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**Beneficial effects of a Cannabis sativa extract treatment on diabetes-induced neuropathy and oxidative stress**

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4 **Beneficial effects of a *Cannabis sativa* extract treatment on diabetes-induced**  
5  
6 **neuropathy and oxidative stress**  
7

8  
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38 Short title: Cannabis extract alleviates diabetic neuropathy  
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## Abstract

Neuropathy is the most common complication of diabetes and it is still considered to be relatively refractory to most of the analgesics. The aim of the present study was to explore the antinociceptive effect of a controlled cannabis extract (eCBD) in attenuating diabetic neuropathic pain. Repeated treatment with cannabis extract significantly relieved mechanical allodynia and restored the physiological thermal pain perception in streptozotocin (STZ)-induced diabetic rats without affecting hyperglycemia. In addition, the results showed that eCBD increased the reduced glutathione (GSH) content in the liver leading to a restore of the defence mechanism and significantly decreased the liver lipid peroxidation suggesting that eCBD provides the protection against oxidative damage in STZ-induced diabetes that also strongly contributes to the neuropathy development. Finally, the nerve growth factor content in the sciatic nerve of diabetic rats was restored to normal following the repeated treatment with eCBD suggesting that the extract was able to prevent the nerve damage caused by the reduced support of this neurotrophin. These findings highlighted the beneficial effects of cannabis extract treatment in attenuating diabetic neuropathic pain, possibly through a strong antioxidant activity and a specific action upon nerve growth factor.

*keywords:* neuropathic pain; *Cannabis sativa*; diabetes; cannabinoid; oxidative stress; nerve growth factor

## INTRODUCTION

Diabetic polyneuropathy, the most common of the peripheral neuropathies, occurs widely in the western countries as a long-term complication of diabetes. Given that diabetes affects approximately 246 million people worldwide, it is estimated that 20-30 million people are affected by symptomatic diabetic neuropathy. The pathogenesis of diabetic neuropathy includes many factors such as metabolic, vascular, autoimmune, neurohormonal growth factor deficiency and oxidative stress (see Zochodne, 1999 for review). Based on these observations, several therapeutic drugs, including antioxidants (Low *et al.*, 1997), selective PKC inhibitors (Nakamura *et al.*, 1999) and neurotrophic factors (Tomlinson *et al.*, 1997) have been used to treat diabetic neuropathy, even if the magnitude of the effects in humans has been smaller than the expected for many of them. In addition, as for other types of painful neuropathies, tricyclic antidepressants, anticonvulsants and opioids are employed in order to obtain pain relief, even if it has been estimated that these therapies led at best to 50% reduction of pain in 50% of patients. Furthermore, the poor efficacy of these drugs is often associated to severe adverse effects. Finally, there is a general consensus that a strict glycemic control may diminish the risk of developing a disabling peripheral neuropathy (Martin *et al.*, 2006), suggesting that the prevention of diabetic neuropathy remains the best strategy. Thus, early diagnosis of diabetic neuropathy, followed by drug therapy combined with glycemic control may be warranted to prevent at least the progression of diabetic neuropathy. However, the future goal in treating diabetic neuropathy should be not only to prevent or delay the painful neuropathic symptoms, but also to completely relieve pain and possibly to promote the regeneration of degenerate nerve fibers after the onset of the pathology. Among the emerging substances very effective against neuropathic pain,

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4 cannabinoids are promising analgesics (see Lever and Rice, 2007 for review).  
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6 Cannabinoids are lipophilic compounds originally obtained from *Cannabis sativa* which  
7  
8 contains more than 60 different cannabinoids. Phytocannabinoids obtained from the  
9  
10 cannabis plant comprise a range of cannabinoid receptor agonists, partial agonists, and  
11  
12 antagonists, and many synthetic cannabinoids have also been developed with specific  
13  
14 receptor affinity and distinct pharmacological profiles. There are two main cannabinoid  
15  
16 receptors, CB1 and CB2, associated with pain modulation. The first is widely expressed  
17  
18 in the CNS and peripheral sensory neurons whereas the latter has been found on  
19  
20 peripheral tissues, including tissues of the immune system and keratinocytes, with  
21  
22 limited expression in sensory and CNS cells (Pertwee, 2006 for review). Synthetic or  
23  
24 pure natural cannabinoids have been found useful in neuropathic pain (Bridges *et al.*,  
25  
26 2001; Fox *et al.*, 2001; Costa *et al.*, 2004b; Costa *et al.*, 2006; Costa *et al.*, 2007) even if  
27  
28 the most of studies were performed employing the injury of the sciatic nerve as animal  
29  
30 model of painful neuropathy. To our knowledge there are only two initial studies  
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32 reporting the efficacy of a synthetic CB1 receptor agonist, WIN55,212-2, on tactile  
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34 allodynia in diabetic rats (Dogrul *et al.*, 2004; Ulugol *et al.*, 2004). We have recently  
35  
36 shown that a controlled cannabis extract, containing multiple cannabinoids, in a defined  
37  
38 ratio, and other non-cannabinoid fractions (terpens and flavonoids) provided better  
39  
40 antinociceptive efficacy than the single cannabinoids given alone when tested in the  
41  
42 chronic constriction injury of the sciatic nerve model of neuropathy (Comelli *et al.*,  
43  
44 2008). We suggested that other than the pharmacological receptor-mediated effect of  
45  
46 cannabinoids, the presence of anti-inflammatory and antioxidant compounds in the non-  
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48 cannabinoid fraction of the natural extract might strongly contribute to analgesia  
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50 (Comelli *et al.*, 2008). This background and especially the multiple aetiologies of  
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4 diabetic neuropathy involving oxidative stress, inflammation and nerve damage,  
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6 prompted us to test the same *Cannabis sativa* extract in the rat model of streptozotocin  
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8 (STZ)-induced diabetic neuropathy, in the attempt to propose a new therapy to  
9  
10 ameliorate this chronic and disabling painful condition after its onset.  
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12

## 13 14 **MATERIALS AND METHODS**

### 15 16 **Animals.**

17  
18 All experiments performed were in accordance with Italian State and the European  
19  
20 regulations governing the care and treatment of laboratory animals (Permission n°  
21  
22 101/2004B) and conformed to the guidelines for the study of pain in awake animals  
23  
24 established by the International Association for the Study of Pain (Zimmermann, 1983).  
25  
26 All efforts were made to minimize the number of animals used and their discomfort.  
27  
28 Male Wistar rats weighing 200-220 g (Harlan, Italy) were housed under controlled  
29  
30 temperature ( $22\pm 1^{\circ}\text{C}$ ), humidity ( $60\pm 10\%$ ) and light (12h/day) and allowed to  
31  
32 acclimatise for at least one week before the tests.  
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### 36 37 **Induction of diabetes.**

38  
39 Type 1 diabetes was induced through chemical pancreatectomy by a single  
40  
41 intraperitoneal injection of STZ (Sigma, Italy) at 60 mg/kg, freshly prepared in citrate  
42  
43 buffer 0.1 M pH 4.5. Diabetes was verified one week later by measurement of blood  
44  
45 glucose concentration by a glucometer (Lifescan One Touch Ultra glucose meter,  
46  
47 Milan, Italy) on a sample of blood obtained from a tail prick. Only rats with a blood  
48  
49 glucose level above 250 mg/dL were selected for the experiments. Control animals  
50  
51 received an intraperitoneal (i.p.) injection of citrate buffer. Blood glucose level and rat  
52  
53 body weight were monitored over the whole period of the experimental study.  
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### 58 59 **Drugs and treatments.**

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*Cannabis sativa* extract with an high cannabidiol (CBD) content (eCBD) is a gift of GW Pharmaceuticals (UK). eCBD contains 64.5% CBD, 4% tetrahydrocannabinol (THC), <4% of other cannabinoids (cannabigerol, cannabichromene, cannabidivarin, cannabidiolic acid) and minor components (terpenes, sterols, triglycerides, alkanes, squalene, tocopherol, carotenoids). The compound was dissolved in a 1:1:18 mixture of ethanol:cremophor:saline.

Diabetic rats, randomly divided in three groups of 6-8 rats each, received orally the drug vehicle or eCBD (15 or 30 mg/kg), once a day for 8 days, starting from the day 28<sup>th</sup> after the STZ injection. The effect of the acute administration of eCBD was studied in diabetic rats treated with vehicle for 7 days challenged with the drug the day of the behavioural evaluations.

#### **Assessment of thermal hyperalgesia and mechanical allodynia.**

Responses to thermal and mechanical stimuli were measured before diabetes induction and subsequently to STZ injection once per week for five weeks. On day 28<sup>th</sup> after STZ injection, the pharmacological treatment began just after the pain behaviour evaluations and at the end of treatment (on day 35) the evaluations were performed 150 min after the last eCBD administration. Heat hypersensitivity was tested according to the Hargreaves procedure (Hargreaves *et al.*, 1988) using the Plantar test (Ugo Basile, Varese, Italy). Briefly, animals were placed in a clear plexiglass box and allowed to acclimatise. A constant intensity radiant heat source was aimed at the midplantar area of the hind paw. The time, in seconds, from initial heat source activation until paw withdrawal was recorded. Mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy). Animals were placed in a test cage with a wire mesh floor, and the tip of a von Frey-type filament was applied to the



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4 middle of the plantar surface of the hind paw. The filament exerted an increasing force  
5  
6 starting below the threshold of detection and increasing until the animal removed its  
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8 paw. Withdrawal threshold was expressed as threshold level in g.  
9

### 10 11 **Sample preparation.**

12  
13 Thirty-five days following STZ, 150 min after the last administration of eCBD or its  
14  
15 vehicle, pain assessment was recorded and animals were sacrificed. The livers and the  
16  
17 sciatic nerves were quickly and carefully removed and washed with ice-cold saline  
18  
19 solution. 2.5 g of liver were homogenized in four volumes of ice-cold 0.15 M KCl and  
20  
21 centrifuged at 9000 g, at 4°C for 10 min. Supernatants were centrifuged at 100000 g, at  
22  
23 4°C for 1 h in order to obtain the cytosolic fractions which were ultracentrifuged again  
24  
25 in presence of HPO<sub>3</sub> (1:4, v:v), used as a protein precipitant. These latter fractions were  
26  
27 used for reduced (GSH) and oxidized (GSSG) glutathione content assay. Part of the  
28  
29 liver was stored at -20°C and used for malondialdehyde (MDA) assay. Sciatic nerves  
30  
31 were immediately stored at -80°C until the neurotrophic factor nerve growth factor  
32  
33 (NGF) was assayed. Protein concentrations of all sample tissues were assayed by the  
34  
35 method described by Lowry *et al.* (1951) with bovine serum albumin as standard.  
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### 42 **GSH and GSSG assay.**

43  
44 The GSH and GSSG content in the hepatic cytosolic fractions was analyzed  
45  
46 fluorimetrically with 350 nm and 420 nm as excitation and emission wavelengths,  
47  
48 accordingly to the method of Hissin and Hilf (1976), using ophthalaldehyde (OPT) as  
49  
50 fluorescent reagent. Briefly, for the GSH measurement, to 0.1 ml of the 100000 g  
51  
52 supernatant, 1.9 ml of a 0.1 M phosphate-EDTA (5 mM) buffer, pH 8, was added. For  
53  
54 the GSSG measurement, 0.5 ml of cytosol was incubated for 30 min with 200 µl of 0.04  
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56 M *N*-ethylmaleimide to prevent oxidation of GSH to GSSG. After the incubation, 4.3  
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4 ml of 0.1 N NaOH was added to this mixture. For both GSH and GSSG, the final assay  
5  
6 mixture (2.0 ml) contained 100 µl of the diluted cytosol, 1.8 ml of phosphate buffer, and  
7  
8 100 µl of the OPT solution (1% in methanol). The GSH and GSSG concentration was  
9  
10 calculated using a standard curve with known amounts of GSH and GSSG (Sigma  
11  
12 Aldrich, Milano, Italy), and expressed as µg/mg protein.  
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#### 15 16 **MDA assay.**

17  
18 The MDA level, an indicator of free radical generation, was estimated in liver  
19  
20 homogenate in a ratio 1 g tissue to 9 ml potassium phosphate (50 mM) plus EDTA (0.1  
21  
22 mM) buffer, pH 7. The lipid peroxide level was determined using the thiobarbituric acid  
23  
24 test of Ohkawa *et al.* (1979). Briefly, 0.2 ml of homogenate was added to 0.8%  
25  
26 thiobarbituric acid, 8.1% sodium dodecyl sulfate (SDS) and acetic acid (20%) in  
27  
28 distilled water. After heating for 60 min in a water bath at 95°C, the mixture was then  
29  
30 cooled and extracted with a mixture of n-butanol/pyridine (15:1, v:v). The absorbance  
31  
32 of the reaction product present in the upper organic layer separated by centrifugation  
33  
34 was measured spectrophotometrically at 532 nm. The MDA content was calculated  
35  
36 employing 0.156 mM/cm as the extinction coefficient and was expressed as nmol  
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38 MDA/g tissue.  
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#### 43 44 **NGF assay.**

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46 Sciatic nerves were homogenized in a cold lysis buffer (250 µl). The homogenates were  
47  
48 centrifuged at 4500 g at 4°C for 10 min, and the resulting supernatants were then diluted  
49  
50 5-fold with Dulbecco's phosphate buffer solution. Samples were acidified to pH < 3.0  
51  
52 by adding 1 N HCl and then neutralized with 1 N NaOH to pH 7.6. NGF protein levels  
53  
54 were determined by enzyme-linked immunosorbent assay (ELISA) using an ELISA kit,  
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56 according to the manufacturer's instructions (Promega, USA). The absorbance at 450  
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4 nm was recorded on a microplate reader (Multiskan® EX, ThermoLabSystem). NGF  
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6 levels were determined by interpolation with standard curve and normalized to protein  
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8 content in each tissue sample.  
9

### 10 11 **Statistical analysis.**

12  
13 All data are expressed as the mean  $\pm$  SEM and analyzed using analysis of variance  
14  
15 (ANOVA) followed by Tukey's post-hoc test for multiple comparison. Student's *t* test  
16  
17 was used to compare the values from two groups. Differences were considered  
18  
19 significant at  $p < 0.05$ .  
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## 22 23 **RESULTS**

### 24 25 **Effect of STZ injection on pain behaviour.**

26  
27 The withdrawal latency and mechanical threshold were tested 7, 14, 21, 28 and 35 days  
28  
29 after a single administration of STZ (60 mg/kg, i.p.). Before STZ administration, the  
30  
31 rats withdrew their left and right hind paws from radiant heat with a latency of about 10  
32  
33 s and sustained a mechanical force of about 38 g. Seven days after the STZ  
34  
35 administration the pain behaviour of diabetic rats was unmodified in respect to control  
36  
37 animals (Fig. 1A, B). Diabetic rats showed a significant increase in the paw withdrawal  
38  
39 latency to thermal stimuli starting from the day 14<sup>th</sup> after STZ (Fig. 1A). This thermal  
40  
41 hypoalgesia further increased during the subsequent days of observation reaching the  
42  
43 maximum between day 28 and 35 (Fig. 1A). Only on day 28<sup>th</sup> after STZ, diabetic rats  
44  
45 developed a significant mechanical allodynia demonstrated by the decrease in the  
46  
47 mechanical force sustained by diabetic rats *versus* non diabetic (Fig. 1B). Mechanical  
48  
49 allodynia was still present the subsequent week, corresponding to the 35<sup>th</sup> day after STZ  
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51 injection (Fig. 1B).  
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4 **Effect of eCBD treatment on diabetes-induced thermal hypoalgesia and**  
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6 **mechanical allodynia.**  
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9 Diabetic rats were orally treated with vehicle or with eCBD once a day for seven days,  
10 starting the day 28<sup>th</sup> after STZ, when both thermal hypoalgesia and mechanical  
11 allodynia were evident. Thermal hypoalgesia and mechanical allodynia were still  
12 present in diabetic rats treated with vehicle, whereas the repeated treatment with eCBD  
13 significantly relieved mechanical allodynia and restored the physiological thermal pain  
14 perception at the highest dose employed (30 mg/kg) (Fig. 2A, B). The single  
15 administration of eCBD at the highest dose evoked only a slight relief of mechanical  
16 allodynia at 150 min (30.42±0.611 g for eCBD treated diabetic rats *versus* 26.20±1.20 g  
17 for diabetic rats) (data not shown).  
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30 **Effect of eCBD treatment on diabetes-induced hyperglycemia and body weight**  
31 **loss.**  
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35 To ascertain whether the relief of diabetic neuropathy induced by eCBD was due to a  
36 glycaemic control, glucose level was measured after the pharmacological treatment (day  
37 35 after STZ) and compared with the basal one (day 28 after STZ) (Fig. 2C). As  
38 expected, by the 2<sup>nd</sup> day after administration of STZ, rats developed hyperglycemia:  
39 their blood glucose level (502.2±42.05 mg/dL) was statistically different from that of  
40 control animals (135.7±14.31 mg/dL) (data not shown). Hyperglycemia became greater  
41 at 28 days after STZ (Fig. 2C). The blood glucose level of rats treated for one week with  
42 eCBD (30 mg/kg) did not differ either from that of diabetic rats treated with vehicle or  
43 from the level before starting the treatment (Fig. 2C), suggesting that the  
44 pharmacological treatment did not affect the hyperglycemia induced by STZ. The body  
45 weight of diabetic rats became significantly lower than that of controls during the four  
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4 weeks after STZ injection; in fact, as shown in Fig. 2D, diabetic rats had lost 20% of  
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6 their original body weight *versus* a physiological increase of about 15% showed by  
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8 control rats, with initial body weights similar in control and diabetic groups ( $224.6 \pm 5.63$   
9  
10 g and  $222.4 \pm 3.92$  g). After the week of treatment, diabetic rats treated with vehicle  
11  
12 showed a further reduction in their body weight; conversely, the repeated administration  
13  
14 with eCBD evoked a significant attenuation of the body weight loss of diabetic rats  
15  
16 (Fig. 2D), showing that the pharmacological treatment led to an amelioration of the  
17  
18 general health of rats.  
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### 23 **Effect of eCBD treatment on oxidative stress.**

24  
25 The content of GSH and GSSG, as well as the MDA level, were measured as hepatic  
26  
27 markers of diabetes-induced oxidative stress. As expected, STZ administration induced  
28  
29 a significant depletion of both GSH and GSSG, with a ratio (GSH/GSSG) that  
30  
31 decreased more than two times in respect to the physiological value necessary to carry  
32  
33 out the glutathione-linked defensive activity (Fig. 3A, B, C). Accordingly, in the same  
34  
35 animals the level of MDA was markedly increased, as sign of free radical generation  
36  
37 (Fig. 3D). The repeated treatment with the eCBD restored the physiological level of  
38  
39 GSH, with a smaller effect upon GSSG (Fig. 3A, B). However, as consequence, the  
40  
41 correct ratio GSH/GSSG was completely restored after eCBD treatment (Fig. 3C). In  
42  
43 addition, the treatment with eCBD significantly reduced MDA level to the control level  
44  
45 (Fig. 3D), highlighting the potent antioxidant and anti-radical effect of eCBD.  
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### 51 **Effect of eCBD repeated treatment on NGF production in the sciatic nerve.**

52  
53 NGF concentration was significantly decreased (35%) in the sciatic nerve of diabetic  
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55 rats compared with controls. This downregulation was completely reversed by eCBD  
56  
57 treatment that brought NGF at a value no more statistically different from that of non  
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4 diabetic animals (Fig. 4), indicating a positive effect of eCBD on diabetes-induced  
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6 impairment of peripheral nerve support by NGF.  
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## 9 10 **DISCUSSION**

11  
12 Diabetes is one of the most frequent pathologies in developing countries and it is  
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14 estimated that the number of people affected with diabetes worldwide is projected to be  
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16 366 million by year 2030 (Wild *et al.*, 2004). Uncontrolled chronic hyperglycemia in  
17  
18 diabetic subjects leads to several complications including nephropathy, retinopathy,  
19  
20 neuropathy. This latter is the most common complication, affecting more than 50% of  
21  
22 diabetic patients. Evidence suggest that most of the neuropathic pain states, including  
23  
24 diabetes-induced neuropathy, are not effectively controlled by first-line therapy with  
25  
26 drugs such as antidepressants, anticonvulsants and opioids. Thus neuropathic pain is  
27  
28 still considered to be relatively refractory to most of the analgesics. Recent treatment  
29  
30 strategies have focused on the action of cannabinoids in neuropathic pain and growing  
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32 evidence highlighted the efficacy of many cannabinoid receptor agonists to exert anti-  
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34 hyperalgesic effect in nerve injury-induced neuropathic pain (Bridges *et al.*, 2001; Fox  
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36 *et al.*, 2001; Costa *et al.*, 2004b; Costa *et al.*, 2006; Costa *et al.*, 2007). In contrast,  
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38 much less is known of the effects of cannabinoids in diabetes-induced neuropathic pain  
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40 (Dogrul *et al.*, 2004; Ulugol *et al.*, 2004). In spite of the efficacy reported, for many  
41  
42 cannabinoids the therapeutic employment is precluded by the concomitant adverse  
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44 effects (hypothermia, sedation, hypomotility) and by the possibility of tolerance and  
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46 dependence development. We recently found that a controlled cannabis extract,  
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48 containing multiple cannabinoids, in a defined ratio, and other non-cannabinoid  
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50 fractions (terpenes and flavonoids) provided better antinociceptive efficacy than the  
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52 single cannabinoid given alone, when tested in a rat model of neuropathic pain elicited  
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4 by sciatic nerve injury (Comelli *et al.*, 2008). In addition to potentiating the  
5 pharmacological efficacy of cannabinoids, the use of controlled extracts could also  
6 decrease the adverse effects following *in vivo* administration. For instance, it was  
7 established in humans that the psychoactive effects of THC were significantly  
8 attenuated when CBD was also present (Dalton *et al.*, 1976). Collectively, the findings  
9 from our previous work strongly support the idea that the combination of cannabinoid  
10 and non-cannabinoid compounds, as present in eCBD extract, provides significant  
11 advantages in the relief of neuropathic pain in terms of efficacy and a lack of central  
12 effects (Comelli *et al.*, 2008). In the present work we showed the therapeutic efficacy of  
13 the same eCBD in relieving diabetes-induced neuropathic pain. The model employed by  
14 us consisting in STZ-induced diabetes, evoked in rats mechanical allodynia and thermal  
15 hypoalgesia. Our observation is in agreement with various studies that demonstrate  
16 profound mechanical hyperalgesia with thermal hypoalgesia or no changes in thermal  
17 withdrawal thresholds in diabetic animals (Malcangio and Tomlinson, 1998; Fox *et al.*,  
18 1999). Similarly, human diabetic patients often develop a compromised ability to  
19 perceive tactile sensation, particularly in the most distal limbs (Norrzell *et al.*, 2001),  
20 and they have longer withdrawal latency than non diabetic patients when exposed to  
21 both warming and cooling thermal stimuli (Navarro and Kennedy, 1991). Here we  
22 demonstrated that the repeated treatment with eCBD evoked a significant attenuation of  
23 mechanical allodynia and restored the physiological thermal nociceptive perception.  
24 These effects were elicited by 30 mg/kg of extract, a dose higher than that necessary to  
25 relieve nerve injury-induced neuropathic pain (15 mg/kg, as previously demonstrated by  
26 us (Comelli *et al.*, 2008)), suggesting that although similar behavioural symptoms are  
27 observed both in nerve injury- and STZ-induced neuropathy, it is possible to assume  
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4 that such symptoms may not share the same aetiology. Of particular relevance for a  
5 possible future clinical employment, it is the ability of eCBD to alleviate diabetic  
6 neuropathy in a therapeutic regimen and through the oral route of administration.  
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8 Concerning the mechanism of action, multiple hypothesis can be postulated in the light  
9 of the miscellaneous of compounds present in the extract and on the basis of the  
10 complexity of the mechanisms underlying diabetic neuropathy. The relief of pain  
11 induced by eCBD might be ascribed to a receptor-mediated action. In our previous work  
12 we demonstrated that eCBD counteracted nerve injury-induced neuropathic pain only  
13 through the transient receptor potential vanilloid subfamily member 1 (TRPV1)  
14 (Comelli *et al.*, 2008) that is highly expressed on primary sensory neurons where it  
15 functions as polymodal nociceptor (see Szallasi *et al.*, 2007, for review); both  
16 antagonists, through the blockade of the receptor, and agonists, through the  
17 desensitization of the receptor, behave as analgesics. In diabetic subjects small fiber (the  
18 unmyelinated C and the thinly myelinated A $\delta$ ) damage is responsible for the allodynia  
19 and hypoalgesia and both type of fibers constitutively express TRPV1. On these bases,  
20 it is possible to speculate a TRPV1 receptor involvement in the relief of diabetic  
21 neuropathy induced by eCBD, even if this hypothesis awaits further experimental  
22 investigation.  
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47 Hyperglycemia has been reported to result in increased polyol pathway activity,  
48 oxidative stress, advanced glycation end product formation, increased activation of  
49 protein kinase C, nerve hypoxia/ischemia and impaired NGF support (see Pop-Busui *et*  
50 *al.*, 2006, for review) and all these pathways contribute to the development of diabetic  
51 neuropathy. Therefore we first tested whether the eCBD-induced relief of diabetic  
52 neuropathy can be due to its action upon hyperglycemia. However, the results showed  
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4 that the repeated treatment with eCBD did not result in a decrease of blood glucose  
5 level, demonstrating that the relief of neuropathy occurred without affecting  
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7 hyperglycemia. One of the important consequences of chronic hyperglycemia is the  
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9 enhanced oxidative stress resulting from imbalance between production and  
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11 neutralization of reactive oxygen species (ROS). Particularly, the diabetes-associated  
12  
13 free radical injury, i.e. accumulation of lipid peroxidation products, depletion of GSH,  
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15 decrease in GSH/GSSG ratio and down regulation of key antioxidant enzymes, have  
16  
17 been detected not only in the liver but also in peripheral nerves, dorsal root and  
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19 endothelial cells in different animal model of diabetes (Nagamatsu *et al.*, 1995; Low *et*  
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21 *al.*, 1997; Romero *et al.*, 1999). Accordingly, we observed a decrease in GSH in the  
22  
23 liver of diabetic rats, probably due to an increased utilization following the diabetes-  
24  
25 induced oxidative stress. Previous studies have reported that there was an increased  
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27 lipid peroxidation in liver of diabetic rats (Yilmaz *et al.*, 2004) that can be due to  
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29 increased oxidative stress in the cell as a result of depletion of antioxidant scavenger  
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31 systems. Lipid peroxide-mediated tissue damages have been observed in the  
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33 development of type I and type II diabetes (Feillet-Coudray *et al.*, 1999). The repeated  
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35 administration of eCBD increased the GSH content in the liver leading to a restore of  
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37 the defence mechanism and significantly decreased the liver lipid peroxidation. eCBD  
38  
39 may help to control free radicals as both CBD and THC offered protection to cells  
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41 against oxidative stress by scavenging free radicals. In fact it has been shown that  
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43 compared with other commonly used antioxidants, CBD and THC protected neurons to  
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45 a greater degree than the dietary antioxidants  $\alpha$ -tocopherol and ascorbate (Hampson *et*  
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47 *al.* 1998). In addition, CBD has been shown to exert antioxidant effects both *in vitro*  
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49 (Chen and Buck, 2000) and in various preclinical models of neurodegeneration and  
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4 inflammatory disorders (Malfait *et al.*, 2000; Costa *et al.*, 2004a) through a receptor-  
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6 independent mechanism. Of interest, many other substances present in the extract  
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8 possess well established antioxidant properties (tocopherol, monoterpenes (Grassmann,  
9  
10 2005)), which could prominently contribute to the antioxidant effect observed after  
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12 eCBD treatment. In conclusion, the protective antioxidant action of eCBD provides the  
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14 protection against oxidative damage in STZ-induced diabetes that also strongly  
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16 contributes to the neuropathy development. In support of this mechanism of action there  
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18 is the demonstration that several antioxidants such as  $\alpha$ -lipoic acid, taurine and  $\beta$ -  
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20 carotene ameliorate nerve function deficit in experimental diabetic neuropathy (Karasu  
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22 *et al.*, 1995).  
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28 There is strong support for the hypothesis that reduced levels or activity of NGF play a  
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30 significant role in the pathogenesis of diabetic neuropathy (Pittenger and Vinik, 2003),  
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32 since the integrity of distinct subpopulations of nociceptive sensory neurons are highly  
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34 dependent on neurotrophic support provided by NGF. In addition, the retrograde  
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36 transport of NGF in the sciatic nerve is also decreased in rats after STZ (Hellweg *et al.*,  
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38 1991). The low level of NGF could be due to either reduced production or transport of  
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40 NGF in diabetes or both, possibly as a result of glucose-induced oxidative stress  
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42 (Pittenger and Vinik, 2003). Clinical studies have confirmed that there are changes in  
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44 NGF mRNA levels in the skin of diabetic subjects (Anand *et al.*, 1996) that may be  
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46 pertinent to progressive loss of epidermal C fibers and subsequent thermal hypoalgesia.  
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48 Unfortunately, clinical trials of NGF therapy in diabetic patients have not been  
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50 successful (Apfel *et al.*, 2000), in part because of the limitation in the exogenous NGF  
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52 delivery and tolerability. We believe that a compound able to induce or enhance  
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54 endogenous NGF production could represent an important therapeutic tool to prevent  
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4 the nerve damage associated to the loss of NGF support. In this sense, we have reported  
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6 here that the NGF content in the sciatic nerve of diabetic rats was restored to normal  
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8 following the repeated treatment with eCBD. It is possible to hypothesize that the  
9  
10 eCBD-induced prevention of diabetes-induced thermal hypoalgesia as well as the nerve  
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12 degeneration consequently to hyperglycemia might be ascribed to the ability of the  
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14 extract to normalize the NGF support, even if the mechanism underlying such an effect  
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16 is still unknown and deserves further investigations.  
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20 In conclusion, the repeated treatment with eCBD in diabetic rats resulted in an  
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22 amelioration of allodynia and of impaired thermal perception along with an  
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24 improvement of oxidative damage and NGF support, suggesting the beneficial effects of  
25  
26 this cannabis extract diabetic neuropathy.  
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31  
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35  
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## Figure legends

**Fig. 1.** Time course of pain threshold values of STZ (60 mg/kg, i.p.)-administered (diabetic) or control (non diabetic) rats, after thermal (A) and mechanical (B) stimuli. Withdrawal latency to heat and mechanical threshold of the paws are expressed as s and g, respectively, and data represent mean  $\pm$  S.E.M. of 6-8 rats. \*\*\* $p$ <0.001, \*\* $p$ <0.01, \* $p$ <0.05 vs non diabetic.

**Fig. 2.** Effect of eCBD (15 and 30 mg/kg, p.o.) given daily to diabetic rats for one week, from day 28<sup>th</sup> after STZ injection, on thermal hypoalgesia (A) and on mechanical allodynia (B). The effective dose of eCBD on pain behaviour (30 mg/kg, p.o.) was also tested on glucose level (C) and on body weight (D) by comparing the values between the beginning (D28) and the end (D35) of the treatment. Data represent mean  $\pm$  S.E.M. of 6-8 rats.\*\*\* $p$ <0.001, \*\* $p$ <0.01, \* $p$ <0.05 vs non diabetic ; °°° $p$ <0.001 vs diabetic.

**Fig. 3.** Effect of eCBD (30 mg/kg, p.o.) given daily to diabetic rats for one week, from day 28<sup>th</sup> after STZ injection, on reduced glutathione (GSH) (A) and on oxidized glutathione (GSSG) (B) in the cytosolic fraction of liver. Panel C represents the ratio between reduced and oxidized glutathione. In panel D is shown the effect of the repeated treatment with eCBD on the level of malondialdehyde (MDA) in the liver. Data represent mean  $\pm$  S.E.M. of 6-8 rats. \*\*\* $p$ <0.001, \* $p$ <0.05 vs non diabetic; °° $p$ <0.01 vs diabetic.

**Fig. 4.** Effect of eCBD (30 mg/kg, p.o.) given daily to diabetic rats for one week, from day 28<sup>th</sup> after STZ injection on nerve growth factor (NGF) production in the sciatic nerve. Data represent mean  $\pm$  S.E.M. of 6-8 rats. \*\* $p$ <0.01 vs non diabetic; ° $p$ <0.05 vs diabetic.