Original Research Article

Anti-inflammatory effect of methanol extracts of hemp leaf in IL-1β-induced synovitis

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Abstract

Purpose: To examine the effectiveness of some hemp (Canabis sativa) leaf extracts as an anti-inflammatory agent on synovitis in vitro.
Methods: Synovial fibroblast cell line SW982 was induced with 5 ng/mL of interleukin 1-beta (IL-1β) to trigger cellular inflammation. The cells were then treated with prepared extracts of hemp (Canabis sativa) leaf originating from three different cultivation sites with varying proportions of terpenoids and cannabinoids, especially cannabidiol (CBD) and tetrahydrocannabinol (THC). Nitric oxide (NO) and prostaglandin E2 (PGE2) production as well as expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and IL-1β genes were determined.
Results: All hemp extracts reduced the production of NO and PGE2 in a dose-dependent manner. The expressions of iNOS, COX-2 and IL-1β genes were significantly decreased by the hemp extracts. This effect was likely related to the amount of sesquiterpenoids and THC. The extract from Huai Hoi (HH) cultivar showed the most promising results for further studies.
Conclusion: The extracts of hemp leaf substantially reduces the level of biomarkers for inflammation in vitro. Therefore, the extracts have a potential application as an inflammatory counteractant in synovitis.

Keywords: Canabis sativa, Hemp, Synovitis, Cannabinoids, Nitric oxide, Interleukin-1 beta

INTRODUCTION

Osteoarthritis (OA) is a well-known inflammation-related disease. It is a chronic disease associated with joint degeneration and reparative bone response on chondrocytes, subchondral bones, and synoviocytes [1]. The inflammation of synoviocytes, or synovitis, is known to be related to OA and can be used as a diagnostic marker [2]. In terms of physiology, synoviocytes manage to protect and maintain the integrity of articular cartilage surfaces, which are altered in OA [3]. In addition, synoviocytes in OA becomes a source

of proinflammatory cytokines including interleukin 1-beta (IL-1β) and nitric oxide (NO). There is strong evidence indicating that IL-1β alone, and probably together with tumor necrosis factor alpha (TNF-α), triggers joint destructive mechanism as both cytokines are dominantly expressed in OA synoviocytes, synovial fluid and cartilage [4].

The production of NO and the expression of inducible nitric oxide synthase (iNOS) gene have also been found to escalate in many inflammatory disorders including OA [5].

Moreover, NO can stimulate the activity of the cyclooxygenase-2 (COX-2) enzyme and enhance production of inflammatory mediators including prostaglandin E_2 (PGE2) [6]. Current OA therapeutic guidelines focus on regulating the activity of related enzymes that produce pro-inflammatory cytokines. The commonly used drugs are glucocorticoids, including dexamethasone, and COX-2 inhibitors, but these therapeutic agents produce undesired side effects [7].

Hemp (*Canabis sativa* L.) is a versatile plant used for industrial, agricultural and medicinal purposes since ancient times [8]. In pharmacology, hemp has been applied to numerous diseases, especially pain and neuronal disorders [9,10]. The active compounds in hemp plant are known as cannabinoids and the most potent one is tetrahydrocannabinol (THC). THC affects the neurons which triggers delusion and delirium, known as the "psychotomimetic effect". Another important substance in hemp is non-psychoactive cannabinoid known as cannabidiol (CBD). THC and CBD together can reduce the progression and severity of pain in various diseases when used in appropriate proportions, e.g., when used in a ratio of 1:1 or 2:1 [11]. The action of the cannabinoids occurs through their specific receptors, cannabinoid receptors 1 and 2 (CB1 and CB2), which are normally expressed in brain, neuron cells and immune cells [12]. The activation of these receptors affect the secretion of cytokines from cells but the exact role of CB receptors, especially CB2, is still unclear [13]. Interestingly, CB2 has been recently reported to be involved in the occurrence and severity of inflammation, including arthritis [14]. Therefore, cannabinoids and other compounds contained in hemp might be effective on synovitis.

Although many parts of the hemp plant possess cannabinoids, most of the researchers and manufacturers are interested in hemp oil or hemp seeds [15]. Hemp leaves are usually disposed in both industrial and pharmaceutical processes. In this study, we examined the phytochemical content, especially THC and CBD, in the leaves of hemp cultivated in Thailand. The phytochemicals in hemp leaf extracts were discovered using gas chromatography and mass spectrophotometry (GC/MS) and the extracts were investigated for their anti-inflammatory effect in synovitis induced by IL-1β.

EXPERIMENTAL

This study was approved by the Narcotics Control Division, Food and Drug Administration, Ministry of Public Health, Thailand (approval ref.no. 210062601400000). All activities undertaken complied with the restrictions of the National Narcotics and Psychotropic Substances Act. The participating institutes have been authorized to import, cultivate, and possess hemp for research and educational purposes under license nos. THAI.FDA.A4.0589033, THAI.FDA.A4.0589026, and THAI.FDA.A4.05890 25 , respectively.

Hemp cultivation and extraction

The hemp seeds in this study were collected from three areas of Chiang Mai Province, which differed from each other in geography and agronomy. The seed varieties were identified by their origins: (1) Huai Hoi (HH) (N18.623974 E98.655991), (2) Mae Sa Mai (MSM) (N18.885391 E98.84600), and (3) Pang Ung (PU) (N18.783784 E98.159269). All hemp seeds were further cultivated in the Pangda Royal Agriculture Station in Samoeng, Chiang Mai (N18.854925 E98.760765). After 90 days of cultivation (June to September), the leaves on the upper third of the main stem were collected. All plant cultivars were sampled and identified by a botanist at the Herb Museum, Faculty of Pharmacy, Chiang Mai University (voucher no. 023209). The leaves were dried at 40 ºC for 48 h and were ground and sieved through 32-mesh sieving. For extraction, 100 g of dried leaf powder was percolated with 400 ml methanol for 24 h. The extracts were collected and filtered through Whatman's filter paper No.1. The extraction process was repeated until the percolated solvent was clear. Methanol was then eliminated using a vacuum evaporator and the yield of collected extracts was measured. All compounds in the hemp extracts were identified using NIST library provided with the GC/MS apparatus [16].

Human synovial fibroblast cell line (SW982) culture and treatment

SW982 was acquired from ATCC® number HTB-93 and was authenticated by DiagCor Bioscience Incorporation Limited. The cells were retained in Leibovitz's L-15 culture media complemented with 10 % fetal bovine serum, 100 U/ml penicillin G sodium and 100 µg/mL streptomycin. The cells were kept in a CO_2 free incubator at 37 ºC. Prior to the experiments, the cells were plated in 6-well plates at a concentration of 2×10^5 cells per well. Phytochemicals at appropriate concentrations were used to treat the cells either with or without 5 ng/mL of IL-1β, which was added for 24 h to induce synovitis. Dexamethasone at a concentration 100 nM was used as the positive

control. After the treatment period, cell lysates were collected for determination of gene expression while culture supernatants were collected for measurement of released cytokine.

Nitric oxide determination

Prior to the experiment, appropriate doses of the hemp extracts were determined using MTT assay [17]. The total amount of released nitric oxide was measured via nitrite which accumulated in the culture supernatant. After the cell treatment, 1 mL of culture supernatant was immediately collected and Griess reagent (0.1 % *N*-(1-naphthyl) ethylenediamine dihydrochloride, 1 % sulfanilamide, and 2.5 % H_3PO_4) was added in an equal volume. The mixture was stored for 10 minutes at ambient temperature and then measured for absorbance at 540 nm. The nitrite concentration in the samples was calculated using the standard curve generated from sodium nitrite concentrations ranging from 1 to 100 µM.

Prostaglandin E2 determination

The level of produced PGE2 was measured using a PGE2 competitive ELISA kit purchased from Thermo Fisher Scientific Co., Ltd., Thailand. The culture media acquired from the treated cells were collected and transferred onto the analytical plate provided with the kit. The experimental procedure was performed following the manufacturer's guidelines. Absorbance was measured at UV 405 nm. The level of PGE2 concentration in the samples was interpreted from the standard curve, which was generated from the standard PGE2 at a concentration range of 0 to 5000 pg/mL.

Gene expression analysis

After treating the cells, total RNA was separated using the Illustra RNAspin Mini RNA Isolation Kit. Reverse transcription reaction was operated using 1.0 µg of total RNA and then reverse-transcribed into cDNA using the Tetro cDNA synthesis kit. Real-time reverse transcriptase polymerase chain reaction technique (RT-PCR) was conducted using SensiFAST™ SYBR® Lo-ROX kit. The reactions were conducted to determine the process of denaturation, annealing and extension on real-time RT-PCR detection system using 7500 Fast Real-Time PCR instrument. The data were collected and the fold changes were measured via the $2^{-\Delta\Delta CT}$ method [18]. The genes of interest were iNOS, COX-2, and IL-1β. β-actin was used as the reference constitutive gene. The primer sequences obtained from Invitrogen™ used in this experiment were as follows: *Homo sapiens*

nitric oxide synthase 2 (NOS2) forward CTGCTTGAGGTGGGCGG*, Homo sapiens nitric oxide synthase 2 (NOS2)* reverse GTGACTCTGACTCGGGACGCC; *Homo sapiens cyclooxygenase-2 (Cox-2)* forward CCCTTGGGTGTCAAAGGTAA*, Homo sapiens cyclooxygenase-2 (Cox-2)* reverse GCCCTCGCTTATGATCTGTC; *Homo sapiens interleukin 1 beta (IL1B)* forward GGACAAGCTGAGGAAGATGC*, Homo sapiens interleukin 1 beta (IL1B)* reverse TCGTTATCCCATGTGTCGAA; *Homo sapiens actin beta (ACTB)* forward CGTGAAAAGATGACCCAGATCA *and Homo sapiens actin beta (ACTB)* reverse CACAGCCTGGATGGCTACGT*.*

Statistical analysis

Statistical differences between the groups were assessed using one-way ANOVA. Data were expressed as mean ± SEM (n = 3). Statistical significance was set at *p* < 0.05.

RESULTS

Cannabinoid content of hemp extracts

After obtaining the extracts from all three cultivars, it was observed that PU cultivar produced the highest amount of extracts, followed by HH and MSM. The yield of PU, HH, and MSM were 26.12, 25.33 and 23.75 %, respectively. All extracts were then analyzed using GC/MS technique. The resulting chromatograms are displayed in Figure 1. After NIST library matching, the compounds present in the extracts were divided into three subgroups, monoterpenoids at retention time less than 18 min, sesquiterpenoids at retention time between 20 and 38 min, and cannabinoids which appeared after 45 min.

Cannabinoids were phytochemicals found in the highest amount in all hemp extracts, followed by sesquiterpenoids and monoterpenoids. The phytochemical composition of each cultivar has been clarified in Figures 2A to 2C. PU produced the highest amount of total cannabinoids and slightly higher amount of sesquiterpenoids compared to others. MSM was the cultivar with the highest amount of monoterpenoids. The major component of sesquiterpenoids was β-caryophyllene, whose peak was found at t~24.1, labeled (1), and humelene at t~25.6, which was labeled (2). The peak of a major cannabinoid, CBD, was observed at t~57.5, labeled (5) while THC was monitored at t~60.2 and labeled (6) compared to authentic standard. Peaks were also detected in PU extracts; the first peak was

at t~51.9, labeled (3), which did not match with any substance in NIST database and the second one was at t~54.8, labeled (4), which probably was tetrahydrocannabivarin (THCV). The other detected peaks in hemp extracts were compared to MS spectra database and are displayed in Table 1.

Quantitative analysis performed on CBD showed that PU produced the highest amount of THC, followed by MSM and HH (1.259, 0.341 and 0.302 % of dry weight, respectively). Remarkably, the amount of THC in PU cultivar was > 1.0 %, which might not be allowed for use due to the restriction in the Acts [19]. HH produced a major amount of CBD, followed by PU and MSM (2.297, 2.256 and 1.879 % of dry weight, respectively). PU produced the highest amount of cannabinoids, followed by HH and MSM (3.515, 2.599 and 2.220 % of dried weight, respectively) (Figure 2D). Since CBD is important

for modulating undesired effects of THC and quality control in term of CBD to THC ratio [20], we determined the ratio of CBD to THC in each cultivar. HH was found have the highest proportion of CBD to THC, followed by MSM, and PU (7.61, 5.51, and 1.79, respectively) (Figure 2E).

Effect of hemp extracts on release of proinflammatory cytokines

In this study, NO and PGE2 were the cytokines of interest as these biomolecules are associated with inflammation including synovitis [1]. It was determined that the appropriate concentrations of hemp extracts used in MTT assay before the experiments on SW982 cells and the appropriate concentrations for further studies were 1, 5, and 10 µg/mL (data not shown). After IL-1β induction, SW982 produced significant amounts of NO compared to the control group (Figure 3).
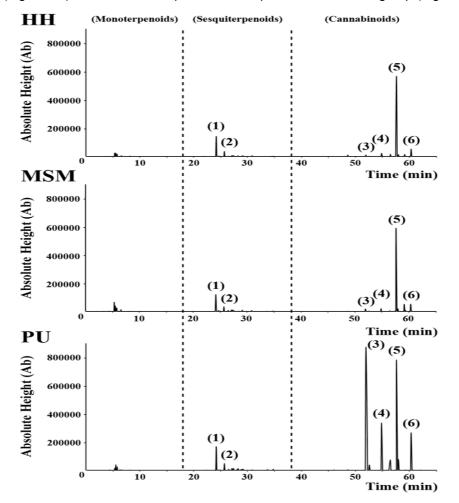

Figure 1: (A) GC chromatogram of all three hemp cultivars used in this study. The peaks detected can be divided into three subgroups using NIST database, monoterpenoids at runtime less than 18 min, sesquiterpenoids at runtime between 20 and 38 min, and cannabinoids after 45 min of injection. For sesquiterpenoids, known peaks are β-caryophyllene at~24.1 min (1) and Humelene at t~25.6 min (2). For cannabinoids, unknown peak can be detected at t~51.9 min (3), while the known peaks are THCV at t~54.8 min (4), CBD at t~57.5 min (5), and THC at t~60.2 min (6).

Table 1: List of phytochemicals that matched with the NIST database. The substances are divided into subgroups related to GC chromatograms. Symbols in the table represent the appearance of substances in each hemp strain; O not detectable, + detectable, ++ detected in 2 folds of + but not more than 5 folds, and +++ detected in 5 folds of +

Compound	HH	MSM	PU
Monoterpenoids			
α-Pinene	++	+++	+
β-Pinene	++	++	+
Limonene	++	+	+
Sesquiterpenoids			
β-Caryophyllene	+	+	+
α-Bergamotene	++	++	+
Humulene	+	+	++
α-Ylangene	+	o	o
β-Copaene	o	+	+
α-Guaiene	+	+	++
α-Farnescene	+	++	++
α-Selinene	+	+	++
α-Cubebene	+	o	++
γ-Eudesmol	o	+	+
β-Guaiene	++	+	++
α-Bisabolene	+	+	+
Caryophyllene oxide	+	+	+
α-Bisabolol	+	+	++
Cannabinoids			
THVC	+	+	+++
Cannabispiran	+	+	+
Cannabivarin	o	o	+
CBD	+	+	+
CBC	+	+	++
Δ^8-THC	+	++	+
Δ^9-THC	+	+	++
CBN	+	0	++

Dexamethasone at a concentration of 100 nM could suppress the increment of NO. Interestingly, treatment with hemp extracts also contained the release of NO from cells in a dose dependent manner (Figure 3). NO level was clearly decreased after treatment at concentrations of 5 and 10 μg/mL (about 50 and 80 % efficacy compared to dexamethasone, respectively). Moreover, the level of NO released after co-treatment with 10 μg/mL hemp extracts was almost the same as the NO level observed in the dexamethasone-treated group. PU was likely to provide the highest inhibitory effect (95 % potency of dexamethasone), although no significant differences were found among the extracts. The same tendency was detected in the determination of PGE2 (Figure 4). Remarkably, 1 μg/ml of HH was found to reduce PGE-2 production compared to other cultivars (42 % potency of dexamethasone) while PU showed a promising result in PGE-2 reduction at 5 μg/mL (83 % potency of dexamethasone). There was no significant difference in PGE-2 production inhibition among the extracts at a concentration of 10 μg/mL (> 90 % efficacy to dexamethasone).

Effect of hemp extracts on iNOS, COX-2, and IL-1β gene expression

In OA, IL-1β induced synoviocytes to produce NO and PGE2 through the iNOS and COX-2 gene upregulation. Furthermore, IL-1β itself can act as an autocrine, stimulating IL-1β gene expression and increasing the IL-1β level [21]. Therefore, the expression of iNOS, COX-2, and IL-1β genes was measured by real time RT-PCR. The results revealed that the extracts from all hemp cultivars affected the expression of inflammatory genes. The iNOS gene expression was increased after the cells were treated with IL-1β, but the iNOS gene expression was gradually reduced in a dose-dependent manner when co-treated with the hemp extract (Figure 5A). The expressions of the COX-2 and IL-1β genes were also affected by the hemp extracts when they were co-treated with IL-1β (Figures 5B and 5C).
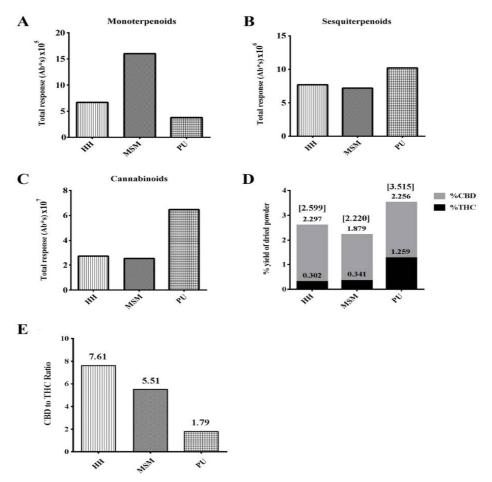
Figure 2: (A) Total monoterpenoid content detected from GC chromatogram of each hemp cultivar. (B) Total sesquiterpenoid content detected from GC chromatogram of each hemp cultivar. (C) Total cannabinoid content detected from GC chromatogram of each hemp cultivar. (D) Amount of cannabidiol (CBD) (light grey), and tetrahydrocannabinol (THC) (black) obtained from hemp extracts of each cultivar. The numbers displayed within the bracket show the total amount of CBD and THC. (E) Ratio of CBD to THC in each cultivar

At a concentration of 5 µg/mL, all the hemp extracts showed promising inhibitory effects (about 60 % potency of dexamethasone), and at 10 µg/mL those effects were nearly equivalent to the effects of dexamethasone (more than 85 % efficacy). Interestingly, the cultivar that contained the highest level of THC (PU) showed the greatest inhibitory effect on the expression of all indicating genes. The values corresponded to the levels of released NO. However, no significant difference in gene expression among all hemp extracts was found.

DISCUSSION

Synovitis is considered to be a major factor of OA pathogenesis. Although the genesis of synovitis is still unclear, the inflamed cells are found to be involved in several symptoms and clinical signs of OA [1,2]. Inflamed synoviocytes produce IL-1β which can orchestrate the production of NO through the induction of iNOS

gene expression. Furthermore, IL-1β stimulates PGE2 synthesis via the upregulation of COX-2 gene expression [21]. NO, PGE2, and IL-1β are pivotal cytokines in the OA inflammatory process and can be used as biomarkers for its diagnosis and treatment [22]. Thus, one of the key concepts in OA therapy is to regulate these proinflammatory cytokines and gene expressions in the synovium. Nowadays, researchers have considered herbal medicine, including hemp, as a substitution for various inflammatory related diseases [10]. Although hemp can be efficiently cultivated in Thailand, this plant has been categorized as a narcotic agent class 5 by the National Narcotic Drug Prohibition Act and is absolutely restricted. It might be necessary to determine the feasibility of hemp using scientific researches. Many parts of the hemp plant can be useful for various purposes; however, the leaves are usually discarded in most of the manufacturing processes regardless of cannabinoids availability [23].
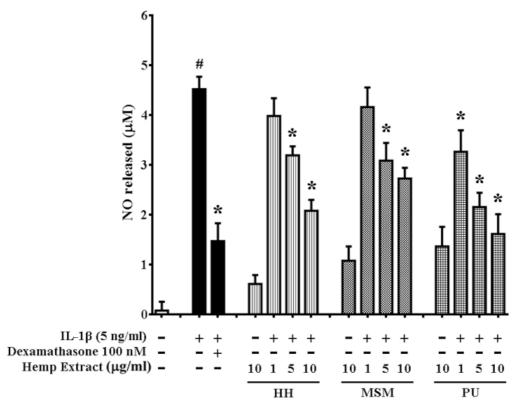
Figure 3: Effect of hemp extracts on NO synthesis from SW982 after IL-1β induction. Statistical significance *at p* < 0.05, compared to IL-1β treated group* and control group#
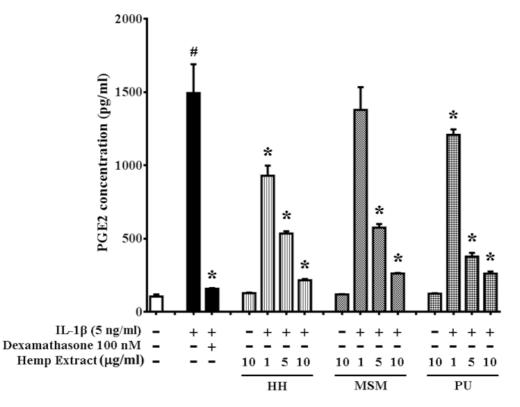

Figure 4: Effect of hemp extracts on PGE2 production from SW982 after IL-1β induction. Statistical significance *at p* < 0.05, compared to IL-1β treated group* and control group#
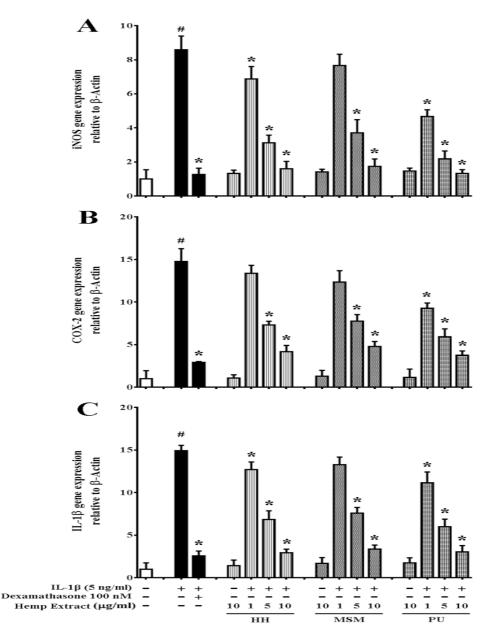
Figure 5: Effect of hemp extracts on inflammatory gene expression: (A) iNOS (B) COX-2 and (C) IL-1β after IL-1β induction. The statistical significance of differences between the groups was determined using one-way ANOVA. Statistical significance at P < 0.05; * when compared to IL-1β treated group, # when compared to control group

Frequently, hemp oil and hemp seed have been used in pharmacology as a neuronal analgesic and an anticonvulsant [9]. Their active ingredients are known to exhibit their effect through their receptors, CB1 and CB2, which are mostly found in the brain, neurons, and immune cells, leading to pain relief and immune balance maintenance. However, these receptors can also be found in some organs and peripheral tissues, such as endocrine glands, spleen, and heart. Moreover, CB2 receptor has been recently informed to enhance the joints of OA patients [14]. Thus, cannabinoids may provide an advantage in the treatment of OA. Moreover, THC and CBD are reported to repress the IL-1β

and TNF-α production in neurons and immune cells [24]. These two cannabinoids have also been notified to inhibit not only NO production but also iNOS gene expression.

Besides, hemp consists of terpenoids, some of which were reported to be useful during inflammatory situations, including suppression of the iNOS gene upregulation [25]. These data provided the goal of this research, i.e., to determine if hemp extracts can reduce the progression of inflammation in synoviocytes. Another objective of our study was to determine whether hemp cultivation sites can affect the amount of cannabinoids in hemp leaves. For that

reason, three hemp cultivars grown in Thailand were included in this study.

In this study, the leaves of hemp cultivars which provided a high ratio of CBD to THC were HH, MSM, and PU. Although PU produced high amounts of cannabinoids, this cultivar also produced a high level of THC (1.259 %) which exceeds the limits specified in the Acts. Therefore, HH was likely to be the ideal choice as this cultivar provides high amounts of CBD and an appropriate CBD to THC ratio, although its biological activities are lower than PU. In addition, there are other cannabinoids contained in hemp plants [23]. Based on GC chromatograms, the amount of THCV was outstanding in PU cultivar, suggesting that THCV might play an important role in anti-inflammation. However, there are very few reports about the effect of THCV on arthritis. Besides, there were unidentified peaks in PU that might need to be investigated in relation to their structures and biological activities. There are also other non-cannabinoid substances contained in hemp plants including terpenoids, alkaloids, flavonoids, and cardiac glycosides [26]. Interestingly, the amount of sesquiterpenoids in each of the hemp cultivar was likely to correlate with the biological responses in induced synoviocytes. This finding indicates that the effect of hemp extracts not only relied on cannabinoids, but also sesquiterpenoids. The major sesquiterpeniods found in this study were β-caryophyllene and humelene, both of which have been reported to reduce PGE2 production and iNOS and COX-2 gene expression in carrageenan injected rats [27]. Both cannabinoids and terpenoids have been reported to possess antioxidant properties which could be useful for diseases that involve reactive oxidation species (ROS), including OA.

SW932 has been identified as the optimal cell line to demonstrate synovitis under arthritis conditions and has been used in several studies [17]. Among the arthritis-related cytokines, IL-1β is the most well-known inflammatory cytokine which can promote self-production (autocrine), stimulate related proinflammatory genes including iNOS and COX-2, and promote synthesis of active cytokines including NO and PGE2 [21]. We initially determined that the concentration of 5 ng/ml of IL-1β provided the most satisfactory condition (data not shown). A positive control used in this study was dexamethasone. The mechanism of action of dexamethasone is to inhibit the transcriptional regulators of proinflammatory genes through glucocorticoid receptor (GR) [28], which therefore suppresses the production of IL-1β, TNF-α and PGE2. More so, dexamethasone reduces the

expression of the iNOS gene and the NO level. From our research, dexamethasone at a concentration of 100 nM could restore inflamed synoviocytes after being triggered with IL-1β, as the level of IL-1β, COX-2 and iNOS gene expressions along with the NO and PGE2 production were restored. Interestingly, when treated with 10 μg/mL of hemp extracts, the level of iNOS and IL-1β gene expressions along with PGE2 production were nearly equivalent to that of dexamathasone-treated cells, regardless of the specific hemp cultivar. This phenomenon might involve activation of CB receptor, which has been found to mediate the function of GR [29].

The difference in cellular responses among cultivars could be clarified when treated at 1 and 5 μg/ml. PU clearly inhibited the production of inflammatory mediators and expression of inflammatory genes compared to the other varieties. Although HH and MSM had different cannabinoid profiles, the responses of inflamed cells were virtually identical to each other. We suggest the relative amount of THC to be the dominant factor related to hemp extract activity.

After converting the CBD:THC ratio to THC:CBD, the proportions of HH, MSM, and PU were 0.131, 0.181, and 0.558, respectively (data not shown). The ratio of THC to CBD of HH and MSM were quite similar which correlated with their cellular responses and was compatible with a previous report indicating an affinity of THC for cannabinoid receptors over CBD, especially CB2 receptor, which was overexpressed in synovitis [12,14]. However, this suggestion could not explain the effect of PU that contained THC about 5 times higher than other cultivars.

This study suggests that the efficacy of hemp extract on synovitis might also relate to sesquiterpenoid content. The level of sesquiterpenoids in HH and MSM were similar while PU contented sesquiterpenoids about 1.5 times greater than others, which corresponded with cellular responses. The level of monoterpenoids did not appear to be involved with biological responses in our study. Nonetheless, hemp extracts with a high proportion of CBD showed favorable outcomes on synovitis. HH cultivar provides promising characteristics for further investigation as it possesses high amounts of CBD, a high ratio of CBD to THC, and is able to confront the inflammation situation. This finding could establish the potency of the hemp extracts in coping with synovial inflammation.

CONCLUSION

The findings of this study show that hemp cultivar extracts significantly reduce proinflammatory cytokines, including NO and PGE2 production, in a dose-dependent manner. In addition, the expression levels of the inflammatory genes, viz, iNOS, COX-2, and IL-1β, declines when hemp extracts are administered to the cells. HH seems to be the most promising cultivar due to its phytochemical contents and biological activities. These findings indicate that hemp extracts or hemp leaf is a potential alternative treatment for inflammation-related diseases including arthritis, but further studies are required in this regard.

DECLARATIONS

Acknowledgement

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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