 700 mg/kg body weight may suggest poor defensive mechanisms against infection resulting to decrease in ability of the body to respond to infection. This decrease in WBC count by both extracts at 700 mg/kg body weight may also indicate a weak immune system. The decrease in level of MCV following administration of both extracts at 700 mg/kg body weight suggests that the animals may be predisposed to anaemia (Coles, 1986). The reduction in monocyte by both extracts at 700 mg/kg body weight could be linked to suppression of leucocytosis from the bone marrow which may account for poor defensive mechanisms against infection (Oyedemi et al., 2010). Lymphocytes are the main effectors cells of the immune system (McKnight et al., 1999). The decrease in lymphocytes by both extracts at 700 mg/kg body weight suggests inhibitory effect on the effectors cells of the immune system.

Liver function tests are often done to ascertain the effectiveness of the hepatocytes (Eze et al., 2015). The concentration of biomolecules like bilirubin in the serum of rats could indicate the state of the liver (Ganong, 2001). The decrease in serum total and direct bilirubin levels observed in this study might be an indication of impairment in the functional capacity of the liver (Moudgil and Narang, 1989) and could be a consequence of severe defects in bilirubin transport, which may cause haemolysis and thus lead to jaundice (Kaplan and Pesce, 1996).

The measurement of the activities of various enzymes in the tissues and body fluids plays a significant role in disease investigation, diagnosis and tissue cellular damage (Malomo, 2000). Changes in the levels of normal range of enzymes localized in specific cells indicate functional toxicity of such cells and these alterations occurs prior to obvious cellular architectural degeneration that are observed on histological examination (Wright and Plummer, 1974) and is required in certain amounts for proper functioning of organs.

The aminotransferases considered in this study are useful markers of liver cytolysis and can be used in assessing damage in the liver and heart (Chapatwala et al., 1982; Shahjahan et al., 2004).

Aminotransferases occupy a central position in the metabolism of amino acids and biochemical regulation of intracellular amino acid pool. The reduction in serum AST and ALT activities observed in present study may be due to damage to the plasma membrane leading to loss of this enzyme into the extracellular fluid (Malbica and Hart, 1971). Alkaline phosphatase is an ectoenzyme of the hepatocyte's plasma membrane. It is one of the enzymes used to assess the integrity of the cells following the administration of chemical compounds (Yakubu, 2006). It also plays an important role in maintaining cell membrane permeability. The significant decrease in serum alkaline phosphatase activity following the administration of aqueous and methanol extract of C. sativa leaves at 700 mg/kg body weight indicates damage on the cell membrane by peroxidation of the polyunsaturated fatty acids present on the membrane and denotes damage to the hepatic cells (Singh et al., 1999).

Histological examination of tissues could serve as complementary evidence to functional indices and enzyme studies by revealing any distortion/damage to the normal structure of the tissues. Neuronal degeneration or cellular damage in neurons has been reported to result in cell death. Cell death could be apoptosis or necrosis, which differ morphologically and cytochemically (Farber et al., 1981; Sarne and Keren, 2004).

The normal histological section of the liver and brain in the control group from week1 to week3 might be due to a defense mechanism exerted by the hepatic and brain cells (Hemieda et al., 2007; El-wenssemy, 2008).

The appearance of acute hepatic necrosis and neuronal degeneration at week1 by the aqueous and methanol extract is an indication of derangement to the hepatocyte by extract toxicity (Neyrinck, 2004). It may also be indicative of degenerated liver tissue and loss of structural integrity by the extract (Bancroft, and Gamble, 2002; Alici et al., 2003).

At week2, the abnormal histological alterations (hepatocellular and neuronal degeneration) observed in the liver and brain respectively of the aqueous and methanolic group is an indication of degenerative and/or disruptive interference on the cellular/structural integrity of the tissues (Martins et al., 1978; Ekong et al., 2008).

The central vein inflammation and multi-focal congestion of vascular channel in the aqueous and methanolic groups, at week3 is an indication of histoarchitectural damage on the liver and brain which would have consequential effect on structure of the tissues (Michael and Wojciech, 2010; Kierszenbaum and Tres, 2011).

In the present study, the aqueous and methanolic extracts of C. sativa at 700 mg/kg body weight may have acted indirectly through generation of high levels of ROS or directly as toxin to the liver and brain thereby affecting their cellular and functional integrity. In cellular necrosis, the rate of progression depends on the severity of insults on tissues. The greater the severity of the insults on tissues, the more rapid the progression of the injury (Martins et al., 1978; Ito et al., 2003).

CONCLUSION

The various alterations in liver function parameters, haematology as well as degeneration in liver and brain histology by aqueous and methanolic extract of C. sativa leaves may suggest that the extract possess functional and structural toxicity in male rats. Findings from the present study therefore support the speculation that consumption of Cannabis sativa may contribute to increasing incidence of liver failure and brain damage.

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