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Antihyperalgesic Effect of a *Cannabis sativa* Extract in a Rat Model of Neuropathic Pain: Mechanisms Involved

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This study aimed to give a rationale for the employment of phytocannabinoid formulations to treat neuropathic pain. It was found that a controlled cannabis extract, containing multiple cannabinoids, in a defined ratio, and other non-cannabinoid fractions (terpenes and flavonoids) provided better antinociceptive efficacy than the single cannabinoid given alone, when tested in a rat model of neuropathic pain. The results also demonstrated that such an antihyperalgesic effect did not involve the cannabinoid CB1 and CB2 receptors, whereas it was mediated by vanilloid receptors TRPV1. The non-psychoactive compound, cannabidiol, is the only component present at a high level in the extract able to bind to this receptor: thus cannabidiol was the drug responsible for the antinociceptive behaviour observed. In addition, the results showed that after chronic oral treatment with cannabis extract the hepatic total content of cytochrome P450 was strongly inhibited as well as the intestinal P-glycoprotein activity. It is suggested that the inhibition of hepatic metabolism determined an increased bioavailability of cannabidiol resulting in a greater effect. However, in the light of the well known antioxidant and antiinflammatory properties of terpenes and flavonoids which could significantly contribute to the therapeutic effects, it cannot be excluded that the synergism observed might be achieved also in the absence of the cytochrome P450 inhibition. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: neuropathic pain; *Cannabis sativa*; cannabinoid; cannabidiol; cytochrome P450; P-glycoprotein.

INTRODUCTION

Cannabis sativa L. has a long history of use both as a medicinal agent and an intoxicant. Several reports highlighted that both the main components of marijuana, Δ^9tetrahydrocannabinol (THC), the psychoactive one, and cannabidiol (CBD), the non-psychoactive one, display many pharmacological effects. THC has antispasmodic, antitremor, antiinflammatory, appetite stimulant and antiemetic properties, whereas CBD has antiinflammatory, anticonvulsant, antipsychotic, antioxidant, neuroprotective and immunomodulatory effects (see Karanian and Bahr, 2006 for review). In addition, the analgesic properties of THC are well characterized (see Lever and Rice, 2007 for review) but they were not useful in therapy because of the concomitant psychoactive effects. Concerning the analgesic properties of CBD, the ability of this compound to alleviate persistent inflammatory and chronic neuropathic pain in rats was recently demonstrated for the first time (Costa *et al.*, 2007). However, this potent therapeutic effect was not associated with a significant decrease in some inflammatory markers, such as tumour necrosis factor α (TNFα), that are crucial for the onset of these pathologies, but it was likely due to a direct action on the pain signalling pathway (Costa *et al.*, 2007).

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Whole plant extracts contain a complex mixture of natural cannabinoids and other non-cannabic compounds that may interact synergically to provide a superior therapeutic profile over that of isolated components. This may explain why cannabis-based medicine made from whole plant extracts may be more effective than single cannabinoid products. In this regard, Russo (2005) reviewed some studies in which combination of THC and CBD and cannabis extracts produced effects greater than that expected from their single components, in both humans and rodents. In addition to potentiating the pharmacological efficacy of cannabinoids, the use of controlled extracts could also decrease the adverse effects following *in vivo* administration. In fact, it was established in humans that the psychoactive effects of THC were significantly attenuated when CBD was also present (Dalton *et al.*, 1976). On these bases, the present study aimed to give a rationale for the employment of phytocannabinoid formulations as therapeutic tools to treat a number of diseases. Among these, neuropathic pain is an area of unmet therapeutic need since it is often refractory or poorly responsive to all currently available analgesic drugs. Synthetic or pure natural cannabinoids have been found useful in this type of chronic pain (Bridges *et al.*, 2001; Fox *et al.*, 2001; Costa *et al.*, 2004b, 2006, 2007) even if the concomitant central unwanted effects limited their employment in humans. The aim of this work was to establish whether a controlled cannabis extract, containing multiple cannabinoids, in a defined ratio, and other non-cannabinoid fractions (terpenes, flavonoids) may provide better therapeutic success and to be better tolerated than

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the single synthetic cannabinoid medicines currently available. With this aim, the antinociceptive benefits of a *Cannabis sativa* extract enriched in CBD (eCBD) were investigated in a rat model of neuropathic pain, the chronic constriction injury of sciatic nerve (CCI), and compared with those evoked by the single components present in the extract. A further aim was to investigate the molecular basis of the synergism achieved with the cannabis extract. In particular, to ascertain what receptor is involved in the pharmacological effect provided by *Cannabis sativa* extract, different cannabinoid and vanilloid receptor antagonists were tested. In fact, both cannabinoid receptor subtypes (CB1 and CB2) are molecular targets for many THC-mediated effects, whereas CBD does not bind to cannabinoid receptors but it displays good affinity for vanilloid TRPV1 receptor (Bisogno *et al.*, 2001; Costa *et al.*, 2004c, 2007). On the other hand, it cannot be excluded that alteration in metabolic phase can modify the bioavailability of drugs and consequently the concentration able to give a pharmacological effect. Therefore, the cytochrome P450 system was studied in the liver of rats orally treated with eCBD, CBD and THC to verify a possible alteration of this metabolic system. Another protein affecting the oral bioavailability of many drugs is the intestinal P-glycoprotein (P-gp), an ATP-dependent efflux transporter coded by the MDR1 gene. Consequently, a further aim was the measurement of P-gp activity in the distal region of the small intestine, where the basal expression levels of this protein are higher than in other regions (Mouly and Paine, 2003).

MATERIALS AND METHODS

Animals. All experiments performed were in accordance with Italian State and European regulations governing the care and treatment of laboratory animals (Permission n° 101/2004B) and conformed to the guidelines for the study of pain in awake animals established by the International Association for the Study of Pain (Zimmermann, 1983). All efforts were made to minimize the number of animals used and their discomfort. Male Wistar rats weighing 200–220 g (Harlan, Italy) were housed under controlled temperature (22 ± 1 °C), humidity (60 ± 10%) and light (12 h/day) and allowed to acclimatize for at least 1 week before the tests.

Induction of neuropathic pain. Painful neuropathy was induced by chronic constriction injury of the sciatic nerve (CCI), as previously described by Bennett and Xie (1988). Briefly, animals were anaesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg, Sigma-Aldrich, Milan, Italy), the right sciatic nerve was exposed at the level of the mid thigh and, proximal to the sciatic nerve trifurcation, four ligatures were loosely tied around it with about 1 mm spacing so that epineural circulation was preserved. Sham animals (sciatic exposure without ligation) were used as controls.

Drugs and treatments. Cannabidiol (CBD), *Cannabis sativa* extract with a high CBD content (eCBD) and Δ^9tetrahydrocannabinol (THC) were a gift of GW Pharmaceuticals (UK). The cannabis extract rich in CBD contained 64.5% CBD, 4% THC, <4% of other cannabinoids (cannabigerol, cannabichromene, cannabidivarin, cannabidiolic acid) and minor components (terpenes, sterols, triglycerides, alkanes, squalene, tocopherol, carotenoids). Compounds were dissolved in a 1:1:18 mixture of ethanol:cremophor:saline.

Sham and CCI rats were randomly selected and divided in six groups of 8–10 rats each. The first group consisted of sham animals treated with vehicle, the second group was the CCI control (CCI rats treated with vehicle), the third group was CCI rats treated with eCBD containing CBD and THC at doses of 10 mg/kg and 0.62 mg/kg, respectively, the fourth and fifth groups consisted of *per se* CBD and THC treated CCI animals at the corresponding extract doses, the sixth group consisted of pure CBD (10 mg/kg) combined with pure THC (0.62 mg/kg) CCI rats. Animals received orally the compounds or their vehicle, once a day for 7 days, starting from day 7 after the surgical technique. The effect of the acute administration of compounds has been studied in CCI rats injected with vehicle for 6 days challenged with the drug on the day of the behavioural evaluations. To study the involvement of cannabinoid and/or vanilloid receptors in the eCBD-induced antihyperalgesic effect, different treatments were tested on the last day of eCBD administration. The cannabinoid CB1 receptor specific antagonist SR141716 (0.5 mg/kg i.p.), the cannabinoid CB2 receptor specific antagonist SR144528 (1 mg/kg i.p.) or the vanilloid TRPV1 receptor specific antagonist capsazepine (10 mg/kg i.p.) were given together with eCBD or its vehicle. SR141716 and SR144528 were kindly supplied by Sanofi-Aventis (Montpellier, France) and were dissolved in a mixture of Tween 80:DMSO:distilled water (1:2:7). Capsazepine was purchased from Sigma-Aldrich (Milano, Italy) and was dissolved in a 1:1:8 mixture of ethanol:Tween 80:saline. The employed doses of SR144528 and SR141716 were selected based on previous works in rodents (Rinaldi-Carmona *et al.*, 1988; Carta *et al.*, 1999), whereas the capsazepine dose was shown by Di Marzo *et al.* (2001) to antagonize the effects induced by the selective TRPV1 receptor agonist, capsaicin, in rats and by ourselves to reverse the antihyperalgesic effect of cannabidiol in carrageenan-induced acute inflammation in rats (Costa *et al.*, 2004a).

Assessment of thermal hyperalgesia and mechanical allodynia. Responses to thermal and mechanical stimuli were measured before surgery, on days 7 (before starting the treatment) and 14 (24 h after the last administration of compounds). In the antagonism studies, heat hypersensitivity was tested on day 13 (90 min after the administration of compounds). 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 acclimatize. 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 middle of the plantar surface of the hind paw. The filament exerted an increasing force starting below the threshold of detection and

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increasing until the animal removed its paw. Withdrawal threshold was expressed as the threshold level in g.

Sample preparation. Fourteen days following surgery, 24 h after the last administration, pain assessment was recorded and the animals were killed. The livers and the distal region of the small intestines were quickly and carefully removed and washed with ice-cold saline solution. 2.5 g of liver was homogenized in four volumes of ice-cold 0.15 M KCl and centrifuged at 9000 × **g**, at 4 °C for 10 min. Supernatants were centrifuged at 100 000 × **g**, at 4 °C for 1 h in order to obtain microsomal pellets which were stored at –80 °C until used for cytochrome P450 assay. The intestinal mucosa was scraped on ice with a slide glass and homogenized using a glass Teflon potter in buffer containing 250 mM sucrose, 50 mM Tris-HCl pH 7.4 and a cocktail of protease inhibitors (Roche Diagnostics, Monza, Italy). The homogenates were centrifuged 10 min at 3000 × **g** and the supernatant was again centrifuged for 30 min at 15 000 × **g**. The pellets containing the crude membranes were dissolved in 0.5 mL of a 50 mM Tris-HCl pH 7.4 buffer containing 50 mM mannitol and a cocktail of protease inhibitors (Roche Diagnostics, Monza, Italy) and stored at –80 °C until used for P-gp assay. Protein concentrations of all sample tissues were assayed by the method described by Lowry *et al.* (1951) with bovine serum albumin as standard.

Spectophotometric cytochrome P450 assay. The total content of cytochrome P450 was determined in the liver spectrophotometrically according to Omura and Sato (1964). Briefly, microsomal pellets were dissolved in Tris-HCl buffer 0.1 M pH 7.4 (1:1 w:v) and then diluted 1:10 in the same buffer. A few crystals of solid sodium dithionite (Sigma Aldrich, Milano, Italy) were added to 2 mL of microsomal suspension and the sample was gently saturated with bubbles of carbon monoxide for approximately 60 s. The reduced-CO spectrum was recorded from 400 to 500 nm using a Jasco V-530 spectrophotometer. The specific content of cytochrome P450 was calculated according to Beer's law using 0.091 nM cm^{-1} as extinction coefficient and expressed as nmol/mg protein.

SDS-PAGE and western immunoblotting. 2B1/2 isoform of cytochrome P450 was immunodetected by semiquantitative western blot analysis. Hepatic microsomal proteins were diluted in Laemmli buffer (0.3 M Tris-HCl pH 6.8, containing 10% SDS, 50% glycerol, 5% dithiothreitol and 0.05% bromophenol blue) to obtain 40 µg of proteins in 20 µL of loaded volume. The proteins were loaded onto a 10% SDS–polyacrylamide gel, separated electrophoretically at a constant voltage (120 V, 0.007 A) and then transferred onto a nitrocellulose membrane (Schleicher & Schuell, BAS 85) with the semidry method for 90 min at room temperature. The membrane was incubated with 5% non-fat dry milk in PBST (16 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 6.7 M NaCl, pH 7.5, 0.1% Tween 20) (blocking solution) overnight at 4 °C and then incubated with primary polyclonal antibody directed against rat 2B1/2 cytochrome P450 (Chemicon International, Temecula, CA, USA) diluted 1:1000 in blocking solution, at 37 °C for 2 h. After washing in PBST buffer, the blot was incubated with secondary antibody (anti-rabbit IgG, peroxidase linked F(ab')2 fragment, 1:1500 in 3% blocking solution) for 1 h at room temperature. After washing in PBST buffer, the blot was detected with an enhanced chemiluminescence detection kit (Roche Diagnostics, Monza, Italy). The blots were then incubated in a stripping buffer (67.5 mM Tris pH 6.8, 2% SDS and 0.7% β-mercaptoethanol) overnight at 4 °C and reprobed with a polyclonal anti-β-actin antibody (1:1000) as loading control. The intensity of each band was quantified by the image analysis software ImageJ (Scion Corporation, Frederick, MD, USA).

P-gp-ATPase activity assay. *Ex vivo* P-gp-dependent ATPase activity was quantified by determining the increased inorganic phosphate (P_i) concentrations in the presence or in the absence of 300 µM of sodium orthovanadate, as described previously by Sarkadi *et al.* (1992) with some modifications. Briefly, the reaction mixture containing membrane suspension (40 µg) and Tris Mes buffer pH 6.8 was incubated at 37 °C for 5 min. The reaction was initiated by adding 12 mM Mg-ATP to the mixture. After incubation at 37 °C for 20 min, the reactions were terminated by the addition of 10% SDS containing 0.1% AntifoamA. Detection reagent (a solution consisting of one part of 35 mM ammonium molybdate in 15 mM zinc acetate and four parts of 10% ascorbic acid pH 5) was then added and after an incubation at 37 °C for 20 min in the dark, the released inorganic phosphate was trapped as phosphomolybdate and reduced by ascorbic acid to form a molybdenum-blue complex, which was detected spectrophotometrically at 630 nm. The vanadate-sensitive ATPase activity (the value in the absence of sodium orthovanadate minus the corresponding value in the presence of sodium orthovanadate) was estimated by the differences in P_i levels in 20 min. A phosphate standard curve was prepared using increasing potassium phosphate concentrations (0–150 nmol) to calculate the nmol of phosphate released. P-gp-ATPase activity was also measured *in vitro*, employing crude intestinal membranes derived from naïve rats. Different concentrations of CBD (0.1, 1, 25, 50 and 100 µM) and eCBD containing CBD 25 µM, were tested following the same experimental procedure adding the drugs to the initial reaction mixture.

Statistical analysis. All data are expressed as the mean ± SEM and analysed using analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparison. Differences were considered significant at $p < 0.05$. Non-parametric data (the values of densitometric analysis) were analysed with Kruskal-Wallis ANOVA followed by Dunn's test.

RESULTS

Effect of eCBD on thermal hyperalgesia and mechanical allodynia

The withdrawal latency to both thermal and mechanical stimuli was tested at 7 and 14 days after the surgery, 24 h after the last administration of compounds. Before surgery, the rats withdrew their left and right hind paws from radiant heat with a latency of about 10 s

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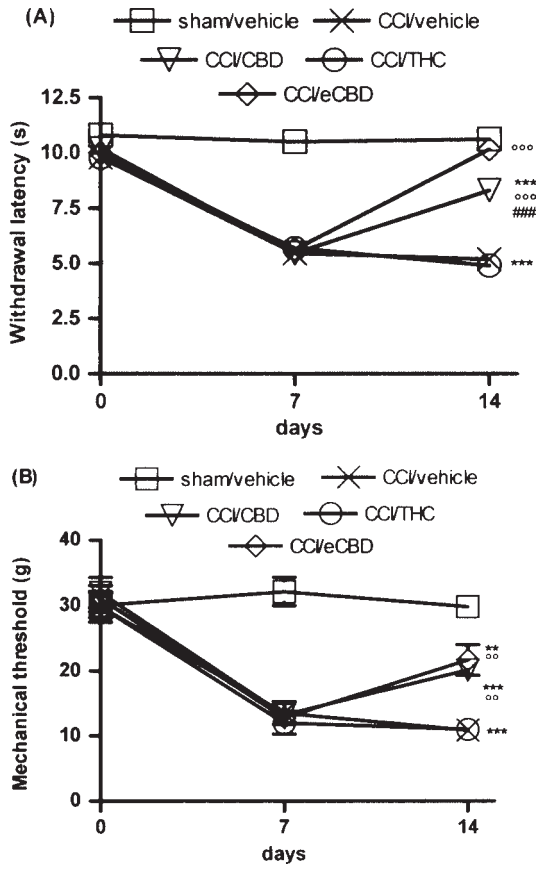

Figure 1. Effect of CBD (10 mg/kg), THC (0.62 mg/kg) and eCBD given orally daily to neuropathic rats (CCI), for 1 week from day 7 after the surgery, on thermal hyperalgesia (A) and on mechanical allodynia (B). Withdrawal latency to heat and mechanical threshold of the injured paws are expressed as s and g, respectively, and data represent mean ± SEM of 8–10 rats. *** $p < 0.001$, ** $p < 0.01$ vs sham/vehicle; °°° $p < 0.001$, °° $p < 0.01$ vs CCI/vehicle; ### $p < 0.001$ vs CCI/eCBD.

and sustained a mechanical force of about 30 g. Seven days after the injury CCI rats developed a significant decrease (about 50%) in both thermal withdrawal latency (Fig. 1A) and mechanical withdrawal threshold (Fig. 1B) of the paw ipsilateral to the injury, compared with sham-operated animals. Thermal hyperalgesia and mechanical allodynia were still present in CCI rats treated for the subsequent 7 days with vehicle. The daily treatment of CCI rats with eCBD for 7 days, completely relieved thermal hyperalgesia and partially attenuated mechanical allodynia (Fig. 1A, B). The repeated administration of pure CBD 10 mg/kg showed a partial effect on both thermal hyperalgesia and mechanical allodynia, while the chronic administration of pure THC (0.62 mg/kg) did not reverse neuropathic pain (Fig. 1A, B). To verify whether the complete relief of thermal hyperalgesia evoked by eCBD treatment could be due to a synergism only between the two main components of the extract (CBD and THC), pure CBD (10 mg/kg) and pure THC (0.62 mg/kg) were mixed together (pCBD + pTHC) and administered to CCI rats following the same scheme of treatment. The data, shown in Table 1, demonstrated that the pharmacological effect evoked by chronic treatment with the association of the two pure compounds was exactly superimposable to that elicited by the chronic treatment with CBD *per se*, clearly indicating that there are other components in the extract necessary for the amelioration of the antihyperalgesia elicited by eCBD compared with pure compounds. The prolonged treatment with eCBD did not affect the response to thermal and mechanical stimuli either of the paw contralateral to the injury or of sham animals (data not shown). The effects of a single administration of eCBD, at the same dose, was assessed in CCI rats treated chronically with vehicle for 6 days and challenged with the drug. Behavioural evaluations were performed at 15, 30, 60, 90, 120, 150, 180 min and 24 h after eCBD administration. The data indicated that the single administration of eCBD reversed thermal hyperalgesia (10.73 ± 0.2963 s vs 10.80 ± 0.1155 s of sham/vehicle) and partially counteracted mechanical allodynia (26.17 ± 3.032 g vs 36.33 ± 1.481 g of sham/vehicle). This effect appeared about 60 min after the administration and completely disappeared 180 min after. A single administration of pure CBD did not alter either thermal nociceptive withdrawal latency or mechanical threshold, at any time (data not shown). On the contrary, pure THC showed an identical time course of antinociception (10.97 ± 0.1453 s vs 10.80 ± 0.1155 s of sham/vehicle; 22.00 ± 2.598 g vs 36.33 ± 1.481 g of sham/vehicle) as after eCBD acute administration.

Effect of CB1, CB2 and TRPV1 receptor antagonists on eCBD-induced antihyperalgesia

The ability of specific CB1, CB2 and TRPV1 antagonists to reverse the effect elicited by eCBD treatment was tested only on thermal hyperalgesia. The antagonism studies were performed on the last day of the chronic treatment with eCBD, 90 min after the coadministration of the drug and the antagonist. The results are shown in Fig. 2 and revealed that neither SR141716 nor SR144528 reversed eCBD-induced antihyperalgesia. On the contrary, capsazepine completely antagonized the effect of eCBD. The antagonists employed, given alone, did not affect the nociceptive response of CCI animals (data not shown).

Table 1. Effect of oral repeated administration of pCBD + pTHC in CCI rats on thermal hyperalgesia and mechanical allodynia

	Sham	CCI				
	Vehicle	Vehicle	CBD	THC	eCBD	pCBD + pTHC
Thermal hyperalgesia	11.1 ± 0.08[a]	5.6 ± 0.12[b]	8.4 ± 0.20[c]	4.9 ± 0.46[b]	10.8 ± 0.21[a]	8.8 ± 0.30[c]
Mechanical allodynia	34.9 ± 1.60[a]	7.9 ± 0.83[b]	20.1 ± 1.59[c]	11.0 ± 1.04[b]	23.2 ± 1.88[c]	16.4 ± 1.25[c]

Data marked by the same letter are not significantly different at $p < 0.05$.

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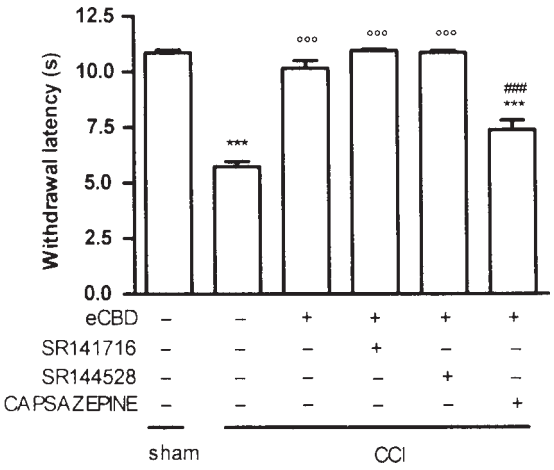

Figure 2. Effect of SR141716 (0.5 mg/kg, i.p.), SR144528 (1 mg/kg, i.p.) and capsazepine (10 mg/kg, i.p.) on eCBD-induced antihyperalgesia in neuropathic rats (CCI), 90 min after the coadministration of drugs and antagonists (on day 13). Withdrawal latency to heat of the injured paws is expressed as s and data represent mean ± SEM of 8–10 rats. *** $p < 0.001$ vs sham/vehicle; °°° $p < 0.001$ vs CCI/vehicle; ### $p < 0.001$ vs CCI/eCBD.

Effect of eCBD treatment on hepatic cytochrome P450 system

At 14 days after the lesion, the total content of cytochrome P450 was measured in liver microsomes. The data revealed no significant differences between sham-operated and CCI rats (Fig. 3A). The repeated treatment with eCBD significantly decreased the cytochrome P450 total content of about 40%, while CBD and THC chronic treatment did not alter the protein content. To assess whether the cytochrome P450 inhibition evoked by eCBD was due to an interaction between CBD and THC, cytochrome P450 levels were determined in the liver of animals treated with pCBD + pTHC. The data showed no significant changes in cytochrome P450 levels (Fig. 3A). The immunoblotting analysis aimed at the evaluation of the cytochrome P450 inducible isoform 2B1/2, revealed a significant inhibition only in the microsomes of rats repeatedly treated with eCBD, as shown by the representative immunoblot in Fig. 3B. The relative densitometric analysis extended to at least four replicates (Fig. 3C), demonstrated a reduction of about 65% of 2B1/2 cytochrome P450 isoform content in the liver of eCBD-treated animal.

Effect of eCBD treatment on P-gp activity

P-gp activity was measured *ex vivo* in the distal region of the intestine of CCI rats chronically treated with eCBD (Fig. 4A). Chronic constriction of the sciatic nerve did not alter P-gp activity. In fact, no significant differences were found in the intestinal P-gp activity of sham-operated and CCI rats. By contrast, eCBD treatment significantly inhibited P-gp activity by about 49%. To better characterize such an effect, an *in vitro* assay of P-gp activity was performed using different concentrations of CBD, the main component of the extract (Fig. 4B). CBD 25 μM was found to be the dose inducing the highest degree of inhibition. For comparison, the *in vitro* assay was also performed using a concentration of eCBD (39 μM) containing CBD 25 μM. The results showed that eCBD induced the same degree of P-gp activity inhibition as the pure CBD.

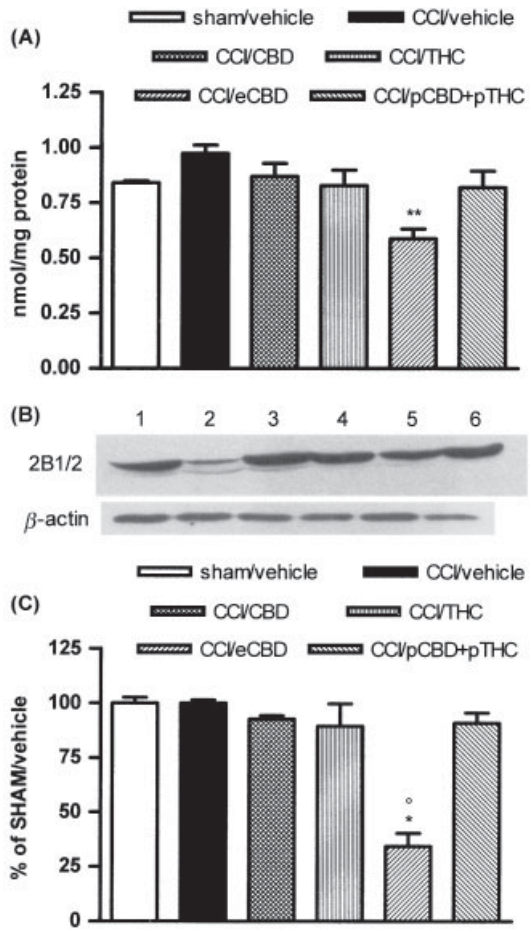

Figure 3. Effect of oral repeated treatment of CBD (10 mg/kg), THC (0.62 mg/kg), eCBD and pCBD (10 mg/kg) + pTHC (0.62 mg/kg) on total hepatic content of cytochrome P450 (A) and on 2B1/2 cytochrome P450 isoform in hepatic microsomal fractions. (A) Data are expressed as nmol/mg protein and represent mean ± SEM of 8–10 rats. ** $p < 0.01$ vs all groups. (B) Representative immunoreactive bands of proteins; each lane was loaded with 40 μg of proteins. Lane 1: sham/vehicle; lane 2: CCI/eCBD; lane 3: CCI/vehicle; lane 4: CCI/CBD; lane 5: CCI/THC; lane 6: CCI/pCBD + pTHC; β-actin is the protein loading control. Relative levels of expression with densitometric analysis of the grey levels using ImageJ software expressed as a percentage of the respective sham/vehicle (C). Each bar represents the mean ± SEM of 2–3 bands. * $p < 0.05$ vs sham/vehicle; ° $p < 0.05$ vs CCI/vehicle.

DISCUSSION

The results reported here supported the idea that the use of a standardized extract of *Cannabis sativa*, containing a large quantity of CBD, the non-psychoactive component of the plant, and a small percentage of THC and other minor cannabic and non-cannabic components, evoked a total relief of thermal hyperalgesia, in an experimental model of neuropathic pain, the chronic constriction injury (CCI) of the rat sciatic nerve, ameliorating the effect of single cannabinoids. In fact, the repeated treatment with only CBD or only THC,

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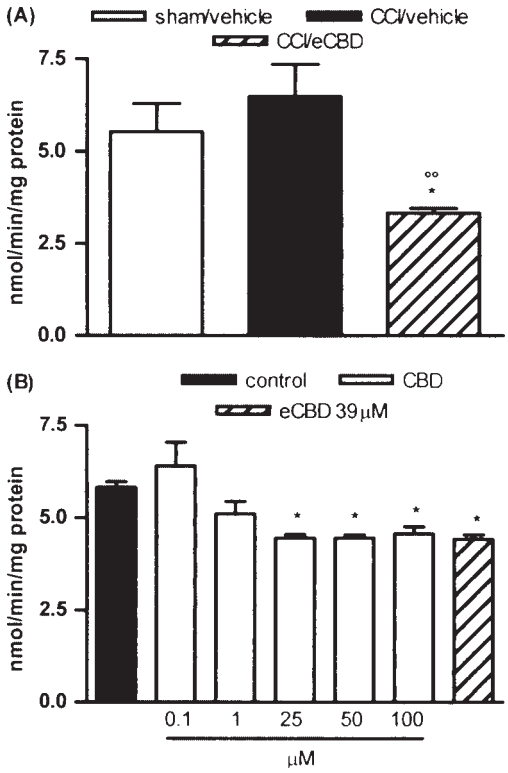

Figure 4. Effect on intestinal glycoprotein-P activity after oral repeated treatment with eCBD measured *ex vivo* (A). Effect of CBD and eCBD on intestinal glycoprotein-P activity evaluated *in vitro* (B). Data represent mean ± SEM of 4–6 rats. * $p < 0.05$ vs sham or control; °° $p < 0.01$ vs CCI/vehicle.

administered at the same dose present in the extract, displayed only a partial effect and a lack of efficacy on nociceptive behaviour, respectively. These data suggested the idea that CBD, THC and perhaps other cannabic and non-cannabic components can act synergistically resulting in the amelioration of pharmacological benefits. The idea that the herbal extract might have a greater effect than its main component, THC, alone was first reported in 1974 when Carlini *et al.*, based on animal and human studies, determined that marihuana produced effects two or four times greater than that expected from the THC content. Then, the presence of unidentified powerful synergism in cannabis extracts causing 330% greater activity in mice than THC alone, has been reported (Fairbairn and Pickens, 1981). In spite of these early studies, further investigations in this area were not reported until a few years ago, when the contribution of CBD, other cannabinoids, terpenes and flavonoids to the clinical cannabis effect has been espoused as an 'entourage effect' by different authors (Mechoulam and Ben-Shabat, 1999; McPartland and Russo, 2001), and, more recently, evidence has been presented indicating a potentiation of THC's antinociceptive effect by a high concentration of CBD (Varvel *et al.*, 2006). The difference between pain responses to the pure cannabinoids and to the extract obtained in our experiments, led to the first consideration that this could be due solely to the combination of CBD and THC in the extract. Therefore, a mixture was tested which did not contain any non-cannabic compounds and additional phytocannabinoids, save THC and CBD. Interestingly, in respect of thermal hyperalgesia relief, the repeated administration to CCI rats of the miscellaneous of CBD and THC at the same doses as present in the extract, was statistically lower than that evoked by the extract, but it was exactly superimposable to that elicited by pure CBD alone. This finding strongly suggests that other constituents present in eCBD, either one alone or several in combination with CBD, account for the greater efficacy of the extract. Surprisingly, CCI rats repeatedly treated with eCBD did not show any amelioration of mechanical allodynia with respect to the treatment with CBD alone. In fact, the partial effect evoked by pure CBD in the Von Frey test was similar to that after eCBD treatment, indicating no potentiation upon mechanical allodynia. We do not have a proven explanation for this, but it might be related to the molecular target of eCBD-induced relief of hyperalgesia, the TRPV1 receptor (as discussed later). In fact, these receptors are mainly expressed on unmyelinated C-fibres, known as polymodal nociceptors, responding to mechanical, thermal and chemical stimuli, whereas relatively few TRPV1 receptors were demonstrated on the thinly myelinated Aδ-fibres that can be activated by intense mechanical stimuli or by noxious heat (Caterina *et al.*, 1997); the Aβ-fibres mediating low threshold afferent mechanical inputs (allodynia) are insensitive to the TRPV1 agonists. When tested in CCI animals after a single treatment, CBD alone was ineffective, while pure THC and eCBD displayed a very similar pattern of antihyperalgesic action. This finding indicates that, although at a very low dose, THC evoked, as expected, an analgesic effect in CCI rats and that the relief of pain after acute eCBD is probably due to its THC content. However, such an effect is short-lasting, because it had disappeared after 3 h. Consequently, the long-lasting (24 h) antihyperalgesia observed in CCI animals after a repeated treatment with eCBD is likely sustained only by CBD. To establish the receptor/s involved in the pharmacological effect provided by eCBD, different cannabinoid and vanilloid receptor antagonists were tested. In fact, both cannabinoid receptor subtypes (CB1 and CB2) are molecular targets for many THC-mediated effects, whereas CBD does not bind to cannabinoid receptors but it displays good affinity for the vanilloid TRPV1 receptor (Bisogno *et al.*, 2001; Costa *et al.*, 2004, 2007). The results demonstrated that the antihyperalgesic effect evoked by eCBD was unaffected by both CB1 and CB2 receptor antagonists, whereas it was completely reversed by capsazepine, a selective antagonist of the vanilloid receptors TRPV1, suggesting that only TRPV1 receptors mediated the antihyperalgesic effect observed (antagonism studies were not performed on the antiallodynic effect because of the partial efficacy). CBD is the only component present at a high level in the extract able to bind to this receptor: thus CBD resulted as the only compound responsible for the antinociceptive behaviour observed. It was previously demonstrated that the CBD antihyperalgesic effect in neuropathic rats was also completely reversed by capsazepine (Costa *et al.*, 2007). It can be hypothesized that CBD, binding to TRPV1 receptors, leads to a desensitization of these receptors, in which the previously excited neurons no longer respond to painful stimuli. These results allow us to exclude that a pharmacodynamic event accounts for the synergism observed. Furthermore, the lack of CB1 activation has to be considered a positive factor in the eCBD efficacy presuming a lack of central side effects due to CB1

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stimulation. Collectively, these findings strongly support the idea that the combination of cannabinoid and non-cannabinoid compounds, as present in eCBD extract, provides significant advantages in the relief of neuropathic pain compared with pure cannabinoids alone, in terms of efficacy and a lack of central effects. The pharmacological contribution of non-cannabinoid compounds to the eCBD effect might be due to the presence of an analgesic (myrcene), some antiinflammatory substances (myrcene, beta-caryophyllene, alpha-pinene) and antioxidants (tocopherol), even if it was not possible to demonstrate whether terpenoid and flavonoid effects were significant.

Phytocannabinoids and endocannabinoids are metabolized by the hepatic cytochrome P450 system (Yamamoto *et al.*, 1995); moreover many studies suggested that cannabinoids are able to modulate this system after acute or repeated treatment. This is particularly described for CBD. In fact, Bornheim and Correia (1989a) showed that acute CBD treatment decreased the mouse hepatic cytochrome P450 content, while repetitive CBD treatment resulted in the induction of cytochrome P450 isozyme indistinguishable from that inducible by phenobarbital, suggesting a 2B family involvement. Similar treatment with THC did not correspondingly result in its induction (Bornheim and Correia, 1989b). However, it was conceivable that THC in combination with CBD could reveal a cytochrome P450 modulation. The results showed that after chronic treatment with eCBD the hepatic total content of cytochrome P450 was strongly inhibited, while neither CBD treatment nor THC alone affected this protein. The western blot analysis performed employing the specific antibody against the 2B1/2 isoform of cytochrome P450 confirmed the data obtained on the total content: a marked inhibition of 2B1/2 protein expression was found only in the liver of eCBD chronically treated rats. The results obtained in the liver of neuropathic rats repeatedly treated with the miscellaneous of pure CBD and pure THC showed a physiological content of cytochrome P450 suggesting that neither CBD nor THC, alone or in combination, are responsible for the inhibition elicited by eCBD. Among the different substances in the extract, terpenes and flavonoids could be those more likely involved in the cytochrome P450 decrease. This hypothesis is corroborated by data reporting that flavonoids modulate the cytochrome P450 activity and inhibit some isoforms (1A, 2E, 3A, 2C) (see Moon *et al.*, 2006 for review). However, the identification of the component/s of eCBD effective in inhibiting the cytochrome P450 requires further investigations. Collectively these findings lead to the hypothesis that the inhibition of hepatic metabolism determined an increased bioavailability of CBD resulting in a greater effect.

Another protein involved in the absorption and disposition of many compounds is the P-glycoprotein. Recently, Zhu *et al.* (2006) demonstrated that CBD enhanced the intracellular accumulation of known P-gp substrates rhodamine123 and doxorubicin in a concentration dependent manner (5–100 μM) in Caco-2 cells and Holland *et al.* (2006) further confirmed these data in a human T lymphoblastoid leukaemia cell line, suggesting an inhibitory effect of CBD on P-gp activity, at least *in vitro*. Our data indicate that the oral administration of eCBD resulted in the inhibition of intestinal P-gp activity. The *in vitro* characterization of such an effect performed on an intestinal crude membrane preparation confirmed the inhibitory effect of the eCBD and highlighted that the only substance present in the extract able to evoke the same degree of inhibition at the same dose is CBD. This result clearly indicates that the P-gp modulation is not the mechanism underlying the synergism observed but opens the important question about the possibility that CBD and eCBD orally administered could potentially influence the absorption of other co-administered compounds that are P-gp substrates.

In the perspective of a possible future therapeutic employment of the *Cannabis sativa* extract, it will be crucial to characterize the compound mediating the inhibition of the cytochrome P450. In the light of the well known antioxidant and antiinflammatory properties of terpenes and flavonoids which could significantly contribute to the therapeutic effects, it cannot be excluded that the synergism observed might be achieved also in the absence of the cytochrome P450 inhibition. Further studies of cannabis-based medicines in neuropathic pain are now required to demonstrate a clinically relevant improvement in the treatment of this condition.

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