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AMELIORATIVE POTENTIAL OF *CANNABIS SATIVA* EXTRACT ON DIABETES INDUCED NEUROPATHIC PAIN IN RATS

Amita Arora*, Rajeev Taliyan and P. L. Sharma

I.S.F. College of pharmacy, Moga, Punjab, India

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Correspondence to Author:

Amita Arora

I.S.F. College of pharmacy, Moga, Punjab, India

ABSTRACT

Diabetes induced neuropathic pain is recognized as one of the most difficult type of pain to treat and conventional analgesics are well known to be partially effective or ineffective. Synthetic or natural cannabinoids are promising drugs to treat neuropathic pain. It has been reported that the effect of cannabinoid analgesics is not compromised in various experimental neuropathic pain model. The aim of present study was to explore the ameliorative potential of cannabis sativa extract in hyperglycemia-induced neuropathic pain. Streptozotocin (STZ) (20 mg/kg, i.p. x 4 days) was administered to induce experimental diabetes in the rats. The pain threshold in diabetic and non-diabetic rats was measured using paw withdrawal latency test. After 28 days of STZ injection, diabetic rats exhibited a significant thermal hyperalgesia and mechanical allodynia along with increased plasma glucose and decreased body weights as compared with controls rats. Moreover, STZ administration was noted to increased oxidative stress (MDA & nitrite level) and decreased antioxidant enzyme levels (glutathione, catalase and superoxide dismutase) significantly in diabetic rats as compared to the age-matched non diabetic rats. Administration of Cannabis extract (25 50 & 100 mg/kg, p.o.) once daily for 14 days, starting from the 3rd week of STZ-injection, significantly attenuated thermal hyperalgesia and mechanical allodynia. Moreover it reduces oxidative stress and concomitantly increased reduced level of antioxidant enzyme levels observed in diabetic rats. This study suggests that cannabis has a beneficial effect in diabetic neuropathic pain.

INTRODUCTION: Neuropathy is the most common complication associated with diabetic patient and recognized as one of the most difficult types of pain [1]. The cause of diabetes induced neuropathic pain is still unclear [2]. However, various mechanisms have been proposed to be involved in the pathogenesis of diabetic neuropathy i.e. increased aldose reductase activity [3], nonenzymatic glycation [4], activation of protein kinase C [5], increase oxidative stress and cytokines levels are the best studied. The chronic pain that follows peripheral nerve injury differs fundamentally from neuropathic pain and conventional analgesic are well reported to be partially effective or in effective in neuropathic pain. Therefore, there is a need to identify an effective clinical treatment. Complementary medicines have gain popularity in recent years. Many indigenous medicinal herbs have been found to be useful to successfully manage pain in various chronic pain models [6, 7].

Cannabinoids have been reported to attenuate nociceptive hypersensitivity in animal models of chronic pain and have shown beneficial effect in preclinical and clinical studies [8, 9]. Various synthetic or pure natural cannabinoids attenuate typical signs of neuropathy, such as allodynia and hyperalgesia in animals [10, 11]. Recently, it has been reported that a controlled cannabis extract, containing multiple cannabinoids, provided better antinociceptive efficacy than the single cannabinoids given alone when tested in chronic constriction injury of the sciatic nerve model of neuropathy [12]. It seems that, the presence of anti-inflammatory and antioxidant compounds in the non-cannabinoid fraction of the natural extract might contribute strongly to analgesia. Therefore, the present study was designed to investigate the effect of *cannabis sativa* extract in diabetes- induced neuropathic pain in rats.

MATERIALS AND METHODS:

Animals*:* Wistar rats (200-260 g) of either sex were used. They were housed in an animal house provided with a 12-h light/dark cycle and free access to water and food. The animal experiments were conducted in accordance with guidelines of Institutional Ethic Committee and study protocol was approved by intuitional ethics committee.

Extraction Method: The leaves of *C. sativa* were collected from Indian plants species, authenticated and dried in shade (25^{o}C) by the air drying method for 8 days and then, were grinded with electrical grinder. The extracts of them were obtained by the maceration method with 80% ethanol in 200 gr/Lit for 48 h. The extracts were concentrated by Rotary Evaporator with a vacuum pump and were dehydrated in desiccators with vacuum pump. Semisolid brownish dried extracts were re-suspended in ethanol and prepared at dose of 100 mg/ml.

Assessment of Thermal Hyperalgesia: The nociceptive threshold was tested according to the Hargreaves procedure [13] using the Plantar test (Ugo Basile, Varese, Italy). The latency to the first sign of paw licking or withdrawal response to avoid heat pain was taken as an index of pain threshold.The withdrawal latency was averaged from at least three trials separated by a 10-min interval and the cut-off was set at 20 s to avoid tissue damage. In brief, each animal was placed in a clear plexiglass box and hind paw was exposed to a constant beam of radiant heat through a plexiglass surface. Time, in seconds, from initial heat source activation until paw withdrawal was recorded. Cannabis extract (25 50 &100 mg/kg, p.o.) was administered in diabetic animals for 14 days, starting after 28 days of STZ-injection, and withdrawal latency

was noted daily 30 min after administration of cannabis extract.

Mechanical Allodynia: Mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy) before diabetes induction and subsequent to STZ injection. In brief, each animal were placed in a test cage with a wire mesh floor, and the tip of a von Frey-type filament was applied to the middle of the plantar surface of the hind paw. Brisk foot withdrawals in response to von Frey type filament stimulation were recorded. Paw-withdrawal threshold was expressed as threshold level in g. Effect of cannabis extract was studied for 14 days, starting after 28 days of streptozotocin injection.

Experimental Design: The animals were randomized into experimental and control groups, and divided into six groups of six animals each. Rats in saline or vehicle treated Group 1 served as control. Rats in group 2 was received intra-peritoneal injection of STZ 20 mg/kg, daily for 4 days to induce experimental diabetes, and after verifying the blood glucose level more than 300 mg/dl considered as diabetic and used in the present study. Diabetic rats, randomly divided in four groups and each consist 6 rats, received orally the drug vehicle or cannabis extracts (25, 50 and 100 mg/kg), once for 14 days, starting from day 28 after the STZ injection.

The experiment was terminated at the end of 42 days and all animals were scarified by cervical dislocation. Blood samples were collected in heparinised tubes and plasma was separated by centrifugation at 3,000 g for fifteen minutes. The liver was removed, cleared of blood and immediately transferred to ice-cold containers containing 0.9% sodium chloride for various estimations. About 2.5 g tissue was weighed and homogenized in appropriate buffer and centrifuged at 7000 × g for 15 min. Clear supernatant were used for measurement of reduced glutathione, superoxide-dismutase and catalase enzyme. A fraction of liver tissue was used to measure malondialdehyde (MDA). Proteins were accessed by Lowry et al. (1951) method [14].

Measurement of Lipid-peroxidation: Lipid-peroxidation, as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) was measured by using the thiobarbituric acid test of Ohkawa *et al.* (1979) [15]. In brief, 0.2 mL of homogenate was added to 0.8% thiobarbituric acid, 8.1% sodium dodecyl sulfate (SDS) and acetic acid (20%) in distilled water. After heating for 60 min in a water bath at 95°C, the mixture was then cooled and extracted with a mixture of *n*-butanol/pyridine (15:1, v:v). The absorbance of the reaction product present in the upper organic layer separated by centrifugation was measured spectrophotometrically at 532 nm.

Estimation of Antioxidant Enzyme levels: Superoxide dismutase (SOD): SOD was assessed by utilizing the technique of Kakkar & Vishwnathan (1984) [16]. A single unit of enzyme was expressed as 50% inhibition of nitroblu tetrazolium (NBT) reduction/min/mg protein. Catalase (CAT) was assayed colorimetrically at 620nm and expressed as mmoles of H_2O_2 consumed/min/mg protein as described by Sinha [17]. In Brief, a mixture (1.5 ml) contained 1.0 ml of 0.01M phosphate buffer pH 7.0, 0.2 ml of tissue homogenate and 0.4 ml of 2M H_2O_2 . The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). Reduced glutathione (GSH) was estimated by the method of Ellman [18]. In brief, 10% TCA was added to the homogenate and the mixture was centrifuged. 1.0 ml of supernatant was treated with 0.5 ml of Ellmans reagent (19.8

mg of 5, 5'-dithiobisnitro benzoic acid in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2M, pH 8.0). The absorbance was measured spectrophotometrically at 412 nm.

Estimation of Nitric Oxide (NO): NO was estimated by using the Griess reagent method 19. To 200 µl of the protein-free supernatant, 30 µl of 10% NaOH was added followed by 300 µl of Tris-HCl buffer and mixed well. To this, 530 µl of Griess reagent (0.3% N-1 [Naphthyl-ethylene-diamine-dihydrochloride] in distilled water + 3% Sulphanilamide in 1M HCl) was added and incubated in the dark for 10-15 minutes, and the absorbance was read at 540 nm.

Data Analysis: All the results are expressed as Mean ± S.D One-Way ANOVA followed by Tukey's test were employed to calculate the statistical significance for multiple comparisons between groups. The level of significance was fixed at $P<0.05$

RESULTS:

Effect of Pharmacological intervention on serum glucose level and body weight: Administration of cannabis extract to non diabetic rats did not modulate serum glucose level or body weight. As shown in **Table 1**, blood glucose levels in rats which had been rendered diabetic by streptozotocin were significantly elevated (403±21.2 mg/dl) as compared to those in age-matched non-diabetic rats (84.25 mg/dl, p<0.01). Body weights were significantly reduced in STZ treated rats (208.56± 7.02g) as compared with age matched control rats (258.42±10.46g, P<0.05). However, body weight was improved dose dependently in diabetic rats after cannabis treatment (25, 50 and 100mg/kg), starting from day 28 after STZ injection, as compared to non treated diabetic rats (P<0.05).

TABLE 1: EFFECT OF STZ AND CANNABIS ON BODY WEIGHT AND BLOOD GLUCOSE

S. No	Blood glucose (mg/dl)	Body weight (g)
Control (citrate buffer)	84.23 + 4.89	258.54 ± 2.43
Streptozotocin treated	403.42 a ± 14.76	208 a ± 7.78
Cannabis (25 mg/kg, p.o)	325.67a ± 9.78	218.82 ± 11.25
Cannabis (50 mg/kg, p.o)	292.21a± 9.21	240.22b ± 12.52
Cannabis (100 mg/kg, p.o)	304.88 a± 13.88	280.90b* ± 10.44

Cannabis treatment started, after 28 days STZ injection, blood glucose and Body weight was measured before and after STZ Injection; Results are expressed as mean ± S.D (n=6). a =P<0.001 vs control: b =P<0.05 Vs control rats

Effect of Experimental Diabetes on Pain Sensitivity: The paw-withdrawal latency and mechanical threshold were tested at 7, 14, 28, 35 and 42 days after administration of STZ Before STZ administration, the rats paw withdrawal latency in both left and right hind paws from radiant heat was about 17 s and sustained a mechanical force of about 39 g. On day 7, following STZ administration the pain sensitivity in diabetic rats was similar to the control animals (16.25). However, diabetic rats showed a significant decrease in the paw withdrawal latency to thermal (**Fig. 1**) and mechanical stimuli (**Fig. 2**) on day 28 after STZ injection, indicating development of thermal hyperalgesia and mechanical allodynia respectively.

Effect of Cannabis treatment on Pain-Sensitivity: Diabetic animals showed mechanical allodynia as indicated by a significant reduction (P<0.05) in the paw withdrawal latency to mechanical stimuli (21.78 ± 3.25g) as compared with age matched control animals (38.75 ± 4.5g) (**Fig. 4**). Thermal hyperalgesia exhibited in diabetic animals (**Fig. 3**) as evidenced by a significant reduction (P<0.05) in the paw-withdrawal latency to heat (9.5 ± 1.25 s) when compared to age matched control

animals (18.52 ± 0.92s). However, cannabis extract (25 50 & 100 mg/kg, p.o) administration, starting after 28 days STZ injection, produced a dose and time dependent increase in both paw withdrawal threshold (mechanical) and paw withdrawal latency (heat) test (Fig. 3) respectively as compared to untreated diabetic rats. The maximum increase in paw withdrawal threshold and paw withdrawal latency was observed with 100mg/kg, p.o. (ethanolic extract) of cannabis in both mechanical and thermal test respectively.

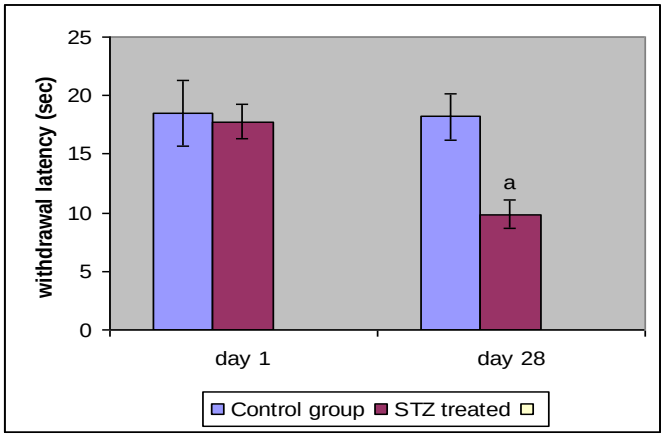

FIG. 1: EFFECT OF EXP DIABETES ON PAIN SENSITIVITY TO THERMAL TEST

Measurement of pain sensitivity on day 1 and day 28 in both STZ treated and control rats subjected to thermal paw withdrawal test. Results are expressed as mean ± S.D, n=6 a=P< 0.05 vs. control

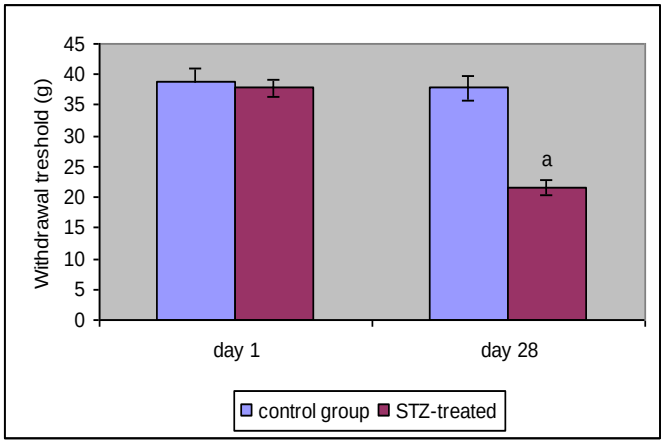

FIG. 2: EFFECT OF EXP DIABETES ON PAIN SENSITIVITY TO MECHANICAL TEST

Measurement of pain thresholds on day 1 and day 28 in both STZ treated and control rats subjected to mechanical paw withdrawal test. Results are expressed as mean ± S.D, n= 6, a= p<0.05 vs. control

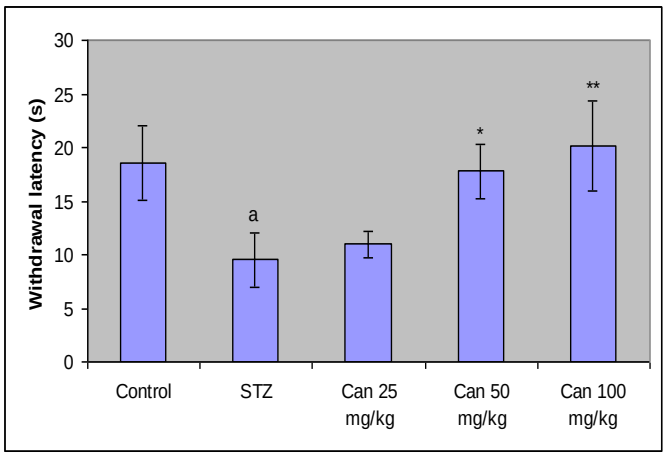

FIG. 3: EFFECT OF CANNABIS TREATMENT ON MECHANICAL ALLODYNIA

Results are expressed as mean ± S.D (n=6), a =P<0.05 vs. control: b =P<0.05 Vs STZ

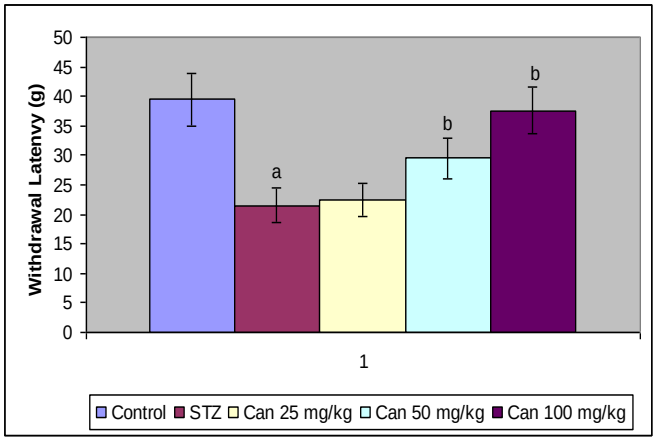

FIG. 4: EFFECT OF CANNABIS TREATMENT ON THERMAL HYPERALGESIA

Results are expressed as mean ± S.D (n=6), a =P<0.05 vs. control: * =P<0.05 Vs STZ, **=p<0.001 Vs STZ

Effect of Hyperglycemia and Pharmacological Intervention on TBARS (LPO) and Antioxidant Enzymes Level: The level of LPO, reduced GSH, SOD and CAT enzyme were measured as markers of diabetes induced oxidative stress in liver. STZ administration induced a significant increase in LPO, as a marker of free radicals generation as compared with vehicle treated control rats. The

activities of SOD, CAT and GSH enzyme in the liver were significantly decreased in STZ-treated rats as compared to age matched control rats ($p<0.05$) (**Table 2**). However, these antioxidant activities were significantly increased in rats treated with cannabis extract in a dose dependent manner when compared to the untreated diabetic rats. Moreover, in diabetic rats, administration of cannabis significantly attenuated the increase in LPO (TBARS) levels when compared with untreated diabetic control rats (Table 2). The maximum increase in the activities of SOD, CAT and GSH enzyme was observed with cannabis extract treatment at a dose of 100 mg/kg, p.o. (Table 2).

Effect of Experimental Diabetes and Cannabis Treatment on Serum and Urinary Nitrite Level: Experimental diabetes markedly increased urinary (40.04 ± 3.28) and serum nitrite (34.24 ± 2.45 concentration as compared with age matched saline or vehicle treated rats. Administration of Cannabis in Non diabetic rats did not alter urine/serum nitrate level (data not shown). However, in diabetic rats, administration of cannabis produced a dose and time dependent decrease in serum nitrite levels, where animals treated with 25, 50 & 100 mg/kg cannabis extracts exhibited significantly lower levels of serum nitrite relative to diabetic controls ($P<0.01$) (**table 3**).

TABLE 2: EFFECT OF HYPERGLYCEMIA AND PHARMACOLOGICAL INTERVENTION ON TBARS (LPO) AND ANTIOXIDANT ENZYMES LEVEL

Treatment	TBARS (nM/100g)	SOD Enz. required for 50% inhibition of NBT reduction (% activity)	CAT (µm of H_2O_2 utilized/min)	GSH (mg/100 g)
Control	78 ± 3.25	10.05 ± 0.58	80.25 ± 5.5	120.89± 6.8
STZ Treated	128a ± 6.45	5.20 a*± 0.20	48.50a# ±3.02	78.50a$± 2.78
Per se Can (100 mg/kg)	76.79 ± 2.8	9.78 ± 0.46	78.8 ± 4.20	122.50± 5.70
Can (25 mg/kg)in DM	118.45 ± 5.10	7.92 ± 0.05	61.5± 3.58	82.45± 3.20
Can (50mg/kg) in DM	85.30 b ± 3.25	8.55 b*± 0.30	72.48 b#± 4.2	94.45a$± 3.5
Can (100 mg/kg) in DM	78.45 b ± 2.5	10.80 b**± 0.62	78.85 b##± 5.20	117.4a$$ ± 5.10

Results are expressed as mean ± S.D (N=6). a, a*,a#, a$ = $p<0.05$ Vs control rats respectively. b, b*,b#, b$ =$p<0.05$ Vs STZ treated respectively. b**,b##, b$$ =$P<0.01$

TABLE 3: EFFECT OF EXPERIMENTAL DIABETES AND CANNABIS EXTRACT TREATMENT ON URINARY AND SERUM NITRITE LEVEL

Treatment	Urinary nitrite (µM/24h)	Serum (µM/l)
Non-DM	15.20±1.74	13.21 ±1.46
DM (STZ treated)	40.40 ± 4.28a	34.24 ± 3.45a
Can 25 mg/kg) in DM	34.90 ± 3.10	30.87 ± 3.02
Can 50 mg/kg in DM	21.45 ± 2.25 b	18.95 ± 2.5b
Can 100 mg/kg in DM	16.05 ±1.76 b*	13.43 ±1.45 b*

Effect of streptozotocin-induced diabetes and SHS treatment on urinary/serum nitrite concentration. Each value is the mean ± S.D. (n=6). a=*p*<0.01 vs. non-diabetic mice. b= P<0.05 vs. SHS of Non DM, b* p<0.01 vs. DM. Non DM = Non diabetic rat, DM= diabetic rats.Can= cannabis extract

DISCUSSION: The present study confirms that streptozotocin-induced diabetes lowers nociceptive threshold as evident by the presence of allodynia and hyperalgesia. Administration of cannabis extract, after 3rd week of STZ administration, for 14 days attenuate diabetes induced thermal hyperalgesia and mechanical allodynia. Modulation of pain thresholds by hyperglycaemia were found to be progressive and were confirmed in various pre clinical and clinical studies [20]. Decrease in pain threshold was observed with mildly noxious stimulus such as mechanical force, which was failed to induce paw-withdrawal in normal rats before the cut off time (20s) indicating that this force is mildly noxious. However, diabetic rats showed a significant reduction in paw withdrawal threshold which indicates the development of allodynia. Moreover, there was also a similar increase in thermal-hyperalgesic activity in diabetic rats as compared with normal rats when subjected to thermal stimuli (Planter test). Our study is fully consistent with earlier report that confirms streptozotocin-induced rats had thermal hyperalgesia and mechanical allodynia, 3-weeks after streptozotocin treatment in a subpopulation of rats [21, 22]. Similar models of mechanical allodynia and thermal-hyperalgesia in streptozotocin-induced rats have been studied previously [21].

The mechanisms responsible for the decreases in pain threshold level in diabetic rats are not yet completely established. It has been reported that hyperglycemia or increase in blood glucose concentration in diabetes profoundly alters hypothalamic-pituitary function, including the activity of endogenous cannabinoid system [23]. Moreover, it has been reported that high glucose concentrations are associated with decreased expression of CB1 receptors in nerve cells, which may contribute to the pathogenesis of diabetic neuropathy [24]. Changes in the concentration of either brain or blood glucose levels was noted to modulate nociceptive processes. Moreover, recently it has been reported that whole plant extract was more effective rather than single chemical constituent of cannabis sativa in combating nerve injury induced chronic pain [11, 25]. In the present study, we have demonstrated a similar effect with ethanolic extract of cannabis sativa in diabetes induce neuropathic pain.

The exact mechanisms by which cannabinoids mediate their antihyperalgesic and anti-allodynic activity are less clear and may involve a combination of central and peripheral activity [26]. In the present study, we have observed that cannabis sativa extract administration for 14 days, starting after 28 days streptozotocin injection, in diabetic rats, dose dependently attenuated thermal hyperalgesia and mechanical allodynia. Our findings are fully consistent with earlier report suggesting antihyperalgesic and anti allodynic effect of cannabinoids in experimental neuropathic pain [11, 27].

Oxidative stress was well reported to modulate pain perception and play a key role in diabetes induced thermal-hyperalgesia and mechanical allodynia [28, 29]. Hyperglycaemia is reported to induce oxidative stress through multiple mechanisms such as redox imbalances, altered PKC [30], increased advanced glycation end product [4], cytokines [31], nitric oxide and mitochondrial overproduction of superoxide [28, 32]. Cannabidiol (CBD) and tetrahydro-cannabinoids (THC) the major constituent of cannabis exhibit antinoceceptive and neuroprotective effect in various animal model [32]. In addition, recently it was observed that β-carophyllene, a constituent of cannabis, which is concentrated in cannabis essential oil, reduces tissue inflammation trough CB2R activation.

Moreover, natural or synthetic cannabinoid have been reported to be powerful antioxidant and neuroprotectant [32, 33]. In the present investigation, we have observed that administration of cannabis extract for 14 days, starting after 28 days STZ injection, attenuated oxidative stress (MDA) and concomitantly increased antioxidant enzyme level. It seems that *Cannabis sativa* extract possesses ant-oxidant properties, that may involved in beneficial effect of cannabis.

In addition, other key mediators of hyperglycaemia- induced oxidative injury are peroxynitrite, which is formed by combination of superoxide with nitric oxide that exerts detrimental effects on the nerve tissue leading to neuropathic pain [29]. A marked oxidative stress and elevation of nitric oxide in diabetic animals was observed previously and in the present investigation in our lab, indicating oxido-nitrosative stress in diabetic rats [22]. Administration of *cannabis sativa* ethanolic extract dose dependently attenuated the increased level of nitrite, which may be attributed to its iNOS and peroxynitite inhibiting potential.

Based on the data in hand, we concluded that cannabis had beneficial effect in experimental diabetic neuropathic pain that may be due to its antioxidant potential.

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