

Original Article

Acetylcholinesterase Inhibitory Activity of Standardized Cannabinoids-rich Fractions



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ABSTRACT

Background: Since cannabis has been legally allowed for medicinal purposes in many countries, it has become the most interesting issue, particularly in neurologic disorders, such as Alzheimer's disease (AD). Inhibition of acetylcholinesterase (AChE) is one of the mechanisms for the treatment of AD.

Objectives: The present study aimed to establish a method for the preparation of cannabinoid-rich extracts and determine their AChE inhibitory activity.

Methods: The cannabinoid-rich extracts were prepared through a green extraction process using microwave-assisted extraction (MAE) followed by hydrophobic column separation. The contents of cannabidiol (CBD) and Δ-9-tetrahydrocannabinol (THC) were determined using high-performance liquid chromatography (HPLC). In vitro AChE inhibitory activity was determined via the photometric method using AChE from *Electrophorus electricus*.

Results: Three cannabinoids-rich fractions were obtained with different concentrations of CBD and THC, namely Fractions I (CBD of 8.1% w/w; THC of 52.2% w/w), II (CBD of 9.2% w/w; THC of 8.0% w/w), and III (CBD 1.3% w/w, THC 33.5% w/w). These cannabinoid-rich extracts exhibited AChE inhibitory activity, with IC₅₀ values of 52.3, 59.8, and 71.2 μg/mL, respectively.

Conclusion: This finding suggests that CBD, but not THC, might be an active compound contributing to AChE inhibitory effect.

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Introduction

Alzheimer's disease (AD), one of the most common forms of dementia, is a brain disorder that is manifested in disruption of thinking skills, memory impairment as well as disorder of language and visuospatial skills. The global prevalence of dementia is expected to increase gradually and may reach 96 million cases in 2050 [1]. In 2020, more than 5 million elderly Americans (>65 years old) had AD and this trend is projected to increase to nearly 14 million cases in the next 40 years. Death rates for AD are also increasing; recently, AD was reported as the sixth leading cause of death among US adults and the fifth leading cause of death among adults aged more than 65 years old [2-4].

The underlying pathophysiology of AD is an accumulation of abnormal neuritic plaques or β -amyloid plaques and neurofibrillary tangles that lead to the loss of function of cholinergic neurons in the brain and neurotransmission process. These processes trigger cognitive impairment in patients [5]. Acetylcholinesterase (AChE) is a cholinergic enzyme that is necessary for terminating neuronal transmission and signaling between synapses to restrict the activation of ACh with the specific receptor [6]. In AD patients, AChE level is increased around β -amyloid plaques; therefore, it may play an important role in β -amyloid fibrillogenesis. Importantly, the inhibition of AChE leads to the accumulation of acetylcholine. Elevated levels of AChE result in increased stimulation of muscarinic and nicotinic receptors, which leads to improvement in some clinical outcomes in AD [7, 8]. Recently, various AChE inhibitors have been discovered and are being used, such as donepezil, galantamine, and rivastigmine. However, there are some limitations in the use of these medications, especially with regard to efficacy as well as an adverse effect when used at a high dose [9].

After the recent announcement of cannabis legalization for medicinal purposes in many countries, medicinal cannabis has gained much attention, particularly in neurologic disorders. The most promising active compounds in *Cannabis sativa* L. are cannabinoids. The two major cannabinoids in *C. sativa* are CBD and THC. Both compounds have been reported to possess diverse pharmacological activities [10]. Cannabis extracts and/or cannabis oils with high content of cannabinoids are increasingly used as alternative treatments for several neurological disorders due to their interactions with the endocannabinoid system through many similar signaling pathways of neurodegenerative conditions [11]. However, the exact

beneficial roles of pure cannabinoids as well as cannabis extract in AD patients are still emerging.

Although several conventional extraction methods, including maceration, percolation, heat reflux, and Soxhlet extraction, used for producing cannabis extracts or cannabis oils are available, these methods possess some disadvantages, such as the need for long extraction time and a large volume of solvent, which some of them are hazardous. Moreover, the heat-accelerated methods still need high temperatures during a long period of extraction leading to the loss of thermally labile compounds. Recently, Microwave-assisted extraction (MAE) has been popularized as a green extraction method for various phytochemicals due to its key advantages, such as less time, solvent, and energy consumption for extraction as well as high phytochemical content in herbal extracts [12, 13]. Therefore, the present study aimed to establish a green MAE method for the preparation of cannabinoid-rich extracts containing different concentrations of CBD and THC and evaluate their role in the modulation of the AChE inhibitory effect. The obtained finding may be relevant to the proper use of cannabinoid extracts in AD.

Materials and Methods

Chemicals and materials

Cannabidiol (CBD) was purchased from Chemfaces (Wuhan, China). Acetylcholine iodide, acetylcholinesterase enzyme from electric eel (*Electrophorus electricus*), 5,5'-dithiobis (2-nitrobenzoic acid), and Trizma® base were obtained from Sigma Aldrich (Singapore), while Silica gel 60 RP-18 F_{254s} plate was from Merck Supelco Sigma-Aldrich (Mainz, Germany). Methanol (analytical and HPLC grades), ethanol, diethyl ether, chloroform, and hydrochloric acid were obtained from RCI Labscan (Bangkok, Thailand). Silica gel (60 Å, 60-200 μ m) was purchased from Vertical Chromatography (Nonthaburi, Thailand), while Diaion® HP-20 was obtained from Mitsubishi Chemical Corporation (Tokyo, Japan). A Luna® C-18 column was purchased from Phenomenex (Bangkok, Thailand). A dried cannabis sample was obtained from the Narcotics Control Board, Bangkok, Thailand.

Preparation of cannabinoids-rich extract

Dried cannabis (Figure 1) was obtained from the Faculty of Natural Resources, Prince of Songkla University, and its voucher specimens (No. SKP 036 03 19 01) were kept at the herbarium of the Faculty of Pharmaceutical



Figure 1. Voucher specimens of cannabis (*Cannabis sativa L.*)

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Sciences, [Prince of Songkla University](#), Hat Yai, Thailand. Dried cannabis (920 g) was reduced to powder using a grinding machine (Retsch, Germany) and the powder was sieved through a No. 4 mesh to produce a moderately fine powder (780 g). The cannabis powder (750 g) was extracted with ethanol (1.5 L) using MAE (900 W) at 63°C for 9 minutes. After filtration, the solvent was evaporated using a rotary vacuum evaporator (Heidolph, Germany) to obtain a dark brown oily extract (106 g) from the filtrate.

The extract (10 g) was chromatographed on a 500 g Diaion® HP-20 column with gradient elution from 50% ethanol to 95% ethanol. Based on TLC analysis, the fractions containing CBD and THC were pooled to produce three cannabinoid-rich extracts, namely Fractions I, II, and III.

Purification of Δ -9-tetrahydrocannabinol

Δ -9-tetrahydrocannabinol (THC) was purified from the cannabinoid extract (2.6 g) using a silica gel column with a step-gradient elution of 2% and 10% diethyl ether in hexane, respectively. Fractions (20 mL) were collected. Based on thin-layer chromatographic (TLC) analysis, fractions 80-83 were the main fractions containing THC. They were pooled, the solvent was evaporated, and the sample was subsequently subjected to a silica

gel column eluted with 40% dichloromethane in hexane. Fractions of 20 mL were collected. The pooled fractions 90-135 were dried and re-chromatographed on a silica gel column eluted with 10% diethyl ether in hexane. Fractions (20 mL) of eluent were collected. Pure THC (90 mg) was obtained from pooled fractions 10-11. The identity of THC was ascertained by ¹H and ¹³C nuclear magnetic resonance (NMR) analysis at 500 MHz using the Ascend™ NMR spectrometer (Bruker/Avance NEO, Switzerland).

TLC method

Standard compounds (CBD and THC) and samples were dissolved in methanol. A reversed-phase TLC technique was used for the detection of CBD and THC. The silica gel 60 RP-18 F_{254s} plate was developed in methanol-water (85:15, v/v). Detection of CBD and THC was performed under UV 254 nm and sprayed with 10% sulfuric acid in methanol. The R_f values of CBD and THC were 0.44, and 0.27, respectively.

Quantitative HPLC method

Quantitative HPLC analysis of CBD and THC was performed using the method previously described by Saingam and Sakunpak [14], with some modifications. Separation was achieved isocratically on a reverse-phase Luna® C-18 column (4.6 mm×250 mm, 5 μm) equipped

with a photodiode-array detector and autosampler (Shimadzu, Japan). The mobile phase consisted of methanol and water (85:15, v/v) and was eluted at a flow rate of 1 mL/min. HPLC chromatogram was determined using a UV-vis detector, at a wavelength of 220 nm. Limits of detection and quantification for THC were 0.12 and 0.40 µg/mL, and for CBD were 0.23 and 0.76 µg/mL, respectively.

Calibration curves of CBD and THC were established using authentic CBD and THC. A working solution of CBD and THC was diluted with methanol to produce six concentrations (6.25, 12.5, 25, 50, 100, and 200 µg/mL). The calibration curves were plotted between concentrations and peak area followed by linear regression analysis. In this study, the calibration curves of CBD and THC were $Y=72615X+72146$ ($R^2=0.9998$) and $Y=54467X+77267$ ($R^2=0.9999$), respectively.

Analyzed samples were prepared by accurately weighing the extracts (2.0 mg) and diluting them with methanol to 10 mL in a volumetric flask. The sample solutions were filtrated through a 0.45 µm filter prior to HPLC analysis.

Acetylcholinesterase inhibitory assay

Acetylcholinesterase inhibitory activity was evaluated using a photometric method modified from Ellman [15]. The incubation mixtures for the assay composed of 3 mM 5,5'-dithiobis (2-nitrobenzoic acid) (125 µL), 1.5 mM acetylcholine iodide (25 µL), 50 mM TRIS-HCl buf-

fer pH 8.0 (50 µL), sample solution in ethanol (25 µL), and AChE enzyme (25 µL) in a 96-well plate. The absorbance of the solutions was measured at 405 nm every 11 seconds for 2 minutes using a PowerWaveX microplate reader (Biotek, USA). AChE inhibitory activity was analyzed using Prism Software and expressed as percentage inhibition and half maximal inhibitory concentration (IC_{50}). Galantamine was used as a positive control. The experiment was performed in triplicate.

Statistical analysis

The results are expressed as Mean±SD. Statistical significance was calculated using a one-way analysis of variance (ANOVA) followed by Tukey's multiple range test ($P<0.05$).

Results

Purification of THC

THC was purified from cannabis extract through three successive silica gel columns. The purity and identity of the purified compound were determined based on the HPLC method and revealed a single peak on the HPLC chromatogram, with a similar UV absorption spectrum (Figure 2) as the previous report for THC [16]. The chemical structure of the obtained compound was determined using 1H and ^{13}C NMR data (Table 1) and compared with the previous report for THC [17].

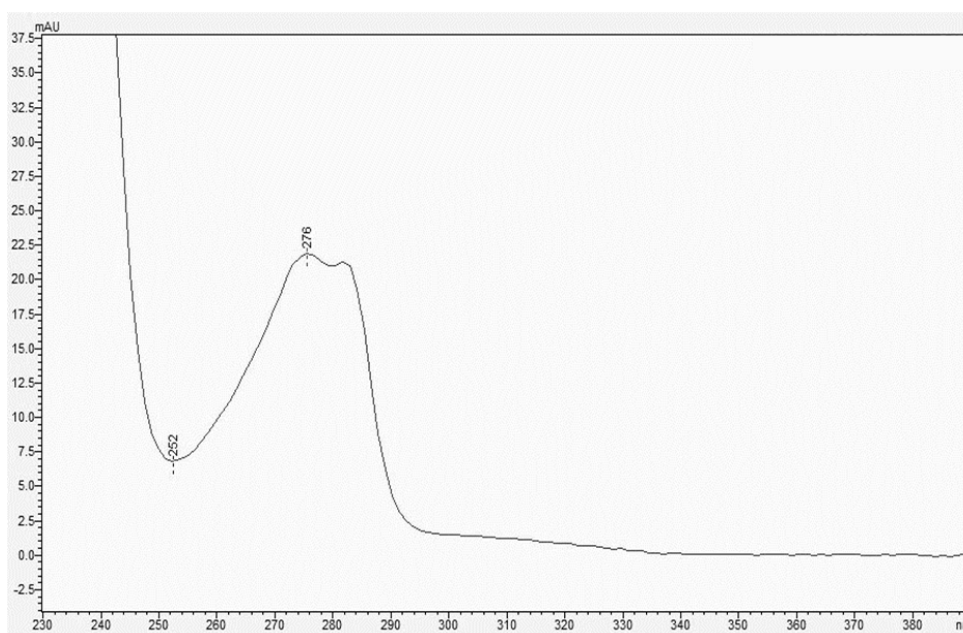


Figure 2. The UV absorption spectrum of the purified Δ -9-THC

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Table 1. ¹H- and ¹³C-NMR assignments for the purified Δ-9-THC

Chemical shifts (in ppm)		
Positions	¹ H-NMR ^a	¹³ C-NMR ^b
1	3.21-3.24(m, 1H)	33.6
2	6.32-6.33(m, 1H)	123.7
3	-	134.4
3-Me	1.68(s, 3H)	23.4
4	2.20(m, 2H)	31.2
5	1.91-1.95(m, 1H), 1.42 (m)	25.0
6	1.55-1.64(m, 1H)	45.8
7	-	76.7
8	1.43(s, 3H)	27.6
9	1.12(s, 3H)	19.3
1'	-	110.1
2'	-	154.8
3'	6.16(d, 1H)	107.5
4'	-	142.8
5'	6.29(d, 1H)	109.0
6'	-	154.2
1''	2.42-2.48(td, 2H)	35.5
2''	1.55-1.61(m, 2H)	30.7
3''	1.26-1.37(m, 2H) ^c	31.5
4''	1.26-1.37(m, 2H) ^c	22.5
5''	0.89-0.92(m, 3H)	14.0
2-OH	4.82(s, 1H)	-

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a: Spectra determined at 500 MHz; b: Spectra determined at 125 MHz; c: Chemical shifts bearing the same symbol overlap.

Preparation of cannabinoids-rich extracts

The cannabinoid-rich extracts were prepared using a green microwave extraction followed by a simple fractionation on a hydrophobic column. Three fractions with different concentrations of CBD and THC, namely Fraction I, II, and III were obtained. Based on HPLC analysis, CBD and THC were eluted at 5.0 and 10.1 min, respectively, with a high degree of resolution. The HPLC chromatograms of cannabinoids-rich extracts and standard compounds are shown in Figure 2 and their content of CBD and THC are presented in Table 2. Fraction I contained a moderate level of CBD (8.1% w/w) with a

very high level of THC (52.2% w/w) with a CBD:THC ratio of about 1:6; Fraction II contained moderate levels of both CBD (9.2% w/w) and THC (8.0% w/w) at a CBD:THC ratio of about 1:1; and Fraction III contained a low level of CBD (1.3% w/w) and a high level of THC (33.5% w/w) with a CBD:THC ratio of about 1:26.

Acetylcholinesterase inhibitory activity

The results of in vitro anti-AChE activity of cannabinoid-rich fractions, CBD, and galantamine are shown in Table 3. Among the cannabinoids-rich extracts, Fraction I exhibited the highest activity followed by Fractions II

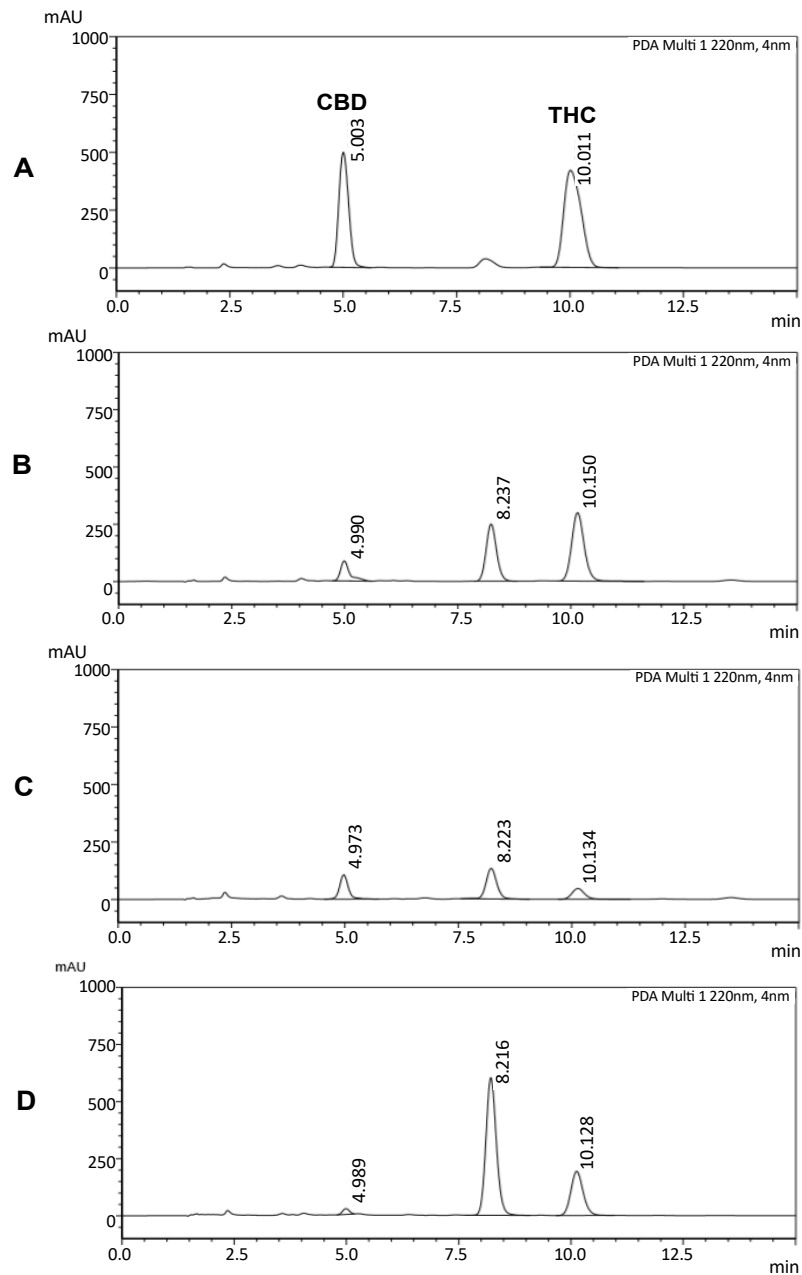

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Figure 3. HPLC chromatograms of standard CBD and Δ -9-THC (A), fraction I (B), fraction II (C), and fraction III (D)

Table 2. CBD and Δ -9-THC content of cannabinoids-rich extracts

Extracts	CBD (% w/w)	THC (% w/w)
Fraction I	8.1 \pm 0.04 ^a	52.2 \pm 0.12 ^a
Fraction II	9.2 \pm 0.05 ^b	8 \pm 0.29 ^b
Fraction III	1.3 \pm 0.07 ^c	33.5 \pm 0.52 ^c

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Values with non-identical letters (a, b, and c) in the same column are significantly different, with the statistic values of $F=1.640 \times 10^4$, $df=2$, and $P<0.05$ for CBD, and $F=1.216 \times 10^4$, $df=2$, and $P<0.05$ for THC.

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Table 3. Acetylcholinesterase inhibition effect of cannabinoids-rich extracts and CBD

Extracts/Compounds	IC ₅₀ (µg/mL)
Fraction I	52.3±8.13 ^a
Fraction II	59.8±3.35 ^b
Fraction III	71.2±3.12 ^c
CBD	13.8±1.13 ^d
Galantamine	0.7±0.08 ^e

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Values with non-identical letters (a, b, c, d, and e) are significantly different, with the statistic values of F=477.450, df=4, and P<0.05.

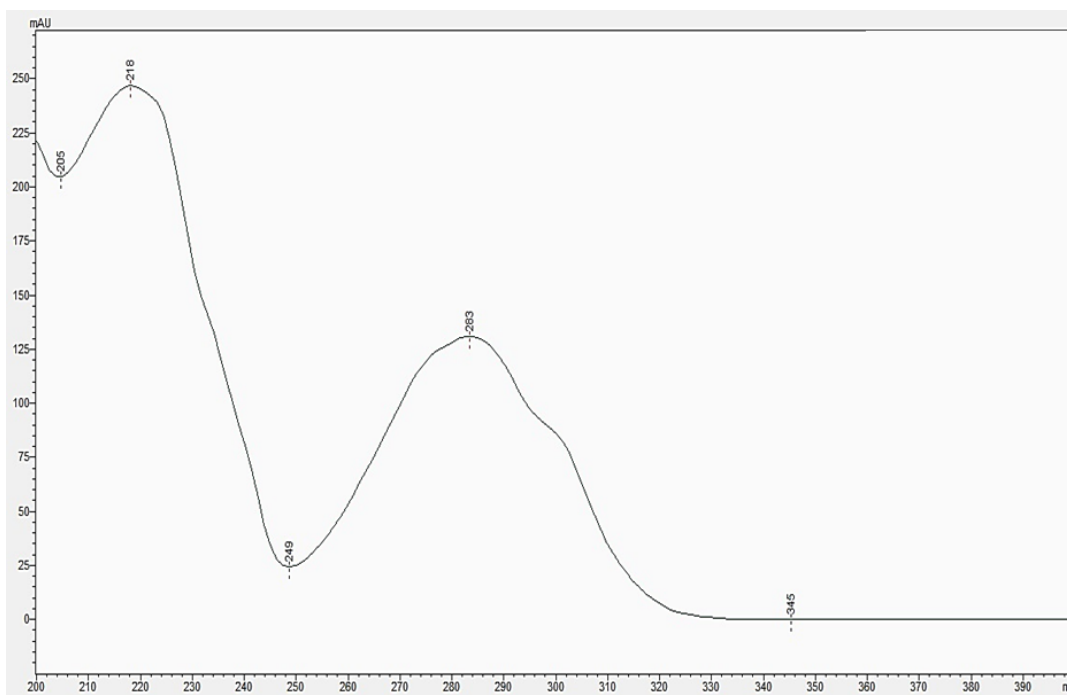


Figure 4. The UV absorption spectrum of the peak at 8.2 min

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and III with IC₅₀ values of 52.3, 59.8, and 71.2 µg/mL, respectively. In addition, galantamine, a positive control, and CBD, a marker compound exhibited good AChE inhibitory activity with IC₅₀ values of 0.7 and 13.7 µg/mL, respectively.

Discussion

Among neurologic disorders, AD has one of the highest incidences and is a top-ranking leading cause of death worldwide. Although there are numerous conventional medicines available for the treatment of AD, they come with some limitations, especially in terms of efficacy and safety. Therefore, more efficacious and safer remedies are still needed. Historically, natural products

or phytochemical compounds have played an important role in the treatment of many human diseases. *C. sativa* extract and its two major cannabinoids, viz; CBD and THC, have been reported to possess diverse beneficial effects in the treatment of many diseases.

The resin extracts from *C. sativa* leaves reportedly demonstrate a dose-dependent inhibitory effect against AChE in both in vitro and in vivo studies. Interestingly, THC and CBD were expected to be potent AChE inhibitors based on in silico studies, with binding affinities of -10.3 and -9.8 kcal/mol, respectively. THC inhibited AChE via hydrogen bonding with Tyr120, pi-pi interaction with Trp286, and Van der Waals interaction with various amino acid residues, while CBD exhibited only

Van der Waals interaction with various amino acid residues [18, 19]. However, no physiological interaction between THC and AChE was found in animal models [20]. Therefore, the present study focused on investigating the anti-AChE potential of cannabinoids-rich fractions containing different concentrations and ratios of CBD and THC, i.e. Fraction I that contained a moderate level of CBD with a very high level of THC at a CBD:THC ratio of about 1:6; Fraction II that contained the moderate levels of both CBD and THC at a ratio of about 1:1, and Fraction III contained a low level of CBD with a high level of THC at a ratio of about 1:26 and compared with CBD, a marker compound.

All three cannabinoid-rich extracts exhibited satisfactory anti-AChE activity, of which Fraction I exhibited the most potent activity, with an IC_{50} of 52.3 $\mu\text{g/mL}$. Fractions II with almost the same content of CBD but markedly lower content of THC than Fraction I exhibited slightly lower activity (IC_{50} of 59.8 $\mu\text{g/mL}$) than Fraction I. It seems to be the case that THC rarely contributes to the anti-AChE effect of cannabinoid-rich fractions. This was also confirmed by the anti-AChE activity of Fraction III (IC_{50} of 71.2 $\mu\text{g/mL}$), which is lower than Fraction II, even Fraction III contained a markedly higher content of THC. Moreover, CBD alone exhibited a good anti-AChE effect with IC_{50} 13.8 $\mu\text{g/mL}$. This finding implied that CBD, but not THC plays a key role in the AChE inhibitory effect of the cannabis extracts. Other than the anti-AChE effect, CBD has been reported to activate the peroxisome proliferator-activated receptor- γ to protect damage from neurotoxicity and oxidation stress, suppress the process of abnormally hyperphosphorylation of tau protein and induce neurogenesis of the hippocampus area, which are beneficial in AD treatment [21, 22]. Additionally, when CBD was administered with THC into mice, it was reported that this combination treatment improved memory function better than treatment with each compound alone [22].

Regarding the relation between the content of CBD in the fractions (8.1-9.2% w/w) and their anti-AChE effect (IC_{50} of 52.3-59.8 $\mu\text{g/mL}$) compared with that of pure CBD (IC_{50} 13.8 $\mu\text{g/mL}$), it seems that the fractions contained other active compounds. Because the extracts contained less than 10% w/w CBD but exhibited only 4-times less activity than CBD. Other cannabinoids, such as cannabinol (CBN), cannabicyclol, cannabitriol, cannabielosin, cannabigerol, cannabichromene, and cannabivarin have previously been recorded to display anti-AChE potential, in silico [19, 23]. In this study, the HPLC chromatograms of the cannabinoid-rich extracts also showed a peak at 8.2 minute (Figure 3), which could

be identified as CBN, a cannabinoid commonly produced by the oxidation of THC. Indeed, the identity of the peak was confirmed by determining its UV absorption spectrum (Figure 4) produced by a photodiode array detector. When the spectrum was compared with that of the previous report for pure CBN, both were found to be similar with identical λ_{max} [24]. Interestingly, although Fraction III contained a low content of CBD (1.3% w/w), its anti-AChE (IC_{50} of 71.2 $\mu\text{g/mL}$) was slightly lower than those of the others. This may be due to a high content of CBN in Fraction III as suggested by its HPLC chromatogram (Figure 3D).

The major pathologies of AD are presented by an inflammatory response in the brain, which is the main cause of the exacerbating β -amyloid burden and neurofibrillary tangles, suggesting that these two mechanisms correlate with neurologic disorder and cognitive decline in AD patients [5, 25]. The anti-AChE effect of cannabinoids possibly leads to an increased accumulation of acetylcholine and enhanced stimulation of cholinergic receptors, which play a crucial role in the cholinergic anti-inflammatory process. In addition, AChE inhibitors may suppress inflammation of the nervous system by diminishing the production of pro-inflammatory cytokines, such as TNF and IL-6 through the $\alpha 7$ nicotinic acetylcholine receptors [26-28]. Therefore, cannabinoids conceivably become an interesting candidate for alleviating the symptoms of AD.

Considering the efficacy of cannabis in AD therapy from pre-clinical studies, the use of cannabinoids, especially when CBD and THC are combined, might have greater benefits over a single pure cannabinoid compound in terms of improving dementia-related symptoms. The positive synergistic effect between cannabinoids as well as other compounds in cannabis, i.e. entourage effect, may play a crucial role in the use of cannabinoids-rich extracts with high content of many phytochemicals, including CBD in the alleviation of AChE-related challenges in AD.

Conclusion

In this cell-free study, we established that CBD as one of the major bioactive compounds in *C. sativa* is the leading agent regarding the inhibition of AChE activity. Although a previous in silico study predicted THC to have a high potential as an anti-AChE agent, our data indicated that THC only plays an adjunctive role, with CBD being the most important AChE inhibitory cannabinoid. Cannabinoids-rich extracts that contain at least 8% w/w CBD with low content THC might be considered as an

alternative AChE inhibitor. However, further studies on the AChE inhibitory activity of the cannabinoids as well as other bioactive compounds in cannabis are needed to clearly identify the exact role of each component on the AChE inhibitory effect. Future high-quality research on animal models and clinical trials will also be needed to investigate the clinical efficacy and safety profile prior to any practical application of cannabinoid-rich extracts in AD patients.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization, data analysis, finding discussion and manuscript preparation: Wiwit Suttithumsatid, Pharkphoom Panichayupakaranan; Providing cannabis raw materials: Charassri Nualsri; Conducting experiments: Wiwit Suttithumsatid; Designing and carry out the acetylcholinesterase inhibitory assay: Jiraporn Kara and Luelak Lomlim.

Conflict of interest

The authors declared no conflict of interest.

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