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สารไฟโตแคนนาบินอยด์ส่งเสริมฤทธิ์ต้านมะเร็งรังไข่โดยกระตุ้นการแสดงออกของตัวรับแคนนาบินอยด์

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Phytocannabinoids Potentially Exert Anti-ovarian Cancer Effects by Increasing Cannabinoid Receptor Expressions

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Received: 13 July 2021 / Edit: 24 August 2021/ Accepted: 16 September 2021

หลักการและวัตถุประสงค์: ภาวะดื้อยาของการรักษามะเร็งถือเป็นปัญหาสำคัญในการรักษามะเร็งรังไข่ จึงมีความจำเป็นที่จะต้องหาการรักษาทางเลือกเพื่อทดแทน มีรายงานว่าสารไฟโตแคนนาบินอยด์มีบทบาทในการต้านมะเร็ง โดยการจับกับตัวรับแคนนาบินอยด์ และสารแคนนาบินอยด์ในร่างกายสามารถกระตุ้นการแสดงออกของตัวรับแคนนาบินอยด์ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาหน้าที่ของสารไฟโตแคนนาบินอยด์ต่อการแสดงออกของตัวรับแคนนาบินอยด์บางชนิด ซึ่งผลการกระตุ้นตัวรับแคนนาบินอยด์จะส่งเสริมฤทธิ์ในการต้านมะเร็งรังไข่เพื่อนำมาพัฒนาใช้รักษาผู้ป่วย

วิธีการศึกษา: นำเซลล์ ES-2 ซึ่งเป็นมะเร็งรังไข่ชนิด clear cell มาศึกษาผลของสารไฟโตแคนนาบินอยด์ 2 ชนิด ได้แก่ cannabidiol (CBD) และ cannabinol (CBN) ต่อการเพิ่มจำนวนและการแสดงออกของตัวรับแคนนาบินอยด์จำนวน 7 ชนิด ได้แก่ *CB1 CB2 GPR3 GPR12 GPR55 TRPV1* และ *TRPV2* โดยวิธี sulforhodamine B และวิธี real-time reverse transcriptase-polymerase chain

ผลการศึกษา: พบว่า CBD และ CBN ยับยั้งการเพิ่มจำนวนเซลล์ ES-2 อย่างมีนัยสำคัญ โดยความเข้มข้นของสาร CBD ที่ยับยั้งการเจริญเติบโตของ ES-2 ลงร้อยละ 50 (IC_{50}) ที่ 24 และ 48 ชั่วโมงเท่ากับ 110.27±4.80 และ 78.90±6.23 µM และค่า IC_{50} ของ CBN เท่ากับ 170.07±3.21 และ 125.80±4.64 µM ที่ 24 และ 48 ชั่วโมง นอกจากนี้ยังพบว่า CBD และ CBN กระตุ้นการแสดงออกของตัวรับแคนนาบินอยด์แตกต่างกัน โดย CBD กระตุ้นการแสดงออกของ *CB1 CB2 TRPV1* และ *TRPV2* ซึ่งการเพิ่มขึ้นของ *CB1 TRPV1* และ *TRPV2* แปรผันตรงกับความเข้มข้นของ CBD ในขณะที่ *CB1 CB2* และ *GPR12* เพิ่มขึ้นตามความเข้มข้นของสาร CBN ที่ใช้

สรุป: CBD และ CBN สามารถยับยั้งการเจริญเติบโตของเซลล์ ES-2 โดยผลดังกล่าวแปรผันตรงกับความเข้มข้นของสารและเวลาที่ใช้ พบการแสดงออกที่เพิ่มขึ้นของตัวรับแคนนาบินอยด์

Background and objectives: A major problem with ovarian cancer (OC) is chemotherapeutic resistance suggesting the need for alternative treatments. The anti-cancer abilities of phytocannabinoids (PCs) have been demonstrated, involving the binding of PCs and cannabinoid receptors (CRs). A few reports suggest that endogenous cannabinoid activates CR expression. Therefore this study aimed to demonstrate the inhibitory effects of PCs on ovarian cancer cell proliferation in association with increased mRNA expression of selected CRs.

Methods: Effects of PCs, cannabidiol (CBD), and cannabinol (CBN) on cell proliferation and seven CRs (*CRs, CB1, CB2, GPR3, GPR12, GPR55, TRPV1,* and *TRPV2*) expressions in ES-2 cells, a clear cell OC subtype, were determined by sulforhodamine B assay and real-time reverse transcriptase-polymerase chain reaction.

Results: CBD and CBN significantly inhibited ES-2 cell proliferation. Half-maximal inhibitory concentrations (IC_{50}) of CBD at 24, and 48 hours, were 110.27±4.80 and 78.90±6.23 µM, and IC_{50} of CBN were 170.07±3.21, and 125.80±4.65 µM. Additionally, CBD and CBN differentially induced CR mRNA expressions. *CB1, CB2, TRPV1,* and *TRPV2* were increased in CBD-treated cells. Increased *CB1, TRPV1,* and *TRPV2* were directly related to CBD doses used, while CBN dose-dependent expressions were observed in *CB1, CB2,* and *GPR12*.

Conclusion: CBD and CBN inhibited ES-2 cell proliferation in a dose- and time-dependent manner. The differential CRs activations were observed in

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ที่แตกต่างกันระหว่างเซลล์ที่ได้รับ CBD และ CBN จึงมีความเป็นไปได้ว่า CBD และ CBN อาจจะกระตุ้นการแสดงออกของตัวรับแคนนาบินอยด์ ซึ่งทำให้ส่งผลเสริมฤทธิ์ต้านมะเร็งของสารไฟโตแคนนาบินอยด์

คำสำคัญ: ไฟโตแคนนาบินอยด์; ตัวรับแคนนาบินอยด์; มะเร็งรังไข่

CBD- and CBN-treated cells. These results suggested the selective CR inductions of CBD and CBN, which later might enhance the anti-cancer effects of the PCs.

Keywords: phytocannabinoids; cannabinoid receptors; ovarian cancer

ศรีนครินทร์เวชสาร 2564; 36(6): 679-686. ● Srinagarind Med J 2021; 36(6): 679-686.

Introduction

Ovarian cancer (OC) is a cancer that originates from the female reproductive organ from the ovary to the endometrium[1]. The incidence and mortality rate of OC are high worldwide. In Thailand, it is the sixth most common female cancer, with estimated incidence of 7.9 per 100,000 , and age-standardized mortality rate of (ASR) 4.7 per 100,000 in 2020[2]. A significant problem in OC treatment is its resistance to chemotherapeutic agents. Hence, alternative OC treatments are urgently required.

The endocannabinoid system (ECS) is a physiological system that controls the physiological functions of body systems, such as the reproductive system[3]. ECS is regulated by the interaction between cannabinoid receptors (CRs) and endocannabinoids (ECs, e.g., anandamide; AEA, and 2-araquidonoglicerol phospholipid; 2-AG). There are at least 7 known CRs; cannabinoid receptor type 1 (CB1), CB2, orphan G-protein-coupled receptors 3 (GPR3), GPR12, de-orphaned G-protein coupled receptor 55 (GPR55), and transient receptor potential cation channel subfamily V-member 1 (TRPV1), and TRPV2[4]. Upon binding to ECs, CR will generate a signal that regulates cell proliferation, differentiation, and survival. There is evidence that ECs induce CR expressions; AEA upregulates CB1 expression[5]. The EC-dependent CR induction might later increase the effects of EC. The anti-cancer effects of EC have already been reported[6], thus, ECS is a potential target for cancer treatment.

Cannabis spp. plant is an alternative source for exogenous cannabinoids or phytocannabinoids (PCs). The well-known Cannabis-derived PCs are tetrahydro-cannabinol (THC), cannabidiol (CBD), and cannabinol (CBN)[7]. The roles of THC and CBD are established, and the effects of these PCs are similar to those of ECs, particularly the anti-cancer properties[8]. There are reports demonstrating the antiproliferative effect of PCs, particularly THC and CBD, on several cancer types, such as breast, cervix, myeloid, brain, stomach, and lung. The proven cancer inhibitory properties of THC and CBD are cell cycle retardation and apoptosis induction[9–13]. There are limited studies regarding the effects of PCs on OC. CBD potentiates the anti-cancer effect of the standard chemotherapeutic drug, paclitaxel but the underlying mechanisms have never been addressed[14]. Therefore, the potential uses of PCs as an anti-OC treatment require further investigation.

The expressions of CRs are observed in the female reproductive system. CR signaling has reproductive control functions, such as oviduct movement, blastocyst implantation, and placental development[15]. Hence, PCs might function in the female reproductive system, and they might be used as an alternative treatment for OC. In the present study, we hypothesized that PCs can inhibit OC cell proliferation and stimulate the CR expressions.

Materials and Methods

Cell Culture and Treatments

ES-2, a cell line derived from an aggressive clear cell OC subtype, was kindly provided by Professor Seiji Okada, Kumamoto University, Japan. ES-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, USA), 1% antibiotic-antimycotic solution (Gibco, USA) and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Cells were maintained in a humidified incubator at 37°C and 5% CO2. CBD and CBN (THC Pharm GmbH, Germany) were dissolved in dimethyl sulfoxide (DMSO, Applichem, UK) and stored at -20°C until use. DMSO was used as vehicle control for each experiment.

Cell proliferation assay

ES-2 cells were cultured in a 96-well plate (5,000 cells/well) for 24 hours and were treated with

increasing concentrations of CBD and CBN, 50, 100, 150, and 200 µM. Comparable DMSO concentration was added as a control (the maximal DMSO concentration was 0.06%). Cell numbers were determined at 24 and 48 hours after treatment using sulforhodamine B assay (SRB, Sigma Aldrich, USA)[16]. Briefly, the cells were fixed with cold 10% trichloroacetic acid (TCA) at 4°C for 30 minutes. Cells were then washed with distilled water for 4 times. The cells were dried and stained with 0.4% SRB in 1% acetic acid at room temperature (RT) for 30 minutes in the dark. After that cells were washed 4 times with 1% acetic acid. . The cells were solubilized by 10 mM Tris-base (pH 10.5) and the absorbance was measured at 540 nm (OD_{540}) using a spectrophotometer (Microplate Reader EZ Read 2000, Biochrom, UK).

Primer design

Messenger RNA sequences of seven CRs were obtained from the National Center for Biotechnology Information (NCBI) Databases (https://www.ncbi.nlm.nih.gov/). The selected sequences are *CB1* (NM_016083.6), *CB2* (NM_001841.3), *GPR3* (NM_005181.4), *GPR12* (NM_005288.4), *GPR55* (NM_005683.4), *TRPV1* (NM_080704.4), and *TRPV2* (NM_016113.5). *ACTB* (NM_001101.5) was used as a control. If the spliced variants are presented, the common sequences of each CR were used to design primer using Primer3 and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The designed primers were checked for melting temperature, GC%, hairpin structure, self-dimer, and hetero dimer by OligoAnalyzer™ Tool (https://www.idtdna.com/calc/analyzer). The primer sequences, melting temperatures and expected PCR product sizes are shown in Table 1. Primers were obtained from Integrated DNA Technologies, Inc., USA.

RNA extraction

ES-2 cells were grown to reach more than 80% confluence prior to CBD or CBN treatment. Cells were incubated with CBD or CBN at final concentrations of 25 or 50 µM for 24 hours. Comparable DMSO concentration was added to a control (the maximal DMSO concentration was 0.015%). Total RNA was isolated from cell lines using TRIzolTM reagent (Invitrogen, USA) according to the manufacturer's recommendations. Cells were washed with cold phosphate buffer saline (PBS), and then 1 mL of TRIzolTM reagent was added directly onto the cells. The cells were lysed by pipetting. After that chloroform was added, vigorously mixed, and aqueous solution was separated by centrifugation. The upper phase was collected and mixed with isopropanol. After washing with 70% ethanol, the pellet was air dried and suspended in 30 µL of RNase-free water. Contaminated DNA was removed by DNase I treatment (New England Biolabs, USA) followed the manufacturer's recommendation. RNA concentration and quality were determined by a UV spectrophotometer (NanoDrop™ 2000/2000c, Thermo Fisher Scientific) and agarose gel electrophoresis. Total RNA was stored at -80°C until use.

Complementary DNA (cDNA) synthesis and real-time PCR

Two µg of total RNA was converted to cDNA by High-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) as recommended by manufacturer. cDNA was diluted to archive the concentration of 20 µg/µL by nuclease-free water. The CR expressions were analyzed using LightCycles®480 real-time PCR system (Roche Diagnostics, Germany). ACTB expression was used for normalization. Each PCR condition contains 1XLightCycles®480 SYBR Green I Master, 2.5 µM of primers, and 10-100 ng of cDNA. The amplification was initiated by incubating at 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds, 62°C for 10 seconds, and 72°C for 10 seconds. Each sample was prepared in duplicate. Mean and standard deviation (SD) of the crossing point (Cp) cycle, and normalized gene expression values were calculated using LightCycler480® Relative Quantification software (Roche Diagnostics). The gene expression levels were expressed as $2^{-\Delta Cp}$, where $\Delta Cp = Cp_{target} - Cp_{ACTB}$. All primers used in the current study are listed in Table 1.

Statistical analysis

The results are expressed as mean±SD from three independent experiments. Statistical significance was determined using one-way ANOVA with $p<0.05$ considered significant.

Results

The effect of CBD and CBN on ES-2 cell proliferation

To determine the effects of CBD and CBN on ovarian cancer cells, ES-2 cells were incubated with indicated concentrations of CBD and CBN for 24 and 48 hours. Cell numbers were measured by SRB

Table 1 List of primers

mRNA	Primers (5'-->3')*	Tm (°C)	Product sizes (bp)
CB1	F: ATAGCCATTGTGATCGCCGT	59.9	137
	R: AGAAGCAGTACGCTGGTGAC	60	
CB2	F: CTGACCGCCATTGACCGATA	59.9	114
	R: TAGTGCTGAGAGGACCCACA	59.9	
GPR3	F: CAAATCTGCCGCATCGTCTG	60	154
	R: AGACAGTGAAGGGCAACCAG	59.9	
GPR12	F: TACCATTCGGAGAGGACGGT	60	150
	R: GTTCTTGGTGAGCGGTCTGA	60	
GPR55	F: GTCAGTGTCTACGGGTGGTG	60	103
	R: TGACGGGCATCACTCAACTC	60	
TRPV1	F: CTGTCCAGGAAGTTCACCGA	59.3	140
	R: TCGTGGCGATTAGGGGTCT	60.4	
TRPV2	F: GTCCCAGGTGCTGTGTTTCC	61.2	127
	R: GACACTGTAGATGCCTGTGTG	58.7	
ACTB	F: GGCTGTGCTATCCCTGTACG	60.3	156
	R: AGGTAGTCAGTCAGGTCCCG	60.3	

*Note: F = forward primer, R = reverse primer

staining. The result showed that CBD and CBN significantly inhibited ES-2 cell proliferation in a dose- and time-dependent manner. CBD at 50, 100, 150, and 200 μM suppressed cell growth to 96.28±8.49% (p=0.3697), 54.28±5.69% (p=4.64x10^{-10}), 31.62±2.81% (p=1.41x10^{-13}) and 8.52±1.91% (p=9.74x10^{-16}) at 24 hours, and 87.02±7.71% (p=0.0022), 26.65±2.12% (p=3.69x10^{-15}), 4.93±1.57% (p=4.68x10^{-17}) and 1.58±0.63% (p=2.02x10^{-17}) at 48 hours when compared with control. CBN treatment at 50, 100, 150, and 200 μM yielded ES-2 cell numbers of 102.02±5.95% (p=0.4977), 93.40±6.93% (p=0.0519), 51.03±5.74% (p=1.06x10^{-11}) and 43.58±2.85% (p=5.04x10^{-14}) at 24 hours, and 95.74±8.94% (p=0.2798), 80.21±8.36% (p=5.81x10^{-5}), 22.17±2.52 (p=1.06x10^{-15}) and 19.62±1.74 (p=3.98x10^{-16}) at 48 hours. Half-maximal inhibitory concentrations (IC50) of CBD at 24 and 48 hours were 110.27±4.80 and 78.90±6.23 μM (Figure 1A), and IC_{50} of CBN were 170.07±3.21 and 125.80±4.65 μM (Figure 1B). The significant difference of inhibitory effect between 24- and 48-hours incubation time was observed from 50 μM of CBD (p=0.0277), while CBN was shown from 100 μM (p=5.81x10^{-5}).

The CR expressions in CBD- and CBN-treated ES-2 cells

To observe the CR expressions in CBD- or CBN-treated cells, ES-2 cells were incubated with 0, 25, and 50 μM CBD and CBN for 24 hours. These doses were selected to observe the direct effects of CBD and CBN on CR expressions without the effects on growth inhibitions. The result showed that CBD and CBN differentially induced CR expressions. Relative expressions of *CB1, CB2, GPR3, GPR12, GPR55, TRPV1* and *TRPV2* in 25 μM CBD-treated ES-2 cells when

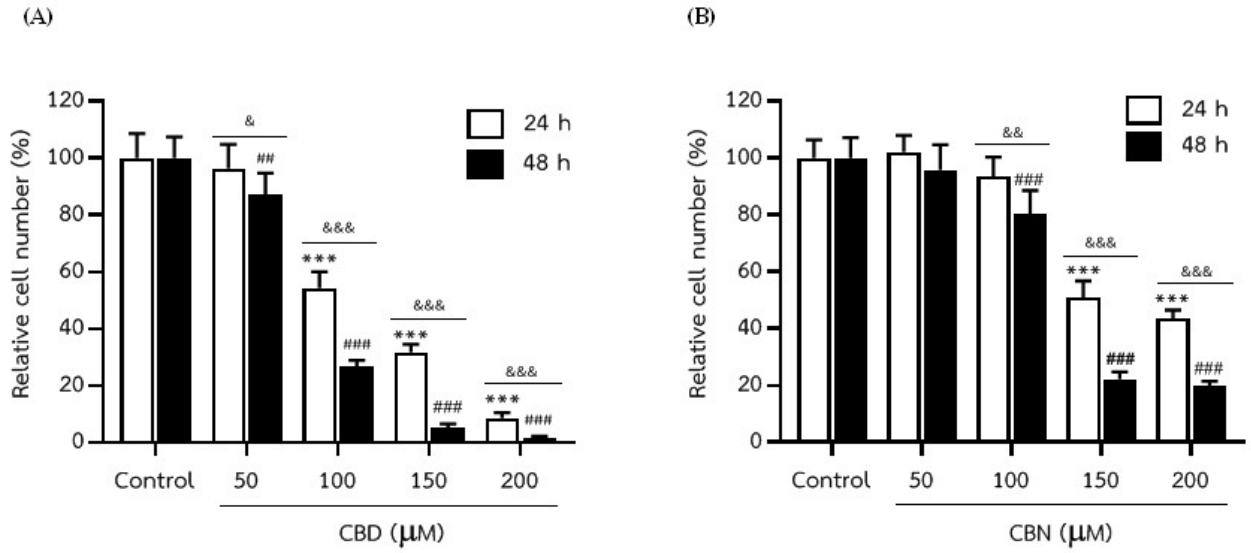

Figure 1 CBD and CBN inhibit ES-2 cell proliferation. ES-2 was treated with 0 (control), 50, 100, 150, and 200 μM of CBD (A) or CBN (B) for 24 and 48 hours and cell numbers were measured by SRB assay. OD_{540} was determined and relative cell number was calculated. OD_{540} of control was set as 100%. Data are presented as the mean±SD of three independent experiments. ***p<0.001, compared to control at 24 hours, ##p<0.01 and ###p<0.001 compared to control at 48 hours, &p<0.05, &&p<0.01, and &&&p<0.001 compared between 24 and 48 hours.

compared to untreated cells (control) were 3.55±0.83 folds (p=0.0060) , 6.06±0.98 folds (p=0.0009), 1.22±0.31 fold (p=0.2921), 3.29±1.52 folds (p=0.0602), 1.69±0.57 folds (p=0.1025), 2.42±0.31 folds (p=0.0012) and 1.26±0.04 fold (p=0.0005) and there were 4.15±1.38 folds (p=0.0167), 2.59±0.98 folds (p=0.0487), 1.79±0.52 fold (p=0.0569), 1.64±0.52 fold (p=0.1001), 0.99±0.15 fold (p=0.9430), 3.67±0.43 folds (p=0.0004) and 1.74±0.31 fold (p=0.0142) in 50 µM CBD-treated cells. In 25 µM CBN treatment, the relative expressions of these receptors were 1.46±0.23 fold (p=0.0264), 3.45±0.94 folds (p=0.0108), 0.79±0.07 fold (p=0.0051), 2.74±1.08 folds (p=0.0496), 1.55±0.43 fold (p=0.0919), 0.57±0.08 fold (p=0.0008) and 1.39±0.48 fold (p=0.2332) while there were 2.11±0.34 folds (p=0.048), 4.20±0.38 folds (p=0.0001), 0.72±0.26 fold (p=0.1427), 4.43±1.41 folds (p=0.0134), 1.66±0.56 fold (p=0.1125), 0.83±0.16 fold (p=0.1349) and 1.01±0.14 fold (p=0.9058) in 50 µM CBN-treated cells. *CB1, CB2, TRPV1* and *TRPV2* were significantly increased in CBD-treated cells. Increased *CB1, TRPV1,* and *TRPV2* were directly related to CBD doses (Figure 2A), and CBN dose-dependent expressions were observed in *CB1, CB2*, and *GPR12* (Figure 2B). Dose-dependent inductions of GPR3 in CBD treatment and GPR55 in CBN treatment were observed but the increased expressions did not reach statistical significance.

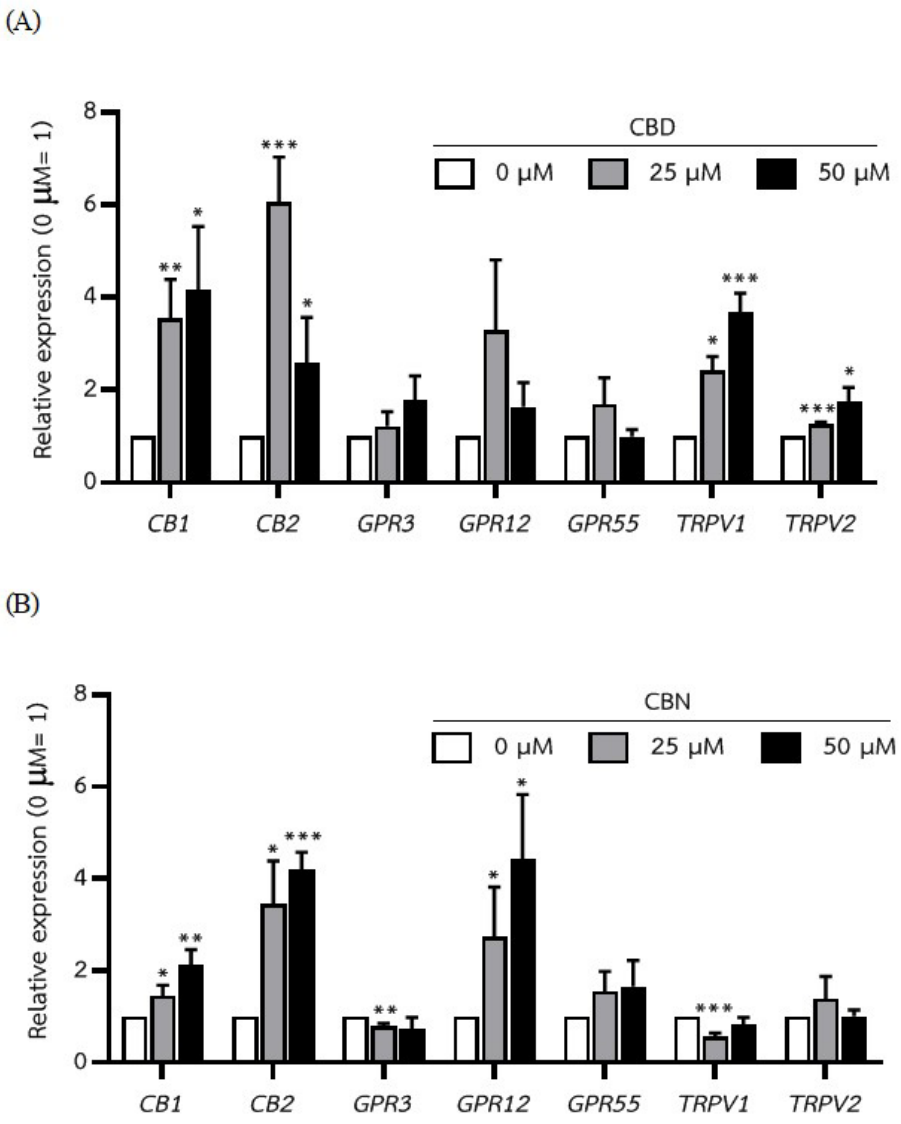

Figure 2 CBD and CBN induce selected CR expressions. ES-2 cells were treated with 25 or 50 µM of CBD (A) and CBN (B) for 24 hours. The CR expressions were measured and were normalized by *ACTB*. All data are expressed as mean±SD from three independent experiments. The expression of CR at 0 µM treatment was set as 1. *p< 0.05, *p<0.01, ***p<0.001, compared to the control (0 µM). CB: cannabinoid receptor, GPR: G-protein coupled receptor, TRPV: transient receptor potential cation channel subfamily V.

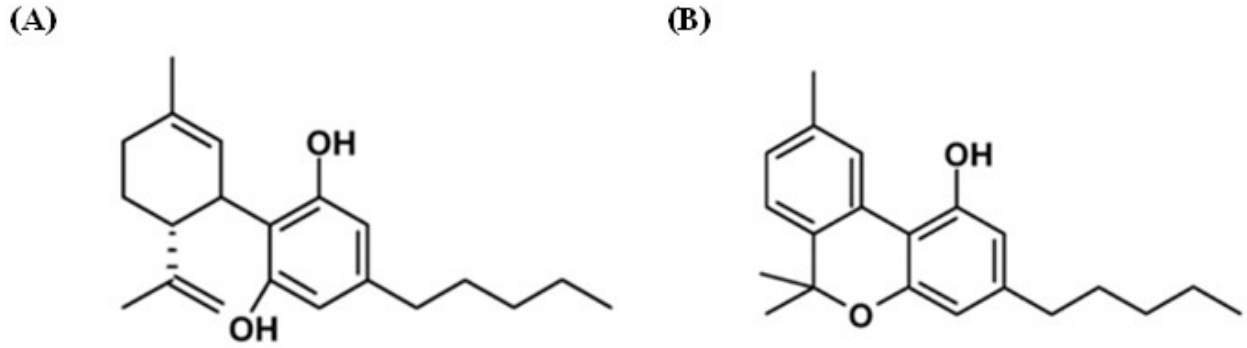

Figure 3 Chemical structures of CBD (A), CBN (B)27. The major differences between CBD and CBN are the closed conformation and higher degree of oxidation observed in CBN structure.

Discussion

OC is a common cancer in Thai women, and anti-cancer drug resistance is a significant problem in OC treatment. A novel treatment option is urgently required. Herbal medicine, particularly PCs, is one promising option. This study showed that CBD and CBN inhibited ES-2, the aggressive OC cell line, in a dose- and time-dependent manner. CBD exhibited a more substantial inhibitory effect with lower IC_{50} when compare to CBN. Additionally, the roles of CBD and CBN on selected CR expressions were demonstrated. CBD induced *CB1, CB2, TRPV1,* and *TRPV2*, while CBN increased *CB1, CB2* and *GPR12* expressions. These results demonstrated the potential effects of CBD and CBN on anti-cancer potentiation through CR inductions.

PCs are a group of C21 terpene phenolic compounds predominately produced by the cannabis plant[17]. CBD and CBN are two major cannabis-derived substances. CBD consists of one aromatic ring connected with alkene ring, and a tert-propyl alkene that links to alkene ring presents stereochemistry. CBN has no double bond isomers nor stereoisomers, and it is an aromatic compound. The chemical structures of CBD and CBN are shown in Figure 3[27]. CBD and CBN bind to CRs with different binding affinities. CBD acquires a higher affinity to CB2, while CBN has a higher affinity to *CB1*[18]. The different structure and CR binding affinity of CBD and CBN might contribute to the different IC_{50} observed in the current study. Furthermore, the potencies of CBD and CBN on ES-2 cell viability were lower than other cell types compared by IC_{50}. For instance, CBD significantly inhibited glioma cell, U87MG, proliferation with the IC_{50} values of 10±2.1 μM at 24 hours and 8.4±2.9 μM at 48 hours[19]. The IC_{50} of CBD in different cervical cancer cell lines at 24 hours are 10.18 μM for HeLa, 4.77 μM for ME-180 and 10.18 μM for SiHa20; SGC-7901 gastric cancer cell line is 74.41 μM13; and two breast cancer cells are 8.2±0.3 μM for MCF-7 and 10.6±1.8 μM for MDA-MB-23121. In human subjects, administration of CBD with a dose greater than 200 mg/kg/day shows adverse effects such as hepatocellular damage, hormone changes, and decreased fertility[22]. CBN has never been tested clinically. In the current study, IC_{50} of CBD were ranging from 78-110 μM. Comparing to the report[22], these concentrations might not be achievable systemically; nonetheless, further study in the animal model with local administration is still required to demonstrate the potential use.

There is an evidence that the expression of CRs is different in each cancer cell type[23,24]. We hypothesized that the different effects of CBD on different cancer types might be due to the differentially expressed CRs. Further investigation on the effects of CBD or CBN on cell lines with differential CR expressions. From the previous report, EC, AEA, induces *CB1* expression[5]. Thus, CBD and CBN might possess CR modulatory effects similar to EC. The effects of CBD or CBN on CR expression were determined. The results demonstrated that both CBD and CBN upregulated *CB1*, and *CB2* expression. CBD, not CBN, significantly induced *TRPV1* and *TRPV2* expression, while only CBN increased *GPR*[12]. The different effects of CBD and CBN might be due to the different chemical structures, and binding affinities to corresponding CR[18]. The mechanisms of CBD and CBN induced CR expressions require further investigation. There are studies reporting anti-cancer effects of CBD are CR-dependent in various cancer cell types; for example, CBD inhibits cervical cancer cell proliferation and invasion and these effects are alleviated when the inhibitors of *CB1, CB2* and *TRPV1* receptor are added[25]. Furthermore, there is a study showed that

patients with non-small cell lung cancer who had high expression levels of *CB1, CB2,* and *CB1/CB2* showed significantly increased survival times[26]. Hence, CBD and CBN-induced CRs expressions might potentiate the effects of ECs and PCs, which would later enhance anti-cancer effects.

However, some limitations in this study should be noted. Firstly, the effect of CBD and CBN was only examined in single cell line *in vitro*; additional ovarian cancer cells should be included. Secondly, another major PC, delta-9-tetrahydrocannabinol or THC, was not tested in the current study because it is psychotropic substance. Lastly, the *in vivo* study was not included, whose results might emphasize the benefit of CBD and CBN in OC treatment.

Conclusion

This study demonstrated the inhibitory effects of CBD and CBN on OC proliferation, using ES-2 cell as a model, in association with increased mRNA expressions of specific CR.

Acknowledgments

The present study was supported by the Research and Graduate Studies Office, Khon Kaen University, Thailand (to KV, grant #RI-Can-62-08) and Faculty of Medicine, Khon Kaen University, Thailand (to KS and KV, grant #IN64223). KS was supported by a scholarship from the Development and Promotion of Science and Technology Talents Project, Royal Thai Government, Thailand (scholarship #582017).

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