

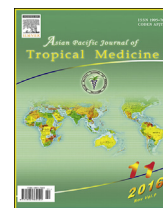
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## Acetylcholinesterase, butyrylcholinesterase and paraoxonase 1 activities in rats treated with cannabis, tramadol or both

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## ABSTRACT

**Objective:** To investigate the effect of *Cannabis sativa* resin and/or tramadol, two commonly drugs of abuse on acetylcholinesterase and butyrylcholinesterase activities as a possible cholinergic biomarkers of neurotoxicity induced by these agents.**Methods:** Rats were treated with cannabis resin (5, 10 or 20 mg/kg) (equivalent to the active constituent  $\Delta^9$ -tetrahydrocannabinol), tramadol (5, 10 and 20 mg/kg) or tramadol (10 mg/kg) combined with cannabis resin (5, 10 and 20 mg/kg) subcutaneously daily for 6 weeks. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities were measured in brain and serum. We also measured the activity of paraoxonase-1 (PON1) in serum of rats treated with these agents.**Results:** (i) AChE activity in brain increased after 10–20 mg/kg cannabis resin (by 16.3–36.5%). AChE activity in brain did not change after treatment with 5–20 mg/kg tramadol. The administration of both cannabis resin (5, 10 or 20 mg/kg) and tramadol (10 mg/kg) resulted in decreased brain AChE activity by 14.1%, 12.9% and 13.6%, respectively; (ii) BChE activity in serum was markedly and dose-dependently inhibited by cannabis resin (by 60.9–76.9%). BChE activity also decreased by 17.6–36.5% by 10–20 mg/kg tramadol and by 57.2–63.9% by the cannabis resin/tramadol combined treatment; (iii) Cannabis resin at doses of 20 mg/kg increased serum PON1 activity by 25.7%. In contrast, tramadol given at 5, 10 and 20 mg/kg resulted in a dose-dependent decrease in serum PON1 activity by 19%, 36.7%, and 46.1%, respectively. Meanwhile, treatment with cannabis resin plus tramadol resulted in 40.2%, 35.8%, 30.7% inhibition of PON1 activity compared to the saline group.**Conclusions:** These data suggest that cannabis resin exerts different effects on AChE and BChE activities which could contribute to the memory problems and the decline in cognitive function in chronic users.

## 1. Introduction

*Cannabis sativa* L (family Cannabaceae) (*C. sativa*) has remained the most widely used and abused drug worldwide [1]. The two most common cannabis preparations are marijuana

which is the dried flowing tops and leaves of the female plants and hashish which is the compressed resin. Cannabis has long been used through the history of mankind for its recreational properties. Cannabis consumers often report the subjective feeling of “being high”, euphoria, altered time perception and increased sensual awareness. With long-term cannabis appears to impair several cognitive functions [2,3]. There is impairment of short-term and working memory that might persist for variable time after abstinence from cannabis [4,5]. There is also aggravation of pre-existing psychosis or even a likeness of developing psychosis in cannabis users [4]. Brain MRI scans indicate structural changes in

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humans with a history of long-term [6] and heavy cannabis abuse while animal models shows neuronal degeneration [7,8] despite neuroprotection against excitotoxic brain injury (glutamate-induced death) being reported [9].

Only recently and with the identification of the cannabinoid receptors and their endogenous ligands, the biological action of cannabis beings to be delineated. The C21 terpenophenolic cannabinoids are the unique constituents of the *C. sativa* plant of which the principal psychoactive ingredient is  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC). Other cannabinoids such as cannabidiol, cannabivarin, cannabichromene, and cannabigerol are devoid of psychotropic action and might even antagonize some of the pharmacological effects of  $\Delta^9$ -THC. This and other cannabinoids exist as their carboxylic acids and are converted (decarboxylated) into their corresponding phenols upon heating [10,11]. Cannabinoids or their endogenous ligands bind to cannabinoid receptors CB1 and CB2 with the former being predominantly expressed in the brain and spinal cord and thus mediates most of the effects of cannabis on the central nervous system. On the other hand, the CB2 receptor is mainly expressed on the surface of the immune cells in the periphery [12].

Tramadol is a frequently prescribed centrally acting analgesic with  $\mu$ -opioid receptor agonist properties. It also inhibits the reuptake of serotonin, and noradrenaline in the brain [13]. It is used to treat acute pain and of moderate to moderately severe chronic pain resulting from musculoskeletal disorders or that due to cancer [14]. The drug is becoming increasingly popular in several countries as a drug of misuse [15–17]. Subjects taking 675 mg or more of tramadol for 5 years or more exhibited an increase in comorbid anxiety, depressive, and obsessive-compulsive symptoms [17]. There is also an evidence of memory impairing action for tramadol [18]. Cannabis users are more likely to report use of other illicit drugs [20] including tramadol [19].

In brain, central cholinergic neurotransmission is crucial for cognitive functions including learning and memory formation [21]. Inhibitors of brain acetylcholinesterase such as donepezil and rivastigmine are the drugs being used to treat the cognitive decline due to aging or Alzheimer's disease by increasing extracellular acetylcholine, the signaling neurotransmitter of the cholinergic system [22]. Changes in central cholinergic activity thus will have an important impact on cognitive functions [21]. The aim of this study was therefore to investigate the effect of cannabis and/or tramadol on the activities of brain acetylcholinesterase and plasma butyrylcholinesterase, the enzymes involved in the hydrolysis of the acetylcholine [23]. We in addition measured the activity of paraoxonase-1 (PON1) in serum of rats treated with cannabis and/or tramadol. The PON1 enzyme is involved in the detoxification of several organophosphorus compounds and many other xenobiotics and changes in its activity have been associated with a number of neurologic disorders [24,25].

## 2. Materials and methods

### 2.1. Animals

Male Sprague–Dawley rats, obtained from Animal House of the National Research Centre, Cairo, weighing between 130 and 140 g were group-housed under temperature- and light-controlled conditions with standard laboratory rodent chow and water provided *ad libitum*. Animal procedures were performed in accordance with the Ethics Committee of the National

Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

### 2.2. Drugs and chemicals

*C. sativa* resin (Hashish) and tramadol were kindly provided by the Laboratory of Forensic Sciences of Ministry of Justice (Cairo, Egypt). Other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

### 2.3. Preparation of cannabis resin extract

Cannabis resin extract was prepared from the dried resin of *C. sativa*. The extraction was performed using chloroform according to the method of Turner and Mahlberg [26] with modification. In brief, 10 g of the resin was grounded in a mortar, subjected to oven heat (100 °C) for 1 h to decarboxylate all its cannabinolic acids content. The resin was extracted in chloroform overnight and filtered. The filtrate was evaporated under a gentle stream of nitrogen and stored at 4 °C and protected from light in an aluminum-covered container. 1 g of the residue (dry extract) was suspended in 2% ethanol-saline.  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC) content was quantified using gas chromatography–mass spectrometry (GC–MS). The resin contained ~20%  $\Delta^9$ -THC and 3% cannabidiol.

### 2.4. Study design

Rats were treated with *C. sativa* resin extract at 5, 10 or 20 mg/kg (expressed as  $\Delta^9$ -tetrahydrocannabinol), tramadol at 5, 10 or 20 mg/kg or tramadol (10 mg/kg) in combination with *C. sativa* resin (5, 10 or 20 mg/kg) subcutaneously daily for 6 weeks. Rats were randomly divided into ten groups, six rats each. Group 1 received the vehicle (0.2 mL saline) daily. Group 2, 3, 4 received *C. sativa* resin at the doses of 5, 10 and 20 mg/kg, subcutaneously daily. Groups 5, 6, 7 received tramadol at doses of 5, 10 and 20 mg/kg subcutaneously daily. Groups 8, 9, 10 received tramadol at 10 mg/kg in combination with *C. sativa* resin (5, 10 or 20 mg/kg, subcutaneously daily). Rats were then euthanized by decapitation under ether anesthesia for tissue collection. The brain of each rat was rapidly dissected and snap-frozen in liquid nitrogen. Tissue samples were stored at –80 °C until further processing. Frozen samples were thawed and homogenized in a glass tube with a Teflon dounce pestle in ice-cold phosphate buffer solution (50 mM Tris–HCl, pH 7.4) and sonicated. Homogenized samples were then centrifuged at 9 000 g for 5 min at 4 °C. The supernatant was stored at –80 °C until further analysis.

### 2.5. Determination of acetylcholinesterase activity

The procedure used was a modification of the method of Ellman *et al.* [27] as described by Gorun *et al.* [28]. The principle of the method is the measurement of the thiocholine produced as acetylthiocholine is hydrolyzed. The color was read immediately at 412 nm.

### 2.6. Determination of butyrylcholinesterase activity

Butyrylcholinesterase activity was measured spectrophotometrically using commercially available kit (Ben Biochemical

Enterprise, Milan, Italy). In this assay, cholinesterase catalyzes the hydrolysis of butyrylthiocholine, forming butyrate and thiocholine. The thiocholine reacts with dithiobis-nitrobenzoic acid (DTNB) forming a colored compound. The increase in absorbance in the unit time at 405 nm is proportional to the activity of the cholinesterase in the sample.

### 2.7. Determination of paraoxonase activity

Arylesterase activity of paraoxonase was measured spectrophotometrically using phenylacetate as a substrate [29,30]. In this assay, arylesterase/paraoxonase catalyzes the cleavage of phenyl acetate resulting in phenol formation. The rate of formation of phenol is measured by monitoring the increase in absorbance at 270 nm at 25 °C. The working reagent consisted of 20 mM Tris/HCl buffer, pH 8.0, containing 1 mM CaCl<sub>2</sub> and 4 mM phenyl acetate as the substrate. Samples diluted 1:3 in buffer are added and the change in absorbance is recorded following a 20 s lag time. Absorbance at 270 nm was taken every 15 s for 120 s. One unit of arylesterase activity is equal to 1 μM of phenol formed per minute. The activity is expressed in kU/L, based on the extinction coefficient of phenol of 1310 m/cm at 270 nm, pH 8.0 and 25 °C. Blank samples containing water are used to correct for the spontaneous hydrolysis of phenylacetate.

### 2.8. Statistical analysis

Data are expressed as mean ± SE. Data were analyzed by one-way analysis of variance, followed by Duncan's multiple range test for *post hoc* comparison of group means. Effects with a probability of  $P < 0.05$  were considered to be significant.

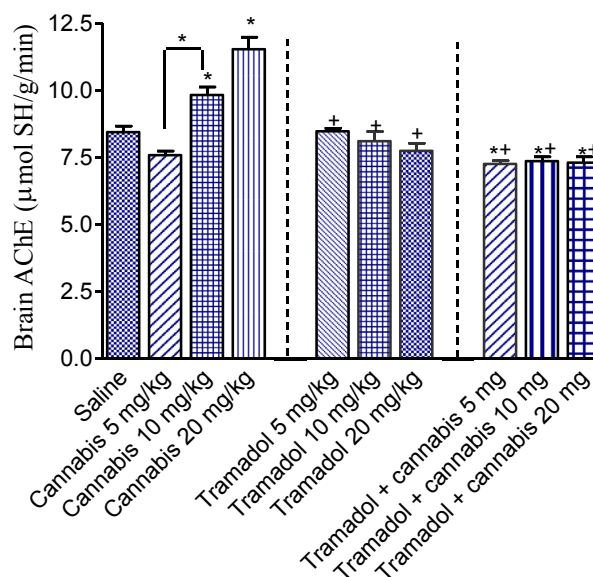
## 3. Results

### 3.1. Acetylcholinesterase activity

Significant increase in brain AChE activity by 16.3% and 36.5% was observed in rats treated with 10–20 mg/kg cannabis resin alone compared to the saline control group [(9.84 ± 0.31) μmol SH/g/min, (11.55 ± 0.45) μmol SH/g/min vs. (8.46 ± 0.22) μmol SH/g/min]. No significant change in AChE activity was found after tramadol. The combined administration of cannabis resin/tramadol was, however, associated with significant decrease in brain AChE activity by 14.1%, 12.9%, and 13.5%, respectively [(7.27 ± 0.22) μmol SH/g/min, (7.37 ± 0.26) μmol SH/g/min, (7.32 ± 0.300) μmol SH/g/min vs. (8.46 ± 0.22) μmol SH/g/min] (Figure 1).

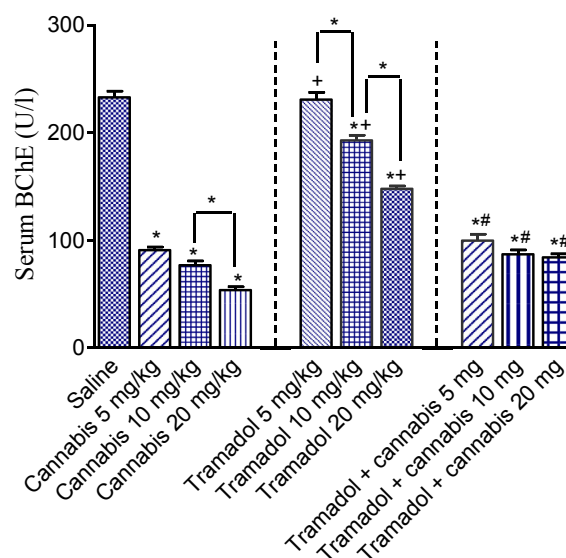
### 3.2. Butyrylcholinesterase activity

Cannabis resin alone at a dose of 5, 10 and 20 mg/kg caused significant inhibition in serum BChE activity by 60.9%, 67.0% and 76.9% compared to the saline control group [(90.91 ± 2.80) U/L, (76.73 ± 4.20) U/L, (53.76 ± 3.10) U/L vs. (232.72 ± 5.90) U/L] (Figure 2). Serum BChE activity was also significantly decreased by 10–20 mg/kg tramadol (17.2% and 36.5% decrease: [(192.71 ± 4.90) U/L, (147.74 ± 2.60) U/L vs. (232.72 ± 5.90) U/L]) and following treatment with both cannabis resin and tramadol (57.2%, 62.6%, and 63.9% decrease: [(99.70 ± 6.00) U/L, (87.00 ± 4.10) U/L, (84.10 ± 3.30) U/L vs. (232.72 ± 5.90) U/L]).



**Figure 1.** Brain acetylcholinesterase (AChE) activity in rats treated with cannabis resin, tramadol or both.

\* $P < 0.05$  vs. saline group and between different groups as indicated in the figure. + $P < 0.05$  vs. only cannabis resin at 10 or 20 mg/kg.

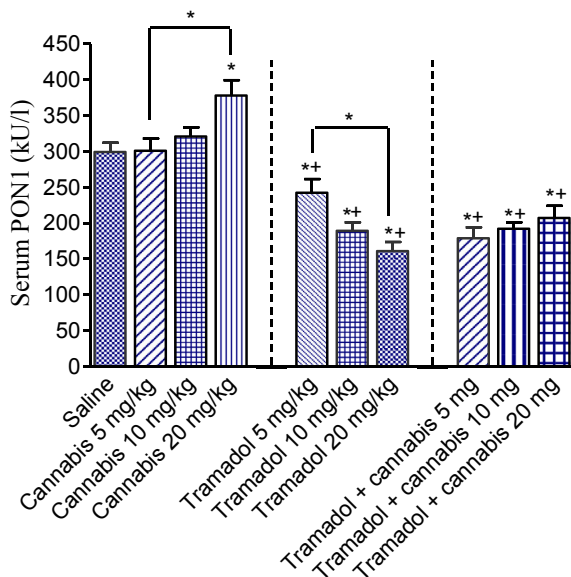


**Figure 2.** Serum butyrylcholinesterase (BChE) activity in rats treated with cannabis resin, tramadol or both.

\* $P < 0.05$  vs. saline group and between different groups as indicated in the figure. + $P < 0.05$  vs. only cannabis resin. # $P < 0.05$  vs. only tramadol.

### 3.3. Paraoxonase 1 activity

In serum, a significant and marked increase in PON1 activity was observed after treatment with 20 mg/kg of cannabis resin compared to the saline group by 25.7%, [(76.1 ± 21.2) vs. (299.2 ± 13.0) kU/L] (Figure 3). A significant decrease in serum PON1 activity by 19.0%, 36.7%, and 46.1% was observed after the administration of 5, 10 and 20 mg/kg of tramadol, respectively [(242.4 ± 19.1) kU/L, (189.3 ± 11.9) kU/L, (161.1 ± 12.7) kU/L vs. (299.2 ± 13.0) kU/L]. Meanwhile, treatment with cannabis resin plus tramadol resulted in decreased PON1 activity by 40.2%, 35.8%, 30.7% compared to the saline group [(179.0 ± 15.4) kU/L, (182.2 ± 8.8) kU/L, (207.4 ± 16.9) kU/L vs. (299.2 ± 13.0) kU/L].



**Figure 3.** Serum paraoxonase-1 (PON1) activity in rats treated with cannabis resin, tramadol or both.

\* $P < 0.05$  vs. saline group and between different groups as indicated in the figure. \*\* $P < 0.05$  vs. only cannabis resin at 10 or 20 mg/kg.

#### 4. Discussion

In the current study, we show that treating rats with a cannabis resin extract rich in delta <sup>9</sup>-THC resulted in an increase in brain acetylcholinesterase (AChE) activity and at the same time caused marked inhibition of serum butyrylcholinesterase (BChE) activity. The serine esterases AChE (EC 3.1.1.7.) and BChE (EC 3.1.1.8.) can catalyze the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid. The enzyme AChE is present in brain, autonomic ganglia, skeletal muscle end-plate and in erythrocyte membrane. AChE hydrolyzes acetylcholine faster than the other choline esters. It is the key enzyme which terminates the action of ACh at cholinergic synapses and is highly efficient in modulating the levels of extracellular acetylcholine and in regulating cholinergic neurotransmission [31,32]. It is not surprising therefore that inhibitors of AChE e.g., donepezil are used for boosting residual cholinergic activity in Alzheimer's disease [33]. In this neurodegenerative disorder, loss of cholinergic innervations and deficits in cholinergic neurotransmission underlie the cognitive deterioration involving memory, language and higher executive functioning [21].

It follows that the increase in brain AChE activity by the cannabis resin observed in this study would result in decreased brain levels of ACh, which could explain at least in part, the cognitive and memory deficits in heavy users of cannabis. In this context, several studies have shown inhibition in extracellular acetylcholine concentration in several brain areas following *i.p.* injection of THC (3–6 mg/kg), the main active ingredient in cannabis [34–36]. In cortical, hypothalamic and striatal rat brain slices, both  $\Delta^8$ -THC and  $\Delta^9$ -THC were found to inhibit the synthesis of 3H-ACh with  $\Delta^8$ -THC being twice as effective as  $\Delta^9$ -THC. Meanwhile, the non-psychoactive cannabidiol did not alter ACh synthesis [37]. In rat hippocampus, inhibition of ACh release also followed *i.p.* injection of synthetic cannabinoids, WIN 55,212-2 (5.0 and 10 mg/kg *i.p.*) and CP 55,94 (0.5 and 1.0 mg/kg *i.p.*) with the effect being blocked by the CB1

antagonist SR 141716A [38]. On the other hand, very low doses of  $\Delta^9$ -THC (10–150  $\mu\text{g}/\text{kg}$ ) or the cannabinoid CB1 receptor agonists WIN 55,212-2 (10–150  $\mu\text{g}/\text{kg}$ ) and HU 210 (1 and 4  $\mu\text{g}/\text{kg}$ ) given intravenously to freely moving rats increased cortical and hippocampal acetylcholine release [39,40].

The chemistry of herbal cannabis, however, is complex, as there are over 70 different cannabinoids identified so far [10]. Some cannabinoids potentiate whilst others e.g., cannabidiol antagonize some of the pharmacological actions of the principal and psychotropic one i.e.,  $\Delta^9$ -THC [41]. Cannabis is not merely cannabinoids and contains several terpenoids and among these  $\alpha$ -Pinene, a bicyclic monoterpene is an AChE inhibitor [42]. Clearly, the net effect will therefore depend on the relative contribution of different cannabis components and it is possible that in high potency cannabis with high content of  $\Delta^9$ -THC, the action of the latter will prevail/predominate.

Butyrylcholinesterase (EC 3.1.1.8; BChE) also known as pseudocholinesterase or plasma cholinesterase is expressed in many tissues but is found primarily in the liver and plasma. The enzyme differs from AChE in substrate specificity. BChE, preferentially hydrolyzes butyrylcholine and also acetylcholine [23,43]. The exact physiological function of BChE is not understood. It can hydrolyze heroin and might function as a detoxifying enzyme for natural compounds. The genetic deficiency of this enzyme in humans results in no apparent physiological consequences [23]. The role of BChE in cholinergic neurotransmission is also much less clear compared with that of AChE. Recent evidence from rodent experiments, however, suggests that, brain-targeted BChE inhibitors elevates extracellular ACh levels and improve the cognitive performance of aged rats [44]. The findings in the present study indicates that the cannabis resin extract exerted marked inhibitory effect on serum BChE activity. It is not clear whether the potent inhibition of BChE activity by the cannabis resin will be reflected in decreased degradation of brain ACh. This is because the hydrolysis of ACh is carried out mainly by AChE, with a minor role for BChE if any [23,43]. Moreover and as shown in this study, brain AChE activity increased by the cannabis resin which will lead to decreased brain ACh availability.

In the present study, we also demonstrated that the repeated administration of cannabis resin extract increased paraoxonase-1 (PON1) activity in serum. This enzyme belongs to the paraoxonase family which also comprises PON2 and PON3 isoforms. Paraoxonase 1 is a calcium-dependent esterase that hydrolyzes the active metabolites (oxons) of several organophosphorus insecticides including parathion, chlorpyrifos and diazinon and an individual's PON1 status determines the sensitivity to these chemicals. Paraoxonase 1 also hydrolyzes aromatic esters such as phenyl acetate (arylesterase activity) and a variety of aromatic and aliphatic lactones (lactonase activity). It is synthesized in the liver and released into blood where it binds to high density lipoproteins and prevents their oxidation [24,30]. The activity of PON1 decreases in patients with Alzheimer's disease and other dementias [45], and autism [46]. PON1 possesses an antioxidant role [24,30] and is inactivated by increased oxidative stress [47,48] which might explain the decrease in enzyme activity in patients suffering from these neurologic disorders.

The present study also investigated the ability of tramadol, an opiate like analgesic with abuse liability and addictive properties [16,19,49] on the activity of AChE, BChE and PON1. In contrast

to the effect of the cannabis resin extract, we found that tramadol did not alter AChE activity in the rat brain. When combined with tramadol, cannabis, however, did not increase brain AChE activity, thereby, suggesting a modulatory action for tramadol on the cannabis-induced increase in AChE activity. In contrast, BChE activity was inhibited by tramadol doses of 10 and 20 mg/kg although to much less extent compared with the cannabis resin. Serum BChE activity might thus serve as a marker in those who abuse tramadol. Meanwhile, there was no additive effect for the combination of cannabis-tramadol in inhibiting BChE activity. PON1 activity, however, was markedly inhibited by tramadol and also by cannabis-tramadol. This finding is important in view of the evidence that the catalytic efficiency of PON1 determines the severity of toxicity following exposure to some of the organophosphorus compounds [50,51]. Thus patients on tramadol might be susceptible to some of the consequences of pesticide exposure and among these lies the risk for developing neurodegenerative disorders like Parkinson's disease or Alzheimer's disease [25].

In summary, the findings of the present study suggest a modulatory effect for cannabis resin extract on the activities of AChE and BChE. This action of cannabis is likely to contribute to the memory deficits and the decline in cognitive function observed in chronic users. The study also demonstrates an inhibitory effect for tramadol on BChE and PON1 activities. It is suggested that the changes in the activities of both enzymes could be a marker for the drug-induced neurotoxicity.

### Conflict of interest statement

The authors declare that there are no competing conflicts of interest.

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