Biochemical, Immunological, DNA and Histopathological Changes Caused by *Cannabis Sativa* in the Rat

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Abstract: The aim of this study was to investigate the effect of repeated administration of *Cannabis sativa* extract on some biochemical, immunological parameters, on DNA damage and on brain and liver histology in the rat. Rats received either saline or *Cannabis sativa* at 5, 10 or 20 mg/kg (expressed as Δ^9-tetrahydrocannabinol), intraperitoneally daily for 30 days. Cholesterol, triglycerides, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), total proteins, glucose were determined in serum. DNA fragmentation was measured in the liver. The level of DNA damage of peripheral blood mononuclear cells (PBMCs) was evaluated by alkaline single cell gel electrophoresis (comet assay). Brain and liver histopathology and caspase-3 immunohistochemistry were performed. Cannabis treatment resulted in increased cholesterol (41.2% by 20 mg/kg cannabis) and triglycerides (17.8-31.8% by 10-20 mg/kg cannabis). HDL-C decreased by 21.9-38.4% while LDL-C increased by 14.7-174.7% by 10-20 mg/kg cannabis. Total proteins were unaltered but glucose increased by 34.2-53.3% by 10-20 mg/kg of cannabis. CD4 increased by 16.6-19.5% by 10-20 mg/kg cannabis. Liver DNA fragmentation increased by 16.7% after cannabis at 20 mg/kg. The comet percentage of PBMCs was higher after 5, 10 and 20 mg/kg cannabis (7.0 ± 0.36, 9.0 ± 0.92 and 21.0 ± 0.97) than that in saline control group (5 ± 0.36). Cannabis caused dark neurons, decreased size of nuclei, cellular infiltration and increased caspase-3 immunoreactivity. In the liver, cannabis treatment was associated with fibrosis, vacuolar degeneration, cellular infiltration in the portal area, dilatation of portal vein and positive reaction to caspase-3 antibody. These effects of cannabis were dose-dependent.

Keywords: Brain, Cannabis sativa, comet assay, liver, metabolic effects.

INTRODUCTION

Cannabis preparations (from the female plant *Cannabis sativa*) are the most common illicit substances worldwide. The most common cannabis preparations are marijuana, hashish and hash oil. Marijuana is prepared from the dried flowering tops and leaves of the plant. Hashish consists of dried cannabis resin and compressed flowers [1]. Cannabis preparations contains more than 70 cannabinoids [2]. One of these, Δ^9-tetrahydrocannabinol (Δ^9-THC) which is found in a resin that covers the flowering tops and upper leaves of the female plant, is believed to be responsible for the psychomimetic properties of marijuana or hashish [1,3,4]. Other cannabinoids include Cannabidiol (CBD), cannabinol, tetrahydrocannabivarin, and cannabichromene, with THC and cannabidiol accounting for 95% of marijuana's active ingredients [2,5]. Δ^9-THC acts at the cannabinoid CB1 receptor to produce a wide-range of biological and behavioral responses e.g., euphoria and relaxation, perceptual alterations, time distortion, intensification of ordinary sensory experiences and increased appetite [1,4]. Cannabinoids have been shown to mediate their effects by interacting with two subtypes of G-protein-coupled cannabinoid (CB1 and CB2) receptors with CB1 receptors being mostly found in the central nervous system and mediates the neurobehavioral effects of cannabis on cognition, memory, reward, nociception, appetite, motor coordination and attention. On the other hand, CB2 receptors are expressed mainly (though not exclusively) on the immune cells in the peripheral tissues and these have been shown to affect cytokine production, lymphocyte phenotype, function and survival, cell-mediated immunity, and balance of Th1/Th2 cells [6].

In recent years, there has been growing interest in the use of cannabis for medical purposes. Dronabinol and nabilone, two oral formulations of a synthetic THC were approved by the US Food and Drug Administration for use in chemotherapy-induced nausea and vomiting refractory to conventional antiemetic therapy [7,8]. Cannabis-based medicinal extracts are being increasingly used in the treatment of spasticity associated with multiple sclerosis. Sativex is an oromucosal spray of whole plant extract [THC: CBD=1: 1] and which has been reported to improve muscle spasticity, neuropathic pain and bladder disturbance in multiple sclerosis [9,10]. The use of cannabis has also been reported by patients with chronic pain in such conditions as multiple sclerosis, arthritis, neuropathy [11], myofascial pain syndrome, discogenic back pain [12], HIV-associated sensory polyneuropathy [13], fibromyalgia [14], sickle cell disease [15], ulcera-tive colitis and Crohn's disease [16]. Cannabinoids might

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E-ISSN: 2309-6179/14

also be of therapeutic value in the treatment of tics in Tourette syndrome, the reduction of levodopa-induced dyskinesia in Parkinson s disease and some forms of tremor and dystonia [17,18].

The present study was therefore designed to investigate the effect of repeated administration of an extract of *Cannabis sativa* for one month on some selected metabolic and immunological parameters and on the level of DNA damage. Histopathological examination and caspase-3 immunohistochemistry (apoptosis) of brain and liver sections were also performed.

MATERIALS AND METHODS

Animals

Adult Sprague–Dawley rats of either sex (120–130 g) were used throughout the experiments and fed with standard laboratory chow and water *ad libitum*. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985).

Equal groups of six rats each were used in all experiments.

Drugs and Chemicals

Cannabis sativa L. plant was supplied by the Ministry of Justice, Egypt. *Cannabis sativa* extract was prepared from the dried flowering tops and leaves of the plant. The method of extraction followed that described by Turner and Mahlberg [19] with modification as described elsewhere [20]. Tetrahydrocannabinol (THC) content was quantified using "GC mass spec". The Δ^9-THC content of the extract was 10%.

Experimental Groups

Rats were randomly assigned into 4 groups, each of 6 animals. Group 1 (normal control) received s.c. saline 0.2 mL/rat. Groups 2, 3 & 4 received *Cannabis sativa* extract s.c., daily at doses equivalent to 5, 10 or 20 mg/kg Δ^9-tetrahydrocannabinol. Rats had free access to food and drinking water during the study. Rats were killed 30 days after drug or saline administration by decapitation under ether anaesthesia.

Biochemical Studies

Determination of total cholesterol, triglycerides, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), total proteins, and glucose in serum was done using commercially available kits (Stanbio Laboratory, Texas, USA).

Determination of DNA Fragmentation

The basis of this method is that extensively fragmented double-stranded DNA can be separated from chromosomic DNA upon centrifugal sedimentation. The protocol includes the lysis of cells and the release of nuclear DNA, a centrifugation step with the generation of two fractions (corresponding to intact and fragmented DNA, respectively), precipitation of DNA, hydrolysis and colorimetrical quantitation upon staining with diphenylamine (DPA) which binds to deoxyribose [21].

Measurement of Comet Assay

Cell Preparation

Peripheral blood leukocytes were isolated by centrifugation (30 min at 1300g) in Ficoll-Paque density gradient (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). After centrifugation, leukocytes were represented as a buffy coat, aspirated and washed twice by phosphate-buffered saline at pH 7.4 (PBS).

Preparation of Cell Microgels on Slides

All the procedures for the alkaline comet assay were done at low temperature to minimize spontaneous DNA damage. The comet assay was performed according to Singh *et al.* [22] with modifications according to Blasiak *et al.* [23]. Cell microgels were prepared as layers. The first layer of gel was made by applying 100 µl of normal melting point agarose (0.7%) onto a precleaned microscope charged slides and coversliped gently. The coverslip was removed after the agarose solidified at 4C. Low melting-point agarose (0.5%) was prepared in 100 mmol/L PBS and kept at 37C. Approximately, 1500 of peripheral blood lymphocytes were mixed with the low melting-point agarose and 100 µl of the mixture was applied to the first gel layer. The slides were then covered with a coverslip and placed at 4C for solidification. After the second layer solidified, the coverslips were removed from the cell microgels. A final layer of low-melting agarose was added followed by coverslips, left to solidify for 10 minutes then the coverslips were removed.

Lysis of Cells, DNA Unwinding, Gel Electrophoresis, DNA Staining

The slides were covered with 100 ml of fresh lysis buffer (2.5 mol/L NaCl, 100 mmol/L EDTA, 1% sodium hydroxide, 10 mmol/L Tris, 1% Triton X-100, 10%

DMSO (pH10) at 4C for 1 h. After draining, microgels slides were treated with DNA unwinding solution (300 mmol/L NaOH, 1 mmol/L EDTA, pH 13) for 30 min at 4C, and placed directly into a horizontal gel electrophoresis chamber filled with DNA-unwinding solution. Gels were run with constant current (300 mA at 4C) for 30 min. After electrophoresis, the microgels were neutralized with 0.4 M Trisma base at pH 7.5 for 10 min. The slides were stained with 20 μl ethidium bromide (10 μg/ml).

Visualization and Analysis of Comet Slides

The slides were examined at 400× magnification using a fluorescence microscope (IX70; Olympus, Tokyo, Japan) equipped with an excitation filter of 549 nm and a barrier filter of 590 nm, attached to a video camera (Olympus). A damaged cell is visualized as each cell had the appearance of a comet, with a brightly fluorescent head and a tail to one side formed by the DNA containing strand breaks that were drawn away during electrophoresis. Samples were analyzed by counting the damaged cell out of 100 cells per slide to calculate the percent of damage.

Quantification of CD4 Concentration in Serum

The soluble CD4 serum concentrations were was determined using a CD4 kit (Glory Sciences Co., Ltd, USA) according to the manufacturer's instructions. The principle of the method was a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA).

Histopathological Studies

The brain and liver from all animals were dissected immediately after death. The sections were fixed in 10 % neutral buffered formal saline for 72 h at least. All the specimens were washed in tap water for half an hour and then dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin. Serial sections of 5 μm thick were cut and stained with haematoxylin and eosin (H&E) for histopathological investigation. Images were captured and processed using Adobe Photoshop version 8.0.

Immunohistochemistry for Caspase-3

Immunohistochemical staining of anti-caspase-3 antibodies was performed with streptavidin-biotin. Sections of 4 μm thick were deparaffinized and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min at room temperature. The specimens were then incubated with anti caspase-3 antibodies as the primer antibody at a 1: 100 dilution. The specimens were counter stained with H & E. Negative controls were prepared by substituting normal mouse serum for each primary antibody.

Statistical Analysis

Data are expressed as mean ± SE. Statistical analysis of the data was done using one-way ANOVA followed by Duncan test for multiple group comparisons. Probability levels of $p<0.05$ were considered statistically significant.

RESULTS

Biochemical Results

Serum Lipids, Proteins and Glucose

Cannabis treatment resulted in increased cholesterol (41.2% by 20 mg/kg cannabis), triglycerides (17.8-31.8% by 10-20 mg/kg cannabis). HDL-C decreased by 21.9-38.4% while LDL-C increased by 14.7-174.7% by 10-20 mg/kg cannabis. Total proteins were unaltered but glucose increased by 34.2-53.3% by 10-20 mg/kg of cannabis (Table **1**).

Liver DNA Fragmentation

DNA fragmentation in the liver was unaltered by 5 or 10 mg/kg of cannabis but significantly increased by 16.7% after cannabis given at 20 mg/kg (Figure **1**).

DNA Damage of PBMCs

The comet percentage of PBMCs was higher after 5, 10 and 20 mg/kg cannabis (7 ± 0.36, 9 ± 0.92 and 21 ± 0.97) than that in saline control group (5 ± 0.36). Moreover, the comet percentage of rats treated with 20 mg/kg cannabis was significantly higher than that of

Table 1: Effect of Cannabis Sativa on Serum Lipids, Proteins and Glucose

	Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL-Cholesterol (mg/dl)	LDL-Cholesterol (mg/dl)	Total proteins (g/l)	Glucose (mg/dl)
Saline	57.5 ± 3.1	57.69 ± 3.61	17.85 ± 0.45	17.0 ± 0.1	7.53 ± 0.47	88.3 ± 5.0
Cannabis (10 mg/kg)	58.61 ± 4.2	67.83 ± 3.2	13.94 ± 0.26*	29.7 ± 0.88*	8.22 ± 0.58	118.5 ± 6.6*
Cannabis (20 mg/kg)	81.19 ± 5.5*	76.02 ± 4.0*	11.0 ± 0.1*	46.67 ± 1.8*	8.29 ± 0.70	135.4 ± 7.2*

Results are mean ± S.E. Six rats were used per each group. Data were analyzed by one way ANOVA and means of different groups were compared by Duncan's multiple range test. P<0.05 was considered statistically significant. *: P<0.05 *vs.* saline control group.

rats treated with 5 or 10 mg/kg cannabis (Figures **2** and **3**).

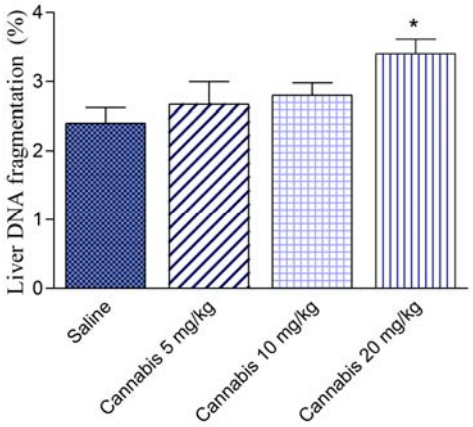

Figure 1: DNA fragmentation in the liver of cannabis-treated rats. Statistical analysis was done using one way ANOVA and Duncan' multiple comparison test. *p<0.05 *vs*. saline control group.

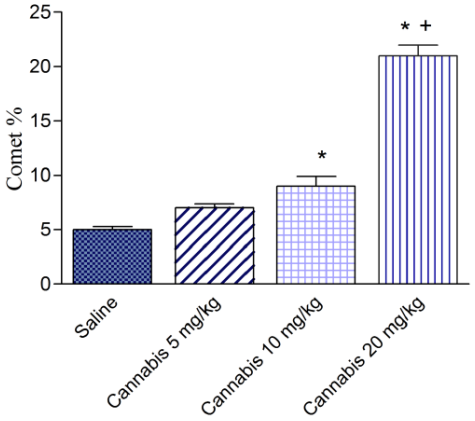

Figure 2: The representative comets for controls and cannabis treated rats. The data are presented as means ± SE. Statistical analysis was done using one way ANOVA and Duncan' test.

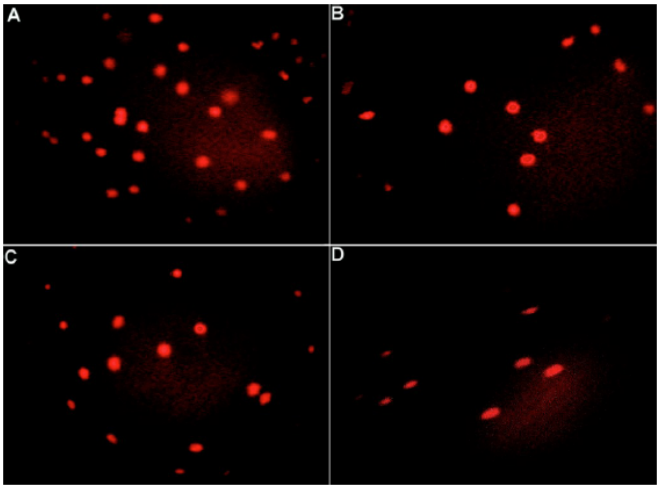

Figure 3: Fluorescence images of comets originating from peripheral blood lymphocytes of saline and cannabis treated rats. **(a)** saline (control); **(b)** cannabis 5 mg/kg; **(c)** cannabis 10 mg/kg; **(d)** cannabis 20 mg/kg.

CD4 Concentration in Serum

Soluble CD4 increased by 16.6-19.5% by 10-20 mg/kg cannabis (Figure **4**).

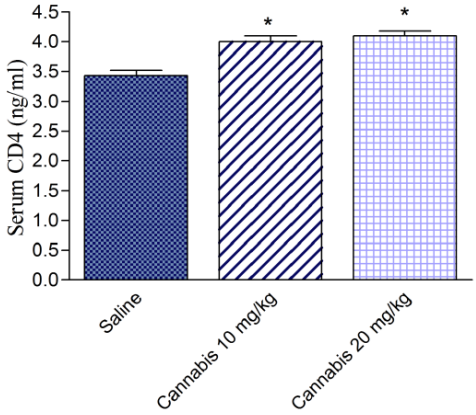

Figure 4: Serum level of soluble CD4 in cannabis and saline-treated rats. Statistical analysis was done using one way ANOVA and Duncan' test. *p<0.05 *vs*. saline control group.

Histopathological Results

Brain

The repeated administration of cannabis was associated with a dose-dependent damage of brain tissue in the form of darkening of neurons, decreased

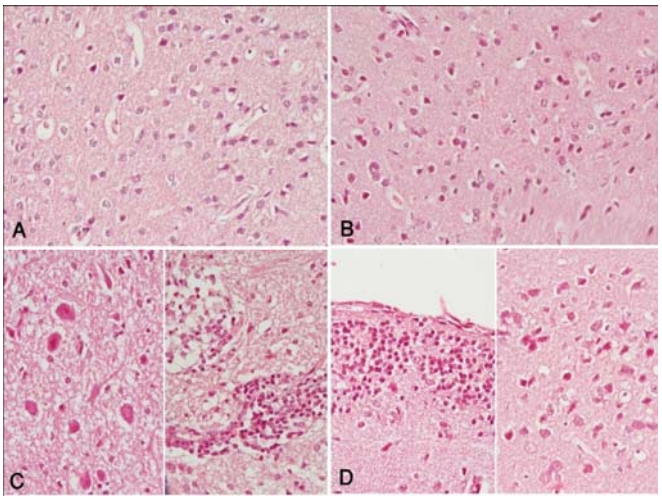

Figure 5: A photomicrograph of a section of H & E stained brain tissue of (**a**) control (saline-treated) rat showing the normal structure of brain tissue; (**b**) cannabis 5 mg/kg showing a few cells with dark nuclei; (**c**) cannabis 10 mg/kg: some neurons in the left part appear having ill defined dendrites with small dark nucleus or with undefined nucleus if compared with normal neuron. The right part shows focal aggregations of immune cells (lymphocytes, plasma cells); (**d**) cannabis 20 mg/kg showing diffuse submeningeal cellular infiltration in the left part. The right one shows multiple neurons with dark small nuclei. These neurons are of small size if compared with the normal ones beside (H & E X 100 & 200).

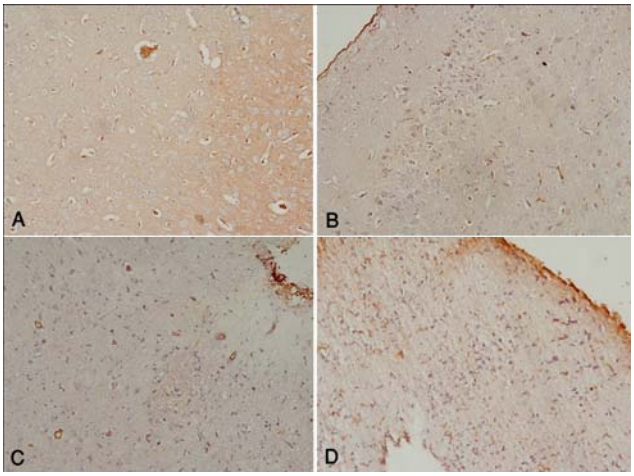

Figure 6: Immunostaining for cleaved caspase-3 in the brain tissue of **(a)** control rat (saline-treated) showing the normal negative reaction of brain tissue to this antibody; **(b)** cannabis 5 mg/kg showing a few cells giving positive reaction; **(c)** cannabis 10 mg/kg showing many neurons with positive reaction to caspase 3 antibody in their cytoplasm; **(d)** cannabis 20 mg/kg showing increased number of positively reacted cells.

size of nuclei which denote increased rate of apoptosis. Sections from rats given of 10 & 20 mg/kg cannabis showed cellular infiltration in some parts of brain tissue denoting necrosis (Figure **5**). These results were confirmed by using immunohistochemical staining with caspase 3 antibody, which revealed increased number of positively reactive cells to the stain denoting increased number of cells that began apoptosis (Figure **6**). Electron microscopy of neurons from a rat treated with 20 mg cannabis is shown in Figure **7** and **8**.

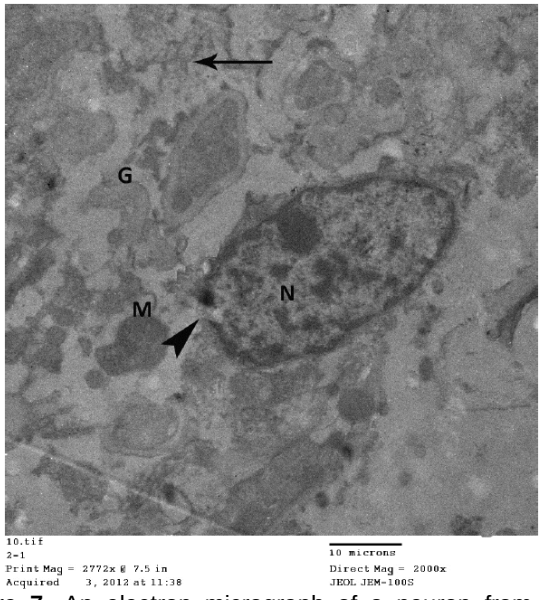

Figure 7: An electron micrograph of a neuron from a rat treated with 20 mg cannabis, showing an elongated nucleus (N) instead of being rounded. The nuclear envelope is discontinued at some parts (arrowhead), however, the nucleolus is eccentric in position giving the nucleus its characteristic shape of the owl eye. In the cytoplasm the mitochondria (M) & the Golgi apparatus are normal in shape, while the rough endoplasmic reticulum is dispersed lacking its regular shape (arrow) and the free ribosomes are very scarce.

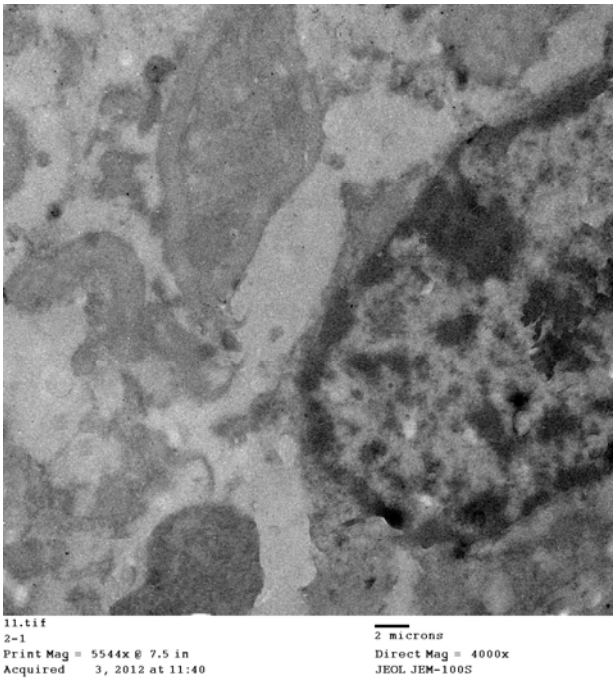

Figure 8: An electron micrograph for the previous section at a higher magnification showing the same findings.

Liver

Cannabis displayed a dose-dependent hepatotoxic effect. Cannabis given at 5 mg/kg, caused fibrosis with cellular infiltration around the central vein with mild degeneration of hepatocytes at the periphery of the lobule (Figure **9B**). A dose of 10 mg/kg caused localized interstitial cellular infiltration (arrow head), dilatation of blood sinusoids with increased cellular infiltration and marked vacuolar degeneration in many hepatocytes. (Figure **9C**). A dose of 20 mg/kg caused localized cellular infiltration especially around the central vein (arrow) (Figure **9D**).

These results were confirmed by using caspase 3 antibody immubohistochemistry, where sections from control rats gave negative results (Figure **10A**); sections from rats treated with cannabis at 5 mg/kg showed a few scattered cells with positive results specially around central veins (Figure **10B**) and sections from rats treated with 10 mg/kg showed increased number of positively stained cells (Figure **10C**). Rats treated with 20 mg/kg of cannabis showed the highest number of positively stained cells distributed randomly all over the hepatic lobule (Figure **10D**).

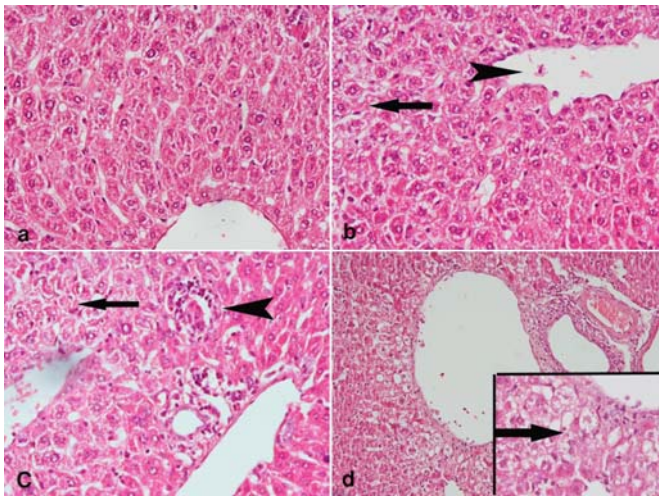

Figure 9: A photomicrograph of H & E stained liver sections from rats treated with (**a**) saline (control) showing the normal structure of this tissue; (**b**) cannabis 5 mg/kg showing fibrosis with cellular infiltration around the central vein (arrow) some hepatocytes at the periphery of lobule show mild degeneration; (**c**) cannabis 10 mg/kg showing dilatation of blood sinusoids with localized cellular infiltration (arrow head) many hepatocytes show vacuolar degeneration (arrow); (**d**) cannabis 20 mg/kg showing focal cellular infiltration at the dilated central vein (arrow) and vacuolar degeneration with varying degrees in many of the hepatocytes.

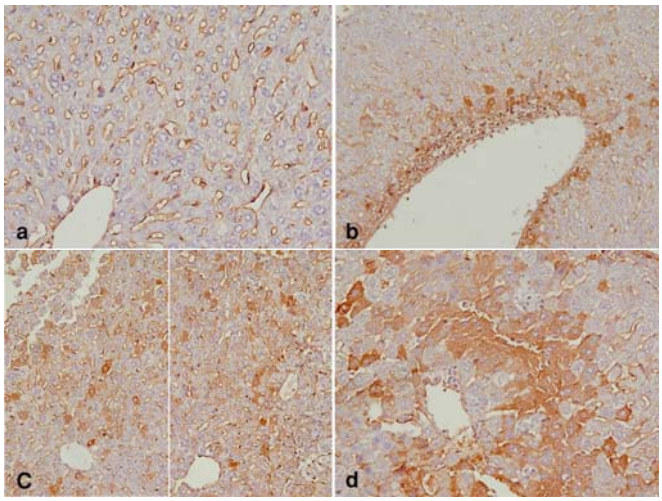

Figure 10: A photomicrograph of a section of liver tissue stained with caspase 3 antibody from **(a)** saline control rat showing negative result for the stain all over the tissue indicating that no apoptotic cells are detected; **(b)** cannabis 5 mg/kg showing few positively stained cells around central vein; **(c)** cannabis 10 mg/kg showing greater number of positively stained randomly distributed all over the hepatic lobule in two sections; **(d)** cannabis 20 mg/kg showing a very great number of positively stained cells in comparison to the previous two sections.

DISCUSSION

Dyslipidaemia is a major risk factor in atherosclerotic disease with its complications of ischaemic heart disease, cerebral infarction and peripheral vascular insufficiency [24-26]. Lowering of elevated LDL-cholesterol levels in plasma results in significant reductions in cardiovascular events in patients with coronary heart disease [27,28] and is advocated for patients with peripheral arterial disease [29]. The findings in the present study indicated that the repeated administration of a cannabis extract in rats resulted in increased cholesterol, triglycerides, LDL-C while HDL-C is decreased. Total proteins were unaltered but glucose increased by 34.2-53.3% by 10-20 mg/kg of cannabis. Cannabis thus is likely to be associated with increased risk of developing atherosclerosis and hyperglycaemia. Blood glucose is a strong independent predictor of cardiovascular- and non- cardiovascular-mortality in nondiabetic subjects with coronary vascular disease [30]. Despite the growing interest in the medicinal cannabis, the metabolic effects of cannabis are less studied and the results are somewhat controversial. In humans, current use of marijuana was associated with lower levels of insulin [and lower homeostasis model assessment of insulin resistance (HOMA-IR) [31]. Chronic cannabis smokers were found to have lower plasma HDL-cholesterol, but normal fasting levels of glucose, insulin and total cholesterol. Adipocyte insulin resistance index and percent free fatty acids suppression during an oral glucose tolerance test was lower [32]. In humans, chronic cannabis use and endocannabinoid system activation might induce insulin resistance, early endothelial damage and vasculogenic erectile dysfunction [33]. In rats, cannabis extract given at 2.5 mg/kg followed by 5 mg/kg for 21 days was associated with increased cholesterol, triglycerides, total cholesterol and HDL-cholesterol in serum [34]. Meanwhile, low doses of Δ^9-THC in mice (1 mg/kg/day) have been reported to significantly inhibit the progression of atherosclerosis. There were decreased proliferation of lymphoid cells and IF-γ secretion as well as macrophage chemotaxis by Δ^9-THC [35]. However, supplementation with 10% dietary hempseed, thought to contain low concentration of Δ^9-THC (1-2%) was found not to prevent arotic plaque formation in rats fed cholesterol-supplemented chow [36]. In healthy humans, supplementation with hempseed also did not alter the concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol [37].

In the current study, the level of DNA damage of peripheral blood lymphocytes was evaluated by alkaline single cell gel electrophoresis (comet assay). The comet assay is a sensitive technique for measuring and analyzing DNA breakage in single mammalian cells. The comet assay was first described

by Singh *et al.* [22] and since then has been used as a basic tool for evaluation of DNA damage/repair, human and environmental biomonitoring and genetic toxicology testing [38]. The comet percentage of peripheral blood lymphocytes was higher in cannabis treated rats than that in control saline treated counterparts. Our results indicate that peripheral blood lymphocytes of cannabis-treated rats have more damaged DNA compared with control rats. The comet test and DNA liver fragmentation assay done in this study clearly suggested that repeated cannabis administration at doses equivalent to 10-20 mg/kg Δ^9-THC induces significant DNA strand breaks.

CD4 is a 60-kDa glycoprotein belonging to immunoglobulin superfamily [39]. It is expressed on the surface of immune cells including T helper cells, monocytes, macrophages and dendritic cells and is involved in T cell activation. CD4+ T cells contribute to protective immunity against viral infection by recruitment of key lymphoid cell populations into secondary lymphoid tissue or sites of pathogen infection, provision of help for expansion or function of other effector cells, or through production of cytokines or cell-mediated cytotoxicity [40]. Serum levels are increased in a number of viral infections such as measles, Kawasaki disease [41], infectious mononucleosis [42] and in inflammatory conditions e.g., polymyalgia rheumatica [43] and autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and ankylosing spondylitis [44]. The levels of serum soluble CD4 appear to correlate with disease activity in some of these conditions [41,42,44]. The findings in current study indicates that CD4 increased by 16.6-19.5% after repeated administration of 10-20 mg/kg cannabis.

Cannabis impairs many aspects of immunity. Δ^9-THC, the main psychoactive component in marijuana inhibits natural killer cell activity [45] and suppresses type 1 T-helper 1 immunity [46]. *In vivo* administration of THC alone in mice decreased CD4 counts and the CD4: CD8 ratio [47]. Δ^9-THC decreases the recruitment of macrophages and lymphocytes, particularly CD4(+) and CD8(+) T cells to the lung after challenge with a nonlethal dose of the influenza virus [48]. Moreover, the antigen-specific IFN-γ production by CD8(+) cytotoxic T cells was reduced by Δ^9-THC which also suppressed the percentage of interferon-gamma (IFN-γ)-producing CD4(+) [49]. Δ^9-THC suppresses inducible costimulator expression in activated T cells and which regulates T cell activation and Th cell differentiation [50]. Δ^9-THC suppressed $CD8^+$ cytotoxic T lymphocytes function and viability independent of CB_1 and CB_2 receptors [51]. On the other hand, cannabidiol, an non-pyschoactive constituent of *Cannabis sativa* has been shown to markedly suppress humoral immunity in mice [52] and induce a fall in lymphocyte subset numbers in peripheral blood following repeated i.p. treatment in rats [53]. It is unclear whether the increased serum CD4 level observed in this study is due to activation of CD4+ T lymphocytes by cannabis and subsequent release of the protein into the circulation. It is also possible that the increased serum CD4 is generated *via* receptor shedding from cell surface by metalloproteinases [44].

Smoking cannabis is common in patients with chronic liver disease including those who are potential candidates for liver transplantation [54-56]. Cannabis use is increasingly emerging as a novel co-morbidity in patients with chronic hepatitis C where studies identified daily cannabis smoking as a novel independent predictor for severity of steatosis and fibrosis progression [57-59]. Cannabis sativa enhanced acute hepatic damage caused by CCl_4 or acetaminophen in rats [60] and enhanced the liver tissue damage and brain degeneration caused by thioacetamide in rats [20]. Cannabis also decreased the hepatoprotective effect of silymarin against carbon tetrachloride (CCl_4)-induced liver damage [61]. In the current study, the histopathological examination of the liver showed that the repeated administration of cannabis exerted a dose-dependent hepatotoxic effect. Cannabis and cannabinoids exert their effects by interacting with cannabinoid CB1 and CB2 receptor subtypes. Studies suggested that CB1 receptors enhance liver fibrogenesis and steatogenesis [62,63] as opposed to CB2 receptors which mediate hepatic protective and antifibrogenic effects [64,65] by mechanisms involving reduction of liver hepatic stellate cell accumulation and activation and reduction of inflammatory cell infiltration [63,64]. The chemistry of cannabis is, however, complex as the plant contains more than 600 different chemical compounds. Cannabinoids are the main biologically active constituents of the cannabis plant of which currently at least 70 are known. Δ^9-THC, however, is the major cannabinoid found in marijuana and hashish [1,2,66]. Δ^9-THC is a CB1 and CB2 receptor partial agonist, while cannabidiol displays high potency as an antagonist of CB1/CB2 receptor agonists and tetrahydrocannabivarin behaves as a CB1 antagonist or, at higher doses, as a CB1 receptor agonist [67]. Other cannabinoids e.g., cannabidiol, cannabinol or tetrahydrocannabivarin can thus result in different

effects from those of Δ^9-THC alone and even have an additive or an antagonistic effect to it [1,68].

Apoptosis or programmed cell death is an orchestrated form of physiological cellular suicide by which multicellular organisms control cell numbers and ensure the removal of damaged or potentially harmful cells [69]. Apoptosis is recognized by a series of morphological events that include condensation of nuclear chromatin, shrinking of cytoplasmic and nuclear compartments, compartmentalization of nuclear material into vesicular “apoptotic bodies”, and degradation of DNA into oligonucleosome-length fragments. This contrasts with necrotic cell death, which involves loss of plasma membrane integrity, cell swelling and cell lysis [70,71]. In cells undergoing apoptosis, apoptotic death stimuli activate the initiator caspases (caspase-8 and -9) and these in turn activate the executioner caspases (capsase-3, -6, and -7). The latter evokes apoptosis by cleaving cellular substrates that disassemble the cell and induce the morphological and biochemical features of apoptosis [72-74]. The findings of the present study indicates that the administration of cannabis results in increased liver DNA fragmentation. Cannabis increased number of caspase-3 positively stained cells. Caspase-3 is a key enzyme to execution stage of apoptotic pathway and the primary activator of apoptotic DNA fragmentation [75, 76].

In the present study, brain sections from rats treated with repeated doses of cannabis showed histological evidence of neurodegeneration, inflammation and apoptosis. Immunostaining for cleaved caspase-3 in the brain tissue showed large number of cells that are positive for cleaved caspase-3 antibody. Apoptosis is also likely to be a contributing pathophysiological mechanism in several neurodegenerative diseases such as such as amyotrophic lateral sclerosis, Parkinson’s disease and Alzheimer's disease, and in neurological injury such as cerebral ischemia and trauma [77,78]. Among the executioner caspases, caspase-3 is most frequently involved in neuronal apoptosis and cleavage of inactive procaspase-3 protein into active caspase-3 subunits is a marker of apoptotic activity [79,80]. The present data indicate that caspase 3 expression occurs after repeated cannabis administration and is associated with evidence of neuronal damage. Neuronal cell loss due to chronic cannabis might thus involve programmed cell death or apoptosis. Smoking cannabis preparations has been associated with memory problems [81] and a tendency for developing or aggravating psychosis [82-85]. Evidence is accumulating that long-term heavy cannabis use is associated with alterations in regional brain volumes where cannabis users had bilaterally reduced hippocampal and amygdala volumes [86] and cerebellar white-matter volume [87]. In first episode schizophrenia patients, cannabis use may be associated with altered brain structure compared with both patients who did not use cannabis and healthy volunteers [88,89]. Psychosis patients and subjects at risk for psychosis might be particularly vulnerable to brain volume loss due to cannabis exposure [90].

In summary, the findings of the present study suggest that long-term use of cannabis is likely to increase the risk of ischaemic heart disease. Cannabis also induced DNA fragmentation in the liver, increased the level of DNA damage of PBMCs and induced apoptosis in brain and liver.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

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Received on 14-12-2013 Accepted on 04-02-2014 Published on 29-05-2014

DOI: http://dx.doi.org/10.12974/2309-6179.2014.01.01.2