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Journal of Toxicological & Pharmaceutical Sciences
NEXUS SCIENCE FORUM

ORIGNAL ARTICLE

Evaluation of hepatoprotective effect of aqueous extract of *Cannabis Sativa* (Marijuana) against Diclofenac sodium induced hepatotoxicity

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Abstract

The most widely used and powerful analgesic, diclofenac sodium, has recently been found to cause hepatotoxicity via oxidative stress. To overcome such negative effects of analgesics, fresh, effective, and safe medicines are of dire need. The hepatoprotective effect of *Cannabis sativa* (Marijuana) aqueous extract against diclofenac sodium-induced hepatotoxicity was investigated in this study. Thirty-two (32) albino rats were randomly divided into four groups (n=8). Group 1 was served as control group and did not receive any drug. Group 2 received only diclofenac sodium (5 mg/kg/day) for 28 days. Group 3 received aqueous extract of *C. sativa* at dose rate of 10 mg/kg/day along with diclofenac sodium (5 mg/kg/day) for 28 days. Group 4 received aqueous extract of *Cannabis sativa* at dose rete of 20 mg/kg/day along with diclofenac sodium (5 mg/kg/day) for 28 days. The results of AST, ALT and ALP clearly indicated that *C. sativa* ameliorated the effect of diclofenac sodium toxicity As a significant increase ($P<0.05$) serum ALT level in group 2 given diclofenac sodium after 14th (193.75+3.19 IU/L) and 28th (195.5± 2.783 IU/L) days when compared with control group. Whereas, an improvement in ALT levels was observed in group 3 (158.5±4.3 IU/L at 14th day and 159.75±.4.81 IU/L at 28th day) and group 4 (139.75±1.25 IU/L at 14th day and 130.5±0.6 IU/L at 28th day) given *C. sativa* at dose rate of 10 mg/kg/day and 20 mg/kg/day, respectively. While, RBCs count, WBCs count, platelets and hematocrit levels were significantly ($P<0.05$) reduced in toxic group and *C. sativa* restored their levels. Thus, this study concludes that aqueous extract of *C. sativa* ameliorates the toxicity induced by diclofenac sodium and it can be used as hepatoprotective agent but further study will be required to investigate the brief underlying mechanism.

Keywords

Cannabis sativa
Diclofenac sodium
Hepatotoxicity

To Cite This Article: Uzma R and Anwar MI, Ambreen A, 2019. Evaluation of hepatoprotective effect of aqueous extract of *Cannabis Sativa* (Marijuana) against Diclofenac sodium induced hepatotoxicity. *J Toxicol Pharmaceut Sci*, **3(1-2),** 1-6.

Introduction

Liver is an important body organ that is not only involved in food and drug metabolism but also removes damaged red blood cells (RBCs), produces bile, blood clotting factors and stores minerals, vitamins and proteins (Ilic *et al*., 2011). Non-steroidal anti-inflammatory drugs (NSAIDs) have been used for management of pain and inflammation but they are thought to be associated with adverse drug events. It has been investigated that diclofenac sodium toxicity involves the mitochondrial protein damage, cytochrome P450 and transporters. Reactive oxygen species initiate toxicity of hepatic cells which is aggravated by cytochrome P450 (van Leeuwen *et al*., 2011). Diclofenac sodium is NSAID that causes gastro-intestinal tract (GIT) and liver toxicity in humans and animals since its metabolites are involved in causing toxicity in various organs (Niu *et al*., 2015). *Cannabis*

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sativa belongs to the family Cannabacaea. Medically it is investigated as a beneficial plant which contains active ingredients which can be actively utilized as revolutionary medicine in future. The active ingredients of *C. sativa* include 9- tetrahydrocannabinol (THC), cannabidiole (CBD), cannabigrol (CBG), and cannabinol (De Backer *et al*., 2009).

THC and CBD are two active constituents of *C. sativa* those have been shown to be analgesic and anti-inflammatory agents in the treatment of multiple sclerosis and pain. *C. sativa* has also been touted as a hepatoprotective, neuroprotective and cardioprotective agent (Morabito *et al*., 2016). The cannabinoid receptors, CB1 and CB2 can be used to treat a number of immune-related diseases. CB1 and CB2 are both G protein-coupled receptors. The responses of rats and humans to the activation of these receptors have been observed and found to be distinct (Gennequin, 2015). The present study aims to see whether an aqueous extract of *C. sativa* could protect rats' livers from diclofenac sodium-induced toxicity.

Materials and Methods

Animals: This was performed on thirty-two (32) healthy albino rats (8-10 weeks old), weighing 150-250 g obtained from animal housing Facility of University of Agriculture Faisalabad (UAF) and kept at 24±2°C in 12 hr dark and light cycles. Standard pellet diet and water was provided *ad libitum* during the experiment (28 days).
Chemicals: Diclofenac sodium injection (5 mg/kg/day) was used for the study with a brand name of Dicloran® by Sami Pharmaceuticals. Dose was calculated for each animal according to their weights. *C. sativa* was obtained from the Department of Botany, UAF.
Preparation of Extract: Dried and ground leaves were macerated in distilled water for 72 hrs with intermittent stirring at room temperature to make an aqueous extract of *C. sativa*. It was filtered through Whatman filter paper No. 1 after maceration (Pandit *et al*., 2010). This filtrate was then dried in an oven at 90-110°C for 8-10 hrs until a powder was obtained. For further dilutions of the medication regimen, dried and sterilized powder of *C. sativa* was used.
Treatment Regimen: 32 albino rats were divided randomly into 4 groups (n=8). Group 1 was control given normal diet and water during the experiment (28 days). Group 2 received diclofenac sodium at dose rate of 5 mg/kg/day for 28 days. Group 3 received aqueous extract of *C. sativa* at dose rate of 10 mg/kg/day along with diclofenac sodium (5 mg/kg/day) for 28 days. Whereas, Group 4 received *C. sativa* extract at dose rate of 20mg/kg/day along with diclofenac sodium (5 mg/kg/day) for 28 days during the experiment. Blood and tissue samples were collected at 14^{th} and 28^{th} day of experiment to do biochemical, hematological and histopathological tests to observe the changes among the treatment groups.
Statistical Analysis: Statistical difference was tested by ANOVA and Duncan's multiple range test using SPSS software.

Results

Effect of diclofenac sodium intoxication and *C. sativa* on liver function: Serum Alanine transaminase (ALT) and aspartate Aminotransferase (AST) are widely used to test the structural integrity of the liver. A Significant increase ($P<0.05$) serum ALT level was observed in group 2 given diclofenac sodium after 14^{th} (193.75+3.19 IU/L) and 28^{th} (195.5± 2.783 IU/L) days when compared with control group (Figure 1a). Whereas, an improvement in ALT levels was observed in group 3 (158.5±4.3 IU/L at 14^{th} day and 159.75±.4.81 IU/L at 28^{th} day) and group 4 (139.75±1.25 IU/L at 14^{th} day and 130.5±0.6 IU/L at 28^{th} day) given *C. sativa* at dose rate of 10 mg/kg/day and 20 mg/kg/day, respectively, as presented in Figure 1a. Similarly, significantly ($P<0.05$) high levels of AST were observed in group 2 after 14^{th} (73.25±1.93 IU/L) and 28^{th} (77± 1.7 IU/L) day when compared with control (18.5±1.32 IU/L at 14^{th} day and 20.25±0.85 IU/L at 28^{th} day) as showed in Figure 1b. While, an improvement in AST levels was observed in group 3 (53.75±5.8 IU/L at 14^{th} day and 42.5±.1.04 IU/L at 28^{th} day) and group 4 (42.5±1.04 IU/L at 14^{th} day and 32.5±1.04 IU/L at 28^{th} day) given *C. sativa.*

Diclofenac sodium has also significantly ($P<0.05$) increased the alkaline phosphatase (ALP) level after 14^{th} (90.75±0.85 IU/L) and 28^{th} (116.75± 5.0 IU/L) day in diclofenac intoxicated rats but *C. sativa* dose dependently ameliorated the effect of diclofenac sodium in treated groups as shown in Figure 2.
Effect of diclofenac sodium intoxication and *C. sativa* on hematological parameters: Hemoglobin levels were reduced significantly ($P<0.05$) in rats treated with diclofenac sodium (12.72±0.15 g/dl at 14^{th} day and 13.64±0.13 g/dl at 28^{th} day) indicating diclofenac sodium induced hepatotoxicity as presented in Table 1. The administration of *C. sativa* extract significantly restored and ameliorated the effect of diclofenac sodium. Further the higher dose of *C. sativa* restored the levels of hemoglobin (14.16±0.7 g/dl at 14^{th} day and 14.84±0.7 g/dl at 28^{th} day). Whereas, RBCs count was also reduced significantly ($P<0.05$) in rats treated with diclofenac sodium. The administration of *C. sativa* extract significantly improved RBCs count as 8.37±0.125 and 8.1±0.17 10^6/µl at 14^{th} and 28^{th} day respectively (Table 1).

Hematocrit levels were reduced significantly ($P<0.05$) because diclofenac sodium reduces the 50% hematocrit concentration in the intoxicated rats. Hematocrit count was reduced significantly ($P<0.05$) in rats treated with diclofenac sodium induced hepatotoxicity.

Table 1: Mean values of hematological parameters in control and experimental rat groups.

Parameters	14th day of study				28th day of study			
	G1	G2	G3	G4	G1	G2	G3	G4
Hb (g/dl)	15.875±0.125	12.72±0.15	14.65±0.2	14.16±0.7	15.675±0.75	13.64± 0.13	14.8±0.18	14.84±0.7
RBC (10^6/µl)	8.5±0.129	7.63±0.177	7.36±0.121	8.37±0.125	8.5±0.108	6.48± 0.18	8.22±0.23	8.1±0.17
HCT (%)	39.65± 0.2	36.69±0.3	37.2±0.2	37.5±0.15	40.4±0.2	38±0.1	38.5±0.1	38.6±0.2
MCV (fL)	55.5±0.5	55.5±0.2	55.51±0.4	55.4±0.6	56.1±0.5	56.9+0.7	54.5±1	55.9±0.7
MCH (pg)	21.29±0.9	22.9+0.8	22±0.7	55.4±	20.2±0.5	20.7±0.73	22±0.5	22.1±0.4
MCHC (g/dL)	29.4±0.18	29.3±0.3	29.4±0.17	29.6±0.1	29.7±0.1	29.5±0.1	29.7±1.2	29.7±0.1
WBC (U/µL)	16067±292	32667±21545	30177±11880	24510±6014	14170±7520	18183±5225	20178±4316	22158±5259
PLT (U/L)	265.25×10^3±2	220.87×10^3±0.7	243.9×10^3±1.3	243×10^3±0.5	234.8×10^3±0.7	217.3×10^3±0.7	228×10^3±1	222×10^3±1.2

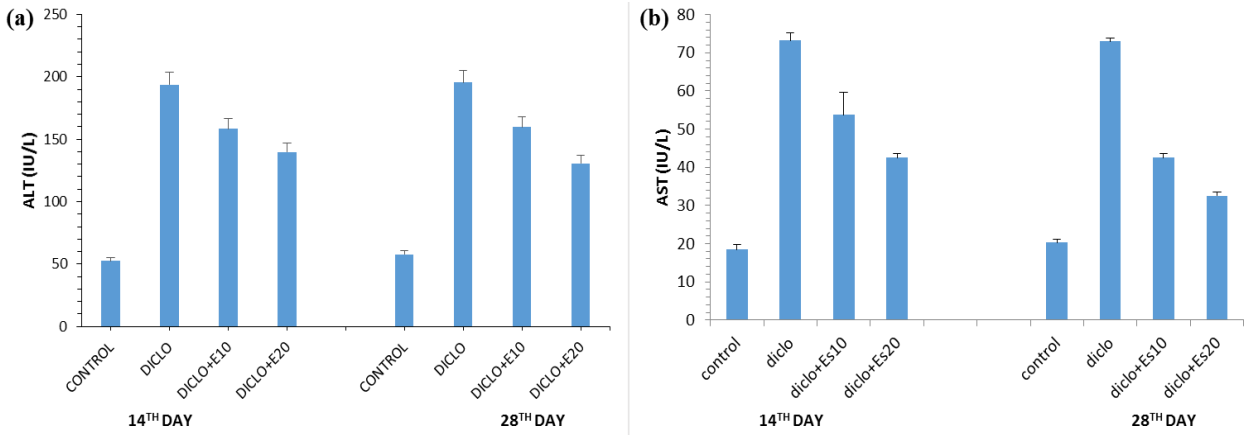

Figure 1: Mean values of ALT (a) and AST (b) in control and experimental groups.

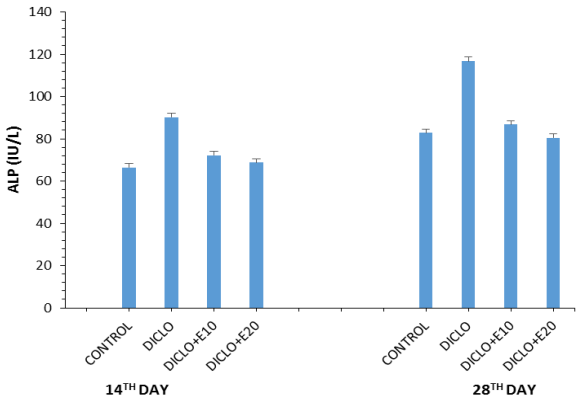

Figure 2: Mean values of alkaline phosphatase (IU/L) in control and experimental rat groups.

Whereas, administration of *C. sativa* extract significantly restored and ameliorated the effect of diclofenac sodium (Table 1). Current study also revealed that there was no significant (P>0.05) difference was observed in MCV between control and experimental groups after 14th and 28th day of study. The results of MCHC were also non-significant (P>0.05) between control and experimental groups. However, MCH levels differs significantly (P<0.05) between control (21.29±0.89 pg at 14th day and 20.2±0.56 pg at 28th day) and experimental groups after 14th and 28th day of study (Table 1).

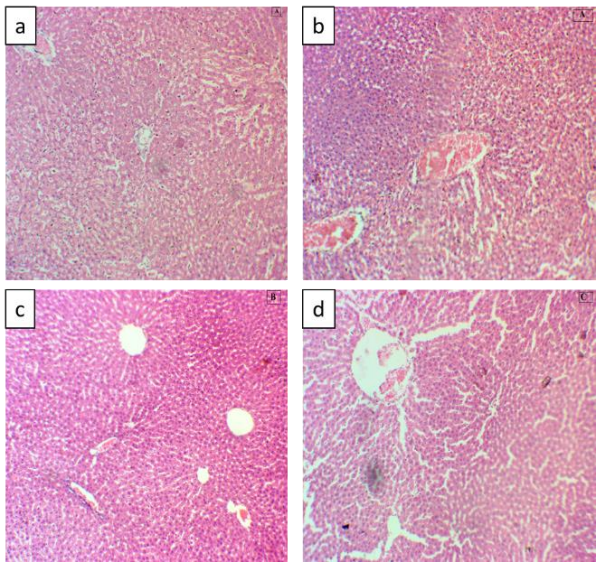

Figure 3: Histopathological changes of liver of rat in control group (a), group treated with diclofenac sodium (b), group treated with 10 mg/kg/day of *C. sativa* (c) and group treated with 20 mg/kg/day of *C. sativa*.

The WBCs count also differ non-significantly (P>0.05) in rats treated with diclofenac and *C. sativa* extract. Further higher dose of *C. sativa* (20 mg/kg/day) reduced the levels of white blood cells. Whereas, platelet count was decreased (220.87×10^3±0.7 and

$217.3\times10^3\pm0.7$ at 14th and 28th day, respectively) in diclofenac sodium treated group significantly ($P<0.05$) as compared to the control group but *C. sativa* extract significantly ($244.9\times10^3\pm0.5$ and $222\times10^3\pm1.2$ at 14th and 28th day, respectively) ameliorated the effect of toxicity induced by diclofenac sodium (Table 1).

Histopathology of liver tissue: Histologically, the liver is divided into lobules. The central vein runs through the lobule's base. Portal triads are located on the lobule's periphery. The nuclei are regular and the sinusoidal spaces are intact. The 10X slide illustrates portal tracts, which receive oxygenated blood from the hepatic arteries, and core veins, which have low oxygenation (Figure 3a). Histopathologic changes in the liver sections stained with hematoxylin and eosin (H&E) in all diclofenac groups included cloudy swelling and hydropic degeneration of the liver cells, focal sinusoidal and vena centralis dilatation, proliferation of the bile duct in portal areas, enlargement of the periportal area with mononuclear cell infiltration, hyperemia and dose-dependent fibrous tissues proliferation and focal necrosis. The tubular epithelial cells of the liver tissue of all diclofenac sodium-treated groups showed cloudy swelling and hydropic degeneration. The researchers discovered necrosis, peritubular lymphocyte invasion, stromal fibrous tissue proliferation, and hyperemia (Figure 3b). These slides show normal nuclei in normal liver parenchyma. Although there was less necrosis than in the diclofenac sodium group, sinusoidal spaces appeared almost regular (Figure 3c). The *C. sativa* (20 mg/kg) group had normal parenchyma architecture, which was similar to the control group, with oxygenated portal tracts, portal arteries, and deoxygenated veins. Hepatocyte nuclei have walled nuclei, suggesting that Cannabis sativa has a protective role in liver cells (Figure 3d).

Discussion

There has been a surge in research into the potential use of phytomedicines for disease treatment in recent years. However, due to the heterogeneity of living cells, performing comparative studies is still difficult. More research is needed in this field, especially on the biocompatibility and distribution of plant extract formulations for normal living tissues. The beneficial function of *C. sativa* aqueous extract was investigated in this report. The efficacy of sativa leaves in shielding the liver from the toxic effects of diclofenac sodium was investigated. The use of diclofenac sodium, which is presently on the market, has been linked to an increase in hepatotoxic risk (Salomone *et al*., 2016).

For 28 days, diclofenac sodium (5 mg/kg) was given intraperitoneally. The doses of diclofenac sodium and cannabis extract used, as well as the routes of administration, were similar to those used in clinical trials. The current study found that diclofenac sodium (5 mg/kg) caused hepatotoxicity in rats, as evidenced by irregular liver function studies, elevations in ALT and AST, and significantly lower levels of biochemical hepatic markers. Aqueous extract of C. sativa leaves substantially normalized hepatic biochemical markers, minimizing the hepatotoxic effects of diclofenac sodium. The change in hepatic biomarkers was more noticeable in rats on day 14 and was more clearly revealed after diclofenac sodium intoxicated rats. This suggests that diclofenac sodium had the most damaging effect on the liver in just two weeks. Histopathological and biochemical studies in laboratory rats model confirmed diclofenac sodium's hepatotoxic impact. As a result, hematological tests showed no major improvements, although there was a substantial drop in hemoglobin red blood cells, white blood cells, and platelets, despite the high dose of *C. sativa* treated groups retaining values close to average. Because of the formation of reactive metabolites, research studies show that diclofenac metabolism by CYP2C9, UGT2B7 and CYP3A4 is the most significant cascade in diclofenac sodium toxicity assessment (Boerma *et al*., 2014). Diclofenac sodium toxicity has previously been related to elevated levels of AST in the serum of animals and humans. Damaged heart tissues release AST, which is commonly used as a diagnostic biomarker in the study of hepatotoxicity. The hepatic function biomarkers were effectively enhanced by co-injecting aqueous extract of *C. sativa* leaves into diclofenac sodium intoxicated rats. The higher dose of aqueous extract of cannabis sativa leaves (20 mg/kg) demonstrated the greatest potential to minimize the hepatotoxic effects of diclofenac sodium as compared to the low dose of aqueous extract of C. sativa leaves (10 mg/kg). The aqueous extract of *C. sativa* leaves' dose-dependent ability to protect the heart and stabilize cellular membranes by controlling diclofenac sodium-induced hepatotoxicity is responsible for this positive reaction. Cannabis has been shown to help prevent lipid peroxidation and the formation of reactive oxygen species (ROS), both of which are major causes of tissue injury and cell death. When the concentration of ROS produced exceeds the anti-oxidant capacity of the cell or when the anti-oxidant capacity of the cell decreases, oxidative damage occurs in the tissues or cells. Various authors have shown that oxidative stress plays a role in diclofenac sodium-mediated tissue toxicity. The current study discovered that diclofenac sodium causes oxidative stress in the blood, making it the leading cause of hepatotoxicity. The oxidative stress caused by diclofenac sodium should be minimized. In comparison to the diclofenac sodium-intoxicated rats, both the low (10 mg/kg) and large (20 mg/kg) doses of aqueous extract of *C. sativa* demonstrated protective effects by lowering serum ALT and AST levels. However, cannabis demonstrated dose-dependent activity, as a

high dose of *C. sativa* aqueous extract (20 mg/kg) significantly reduced oxidative stress. It has the ability to boost antioxidant activity, boost antioxidase activity, and lower free radical levels. It prevented the antioxidant depletion that was more apparent in diclofenac sodium-intoxicated rats. Hematological parameters show that rats intoxicated with diclofenac (5 mg/kg), but cannabis sativa 10 mg/kg and cannabis sativa 20 mg/kg substantially restored hemoglobin and hematocrit concentrations. The average amount of hemoglobin in red blood cells is represented by mean corpuscular hemoglobin (MCH). MCH is higher in RBCs with higher hemoglobin, while MCH is lower in RBCs with lower hemoglobin. Hemoglobin and MCH have a direct relationship (George-Gay & parkar, 2003). Hemoglobin depletion causes iron deficiency anemia, which can be due to hematopoiesis disturbances, and certain medications, such as diclofenac sodium, impair the normal range of hematological parameters. The hyperactivity of the bone marrow, which contributes to the creation of red blood cells with damaged integrity that are easily killed in the circulation, may be to blame for the decrease in blood parameters such as hematocrit concentration (Aziz & Zabut, 2011). The hematopoietic system is exceptional as a target organ because of the essential role of blood cells and the sensitivity of highly proliferative tissue to xenobiotic intoxication (Palani *et al*., 2009). Various blood cells, such as red blood cells, are produced at a rate of 1 to 3 million per second, and certain diseases, such as liver damage, can affect this rate. The decrease in RBC count may be attributed to hematopoeisis disturbances, erythrocyte degradation, a decrease in the rate of erythrocyte formation, or their increased removal from the circulation (Essawy *et al*., 2010). In diclofenac sodium-induced toxicity, there was a substantial reduction in red blood cells. The average size of red blood cells is determined by mean corpuscular volume (RBC). The MCV of small RBCs is low, whereas the MCV of larger RBCs is higher (George-Gay & Parker, 2003) The level of MCV, however, did not display any significance. During the 14^{th} and 28^{th} days, lower levels of platelets and white blood cells were detected, and these increased levels were restored by the low (10 mg/kg) and higher (20 mg/kg) doses of *C. sativa*. Control and cannabis-administered rats had normal hepatic tissue, while diclofenac sodium-intoxicated rats had inflammation and infiltration as revealed by histopathological analysis. Low and high doses of cannabis also had a protective effect and normalized the hepatic tissues. *C. sativa leaves* were effective in reducing diclofenac sodium-induced hepatotoxicity, as evidenced by a substantial decrease in ALT and AST. As a result, Cannabis sativa tends to have a protective effect. Research studies are needed further to explore the constituents of plant responsible for this protective effect. Possibly the existence of alkaloids, flavonoids etc. are responsible for protective potential. In liver function test, a significant rise in biochemical markers of liver which shows the hepatotoxicity induced by diclofenac sodium. A significant difference was observed in lower and high dose of *C. sativa* treated groups while indicating the protective effect. Thus an extensive and detailed study is required to explore the lucid mechanism of protective potential of *C. sativa.*

Conclusion: *C. sativa* leaves proved to be efficient in lowering hepatotoxicity induced by diclofenac sodium and evidenced the significant decrease of AST and ALT. Histopathological findings have also indicated the protective effect of cannabis sativa against diclofenac sodium induced hepatotoxicity. Diclofenac sodium and cannabis sativa treatment have affected the blood count and platelets in different treatment groups. Diclofenac sodium reduces platelets count in albino rats that was effectively improved with the aqueous extract of *C. sativa***.** This concludes that *C. sativa* has protective effect. More research is required to determine which plant constituents are responsible for this protective effect. It's possible that the presence of alkaloids is to blame for the defensive potential.

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