DEVELOPMENT OF MOUTHWASH CONTAINING HEMP EXTRACT FOR MOUTH ULCER

KHESKANYA KONGKADEE

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Cosmetic Sciences
2021

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has been approved by the Graduate School as partial fulfillment of the requirements

for the Master of Science in Cosmetic Sciences of Naresuan University

Oral Defense Committee

..... Chair
(Associate Professor Watcharee Khunkitti, Ph.D.)

..... Advisor
(Associate Professor Neti Waranuch, Ph.D.)

..... Co Advisor
(Associate Professor Kornkanok Ingkaninan, Ph.D.)

..... Co Advisor
(Wudtichai Wisuitiprot, Ph.D.)

..... Internal Examiner
(Associate Professor Tasana Pitaksuteepong, Ph.D.)

Approved

.....
(Associate Professor Krongkarn Chootip, Ph.D.)

Dean of the Graduate School

Title	DEVELOPMENT OF MOUTHWASH CONTAINING HEMP EXTRACT FOR MOUTH ULCER
Author	KHESKANYA KONGKADEE
Advisor	Associate Professor Neti Waranuch, Ph.D.
Co-Advisor	Associate Professor Kornkanok Ingkaninan, Ph.D. Wudtichai Wisuitiprot, Ph.D.
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ABSTRACT

Oral inflammation and ulceration occur when head and neck cancer patients undergo chemotherapy and radiation treatment. The damaged cells generate reactive oxygen species (ROS) and inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukins; IL-1β, IL-6, IL-8, and prostaglandin E_2 (PGE_2) in response to the inflammation. The infection from gram-positive bacteria and yeast can occur in the ulcerative area before the stage of wound remodeling. The use of a non-irritating mouthwash is the simplest method, which can do in a routine, to deal with those oral conditions. Recently, the use of plants either for cosmetic or pharmaceutical purposes is popular worldwide. *Cannabis sativa* L. subsp. *sativa* (hemp) is one of the interesting plants that contains, a major non-psychoactive compound, cannabidiol (CBD) which demonstrates the anti-inflammatory activity in several models of inflammation.

The objectives of this study were, firstly, to investigate the antioxidation, anti-inflammation, collagen type I production, wound healing, and antimicrobial activities of the hemp extract and CBD. As well, the hemp extract was used as an active ingredient in a mouthwash formulation and the stability of the finished product was investigated.

The hemp extract was obtained by ultrasonic-assisted extraction. The significant compound of interest found in this extract was CBD. The IC_{50} of the hemp extract, which was examined by DPPH radical scavenging assay, was 216.23 ± 5.80

µg/mL. However, the CBD standard showed more potent antioxidant activity than the extract with an IC_{50} value of 107.67 ± 5.58 µg/mL. The non-cytotoxic concentrations of the hemp extract and the CBD were investigated by MTT assay before measuring other cellular biological activities. The treatment of the hemp extracts 50 µg/mL and CBD standard 1 µg/mL greatly inhibited TNF-α and IL-1β production in LPS-induced inflammation in murine macrophage (RAW 264.7) cells. To further explore the inhibition of PGE_2 release, inflammation in human gingival fibroblast (HGF-1) cells was induced by adding IL-1β to the cells. The treatment using the hemp extracts 10 µg/mL greatly inhibited PGE_2 production. As well, the hemp extract at 25 µg/mL was the potential concentration that enhanced pro-collagen type I synthesis.

The ability of hemp in oral wound healing was then determined using a scratch test assay which showed that the hemp extract (5 µg/mL) increased wound closure by 27.92 ± 1.21%, while the treatment with CBD (0.5 µg/mL) also enhanced wound closure by 33.49 ± 1.67% at 48 hours after wounding. Unfortunately, neither the hemp extract nor the standard CBD showed antimicrobial activity.

Based on the results of the cellular biological activity tests that we undertook, the recommended dose of the hemp extract to develop a mouthwash is 5 µg/mL. The stability study of the tested mouthwash indicated chemical stability and stable pH, but the color changed due to the oxidation of the chlorophyll in the plant material. A preservative efficacy test of the mouthwash showed that it was effective against microbial growth during the manufacturing and storage periods.

It can be concluded, therefore, that hemp extract provides antioxidation and anti-inflammation protection and induces collagen synthesis, as well as gingival wound healing activity. Also, the biological activities of hemp extract from the inflorescences are influenced by CBD. The results suggest that mouthwash containing hemp extract may have potential in the treatment of oral inflammation and ulcers.

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CHAPTER I

INTRODUCTION

Background and significance of the study

In head and neck cancer patients, receiving radiotherapy with or without chemotherapy, have frequently encountered side effects to oral tissue. These patients usually have severe pain due to oral mucositis and significant effects on speaking, eating, drinking, as well as quality of life. Particularly, the discontinuity in oral mucosa and underlying tissues lead to oral inflammation and ulceration (Sumita et al., 2014). More than 80% of patients who received radiotherapy alone or in combination with chemotherapy experience these problems (Rodríguez-Caballero et al., 2012). The pathogenesis of oral mucositis is associated with complex mechanisms (Carrozzo et al., 2019). Chemotherapy and radiotherapy induce ROS generation and damage to the DNA of oral cells. Inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and PGE_2, are produced by damaged cells, leading to inflammation and ulceration. Normally, there are many treatments used in the management of irradiation-induced mucositis, including the use of topical applications, anti-microbial agents, anti-inflammatory agents as well as physical therapies (Alterio et al., 2007; Rodríguez-Caballero et al., 2012). However, there is no intervention or treatment used to prevent or treat these conditions. The use of combining agents is more effective. Especially, good oral hygiene is necessary for minimizing or preventing the incidence and severity of oral mucositis in patients. Therefore, using a mouthwash, which is basic, simple oral care, is not only used to clean the whole mouth, prevent bad breath, and eliminate bacteria but also to relieve inflammation.

Plants have been the basis for medical treatments throughout much of human history. Traditional medicine is still widely utilized today, and many plant-derived compounds are used as the basis for evidence-based pharmaceutical drugs. Due to the belief that such products are safer than using synthetic chemicals, the applications and development of herbal medicine or products are an interesting topic for future research.

Cannabis plants including *Cannabis sativa* L. subsp. *sativa* (hemp) and *Cannabis sativa* L. subsp. *indica* (marijuana) have become of interest in Thailand. However, hemp is still under legal control and the inflorescences are still legally considered narcotics. Notwithstanding these legal strictures, hemp inflorescences, which contain high cannabidiol (CBD) and low delta-9-tetrahydrocannabinol (THC), are allowed to be used for research and study. Hemp is also an important source of fiber for producing textiles and various forms of fabric. Cannabis plants share the main phytochemicals; cannabinoids, flavones, and terpenes, which have pharmacological properties.

A lot of research is now being undertaken on CBD, a major non-psychoactive constituent of the cannabis plant (Hartsel, Eades, Hickory, & Makriyannis, 2016), used as an anti-inflammation in both *in vivo* and *in vitro* models (Burstein, 2015; Milando, & Friedman, 2019; Petrosino et al., 2018; Pisanti et al., 2017). It has been reported that CBD has completely inhibited the production of inducible nitric oxide synthase (iNOS) (Esposito et al., 2007; Rajesh et al., 2007) and nitric oxide (NO) (Borrelli et al., 2009; Costa et al., 2004; Costa, Trovato, Comelli, Giagnoni, & Colleoni, 2007), TNF-α (Ben-Shabat, Hanuš, Katzavian, & Gallily, 2006; Hammell et al., 2016; Muthumalage, & Rahman, 2019; Ribeiro et al., 2015) and IL-1β (Borrelli et al., 2009; Esposito et al., 2007; Horváth, Mukhopadhyay, Haskó, & Pacher, 2012; Kozela et al., 2010; Pan et al., 2009). Also, hemp leaf methanolic extract is reported to inhibit NO, iNOS and IL-1β (Duangnin et al., 2017). The bioactivity of CBD includes not the only anti-inflammatory activity but also exhibits antimicrobial activity. It has been found to inhibit gram-positive bacteria (Van Klingeren, & Ten Ham, 1976). In addition, CBD exposure up-regulated the COL I and COL III gene expression in human dental pulp cells (HDPCs) (Qi et al., 2021). In addition to CBD, collagen production and wound healing of hemp seed extract have also been reported in human fibroblast cell lines (Hs68) (Jin, & Lee, 2018).

The therapeutic effects of plants have been increasingly studied over recent times. Many studies have evaluated the effects of plants on oral-associated diseases and other applications in dentistry. Given the many pharmaceutical properties of CBD in hemp, this research focused on the biological activities of CBD contained in hemp,

including antioxidant activities, anti-inflammation activities, collagen production, wound healing and anti-microbial activity. Also, a mouthwash containing the optimal effective hemp extract dose was developed. The bioactivities were used for evaluating the potential of oral health care applications containing hemp extract development. The knowledge gained from this research will also be the basis of future oral care products as well as cosmeceutical products.

Objectives of the study

1. To determine the antioxidant activity of hemp extract and CBD
2. To investigate the following cellular bioactivities of hemp extract and/or CBD
    2.1 the cytotoxicity to murine macrophage (RAW 264.7) cells and human gingival fibroblast (HGF-1) cells
    2.2 the inhibition of inflammatory cytokines (TNF-α, IL-1β and PGE_2)
    2.3 the production of pro-collagen type I
    2.4 the wound healing property
3. To study an anti-microbial activity of hemp extract and CBD
4. To develop the mouthwash containing hemp extract
5. To evaluate the stability of the mouthwash

Scope of the study

The cytotoxicity, anti-inflammatory activity, collagen production, wound healing effectiveness and anti-microbial of hemp ethanolic extract and CBD were examined. The anti-inflammatory activity in RAW 264.7 and HGF-1 cells was also studied to identify the ability of the hemp extract and CBD to reduce the inflammatory mediators, including TNF-α, IL-1β, and PGE_2. In addition, the production of collagen type I was measured in the HGF-1 cells, and the wound healing properties were evaluated by a scratch wound healing assay. Further, the antimicrobial properties of the hemp extract and CBD against gram-positive cocci bacteria (*S. aureus* and *S. mutans*) and Fungi (*C. albicans*) were studied. Finally, a mouthwash containing hemp extract was developed and its stability was investigated.

Expected outcome of the study

1. This study provided information about the antioxidant, anti-inflammation, anti-microbial, wound healing activities, and collagen type I production of the hemp extract and CBD.

2. The mouthwash containing hemp extract was developed and will effectively use to reduce inflammation and enhance wound healing by oral ulcer patients.

CHAPTER II

LITERATURE REVIEW

Cannabis sativa L. subsp. *sativa*

Figure 1 The appearance of *Cannabis sativa* L. subsp. *sativa*

Source: https://hempoint.cz/en/the-chance-to-remove-the-barriers-of-the-hemp-industry-development/

Cannabis sativa L. is a plant in the cannabaceae family and Cannabis genus, also known as marijuana or hemp. This plant and its extracts have been an important source of food, textile fiber, medicine, as well as psychoactive drugs, since ancient times (Russo, 2007). Firstly, Cannabis has been divided into three main species: a fiber-type is *C. sativa* L. subsp. *sativa* or hemp or industrial hemp, a drug-type is *C. indica* Lam. subsp. *indica* or marijuana, characterized by high levels of the delta-9-tetrahydrocannabinol (Δ9-THC), and another one is *C. ruderalis* Janisch (Hartsel et al., 2016). Generally, hemp contains high fiber content. So, it is used for the fabric and textile industry.

The morphology of hemp

Figure 2 The leaves of *Cannabis sativa* L. subsp. *sativa*

Source: https://www.britannica.com/plant/cannabis-plant.

Figure 3 The cross sections of the stem of marijuana (left) and hemp (right)

Source: Small, 2015

The differences between hemp and marijuana are morphology and phytochemical constituent. The main morphologies of hemp are leaves which long, small, thin, and usually 5-9 leaflets per leaf. The stem is thick, hollow and long, and there are few branches, all of which contain both cellulosic and woody fibers. The

core is lignified, while the cortex has long cellulose-rich fibers, known as bast fibers (Guerriero, Sergeant, & Hausman, 2013). Normally, the height varies from 6 to 12 feet. The buds are around 3 feet in length and thin (Thomas, & Elsohly, 2015). The separation of sex appears in their flowers.

The chemical compositions of the cannabis plant

The cannabis plant produces more than hundreds of natural compounds of different chemical classes (Hartsel et al., 2016; Upton, & ElSohly, 2014) and is characterized by a complex of chemical compositions, including terpenes, carbohydrates, fatty acids and their esters, amides, amines, phytosterols, phenolic compounds. Particularly, the specific compounds of this plant are cannabinoids or phytocannabinoids that have not been detected in any other plant (Andre, Hausman, & Guerriero, 2016; Raphael Mechoulam, Peters, Murillo-Rodriguez, & Hanuš, 2007).

1. Non-cannabinoids

1.1 Terpenes

Terpenes are the most abundant classes with volatility and are responsible for the odor and flavor of cannabis. They are derived from the repeating pattern of isoprene units (5-carbons); such as monoterpenes, sesquiterpenes, and triterpenes. Both mono and sesquiterpenes are detected in fresh flowers at glandular trichomes, roots and leaves of the plant (Andre et al., 2016; Da Porto, Decorti, & Natolino, 2014). The examples of monoterpenes that are produced by cannabis, are β-myrcene, D-limonene, α- and β-pinene as well as linalool. As regards monoterpenes, Myrcene is known to permeate the cell membrane including the blood-brain barrier and increase transdermal absorption (Schmitt, Schaefer, Doebler, & Reichling, 2009). β-myrcene possesses anti-inflammatory, analgesic, and anxiolytic properties (Andre et al., 2016). Moreover, it shows anti-inflammatory and anticatabolic effects in human chondrocyte models of osteoarthritis (Rufino et al., 2015). In addition, α-Pinene is an acetylcholinesterase inhibitor (Kennedy et al., 2011). As for sesquiterpenes, cannabis plants produce β-caryophyllene, α- humulene as well as carophyllene oxide. Particularly, β-caryophyllene was found to be an anti-inflammatory agent and a gastric cytoprotector (Andre et al., 2016). Further, Limonene also has anti-inflammatory effects in models of osteoarthritis (Rufino et al., 2015) and asthma

(Hirota et al., 2012). Additionally, triterpenes are abundantly found in hemp root, hemp fiber and hemp seed oil, such as β-amyrin, cycloartenol, and dammaradienol (Andre et al., 2016).

1.2 Flavonoids

Flavonoids are found in many of the parts of cannabis. For example, flowers (not present in glandular trichrome (Pollastro, Minassi, & Fresu, 2018)), leaves, twigs and pollen (Vanhoenacker, Van Rompaey, De Keukeleire, & Sandra, 2010). Especially, cannflavin A and B are found to inhibit inflammation via the inhibition of PGE_2 and 5-lipoxygenase (Werz et al., 2014). In addition, Cannflavin A is found to be a good antileishmanial activity and a moderate antioxidant. Furthermore, Cannflavin B also possesses an antimicrobial and antileishmanial activity (Pollastro et al., 2018).

2. Cannabinoids

Cannabinoids or phytocannabinoids are a group of C_{21} or C_{22} (meroterpenoid) terpenophenolic compounds. There are more than 86 cannabinoids identified in *C. sativa*, including delta-9-tetrahydrocannabinol (Δ9-THC), cannabidiol (CBD), cannabichromene (CBC), cannabigerol (CBG), cannabinol (CBN), cannabicyclol (CBL), cannabielsoin (CBE), and cannabitriol (CBT). They are mostly synthesized in glandular trichomes, especially in female inflorescence. The most abundant cannabinoids found in hemp are cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA) and are further decarboxylated to CBD and CBG, respectively (Brighenti, Pellati, Steinbach, Maran, & Benvenuti, 2017).

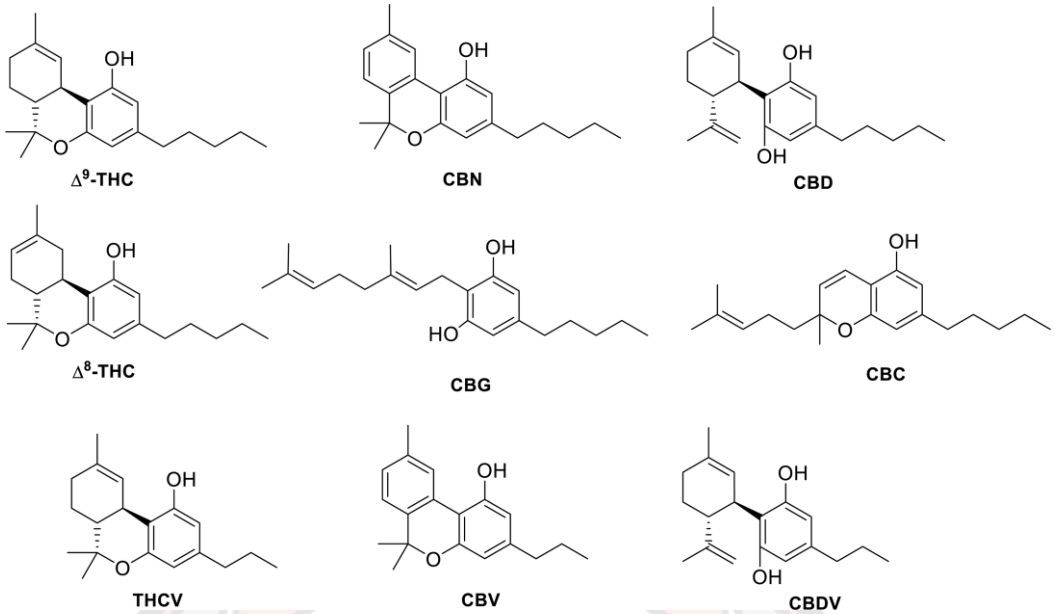


Figure 4 The structures of well-known cannabinoids

Source: Morales, Hurst, & Reggio, 2017

3. The biosynthesis of cannabinoids

The biosynthesis of cannabinoids is illustrated in Figure 4. The geranyl pyrophosphate (GPP) from deoxyxylulose phosphate/methylerythritol phosphate ((DOXP)/MEP) pathway and olivetolic acid (OLA) from polyketide pathway are catalyzed by geranylpyrophosphate: olivetolate geranyltransferase, leading to the formation of cannabigerolic acid (CBGA). Then, the specific enzymes, including tetrahydrocannabinolic acid (THCA) synthase, cannabichromenic acid (CBCA) synthase and cannabidiolic acid (CBDA) synthase, oxidocyclized CBGA to THCA, CBCA, and CBDA, respectively. Finally, they are decarboxylated by heat or light resulting in the formation of THC, CBC, and CBD, respectively. (Small, 2015; Thomas, & Elsohly, 2015)

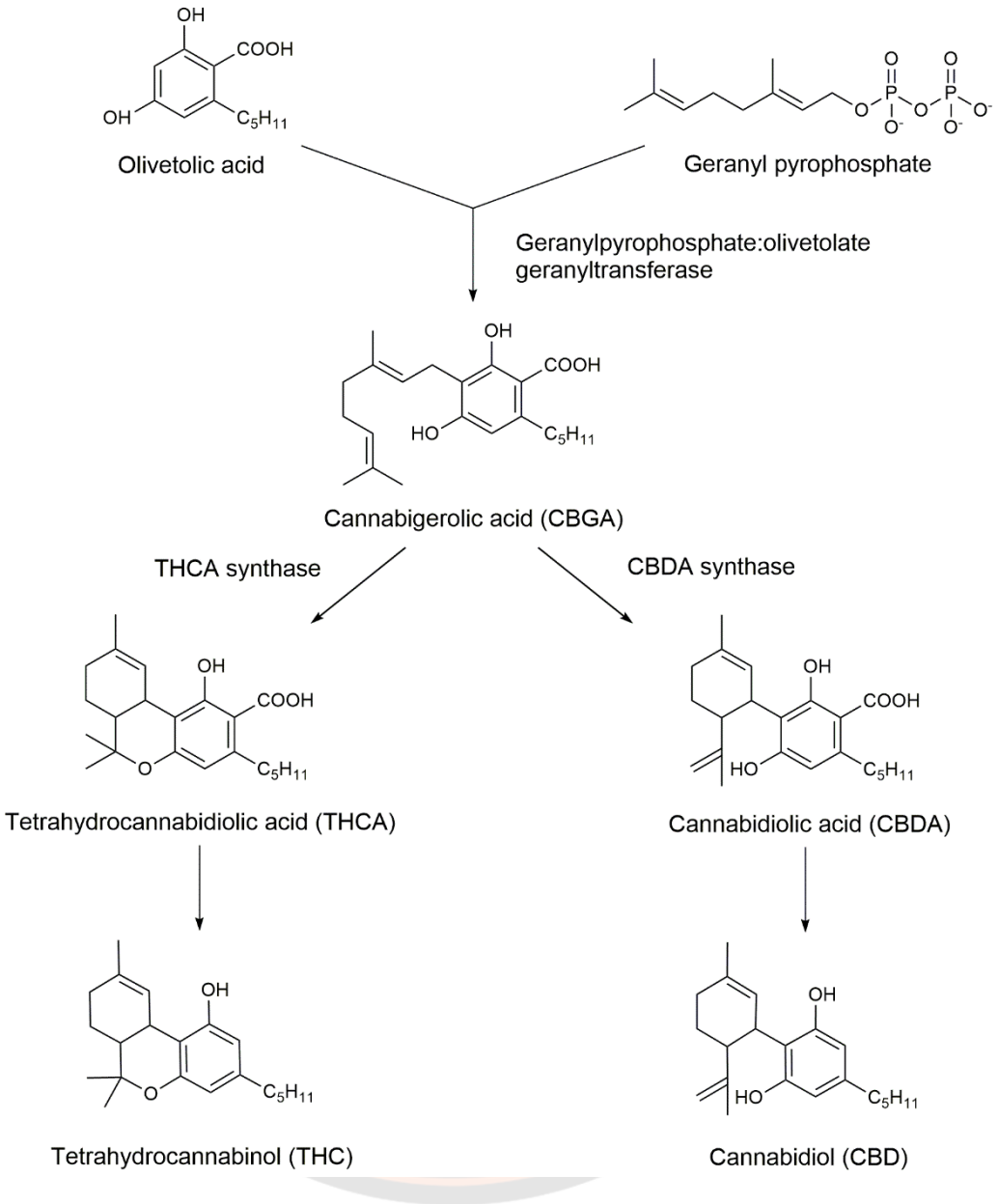


Figure 5 The biosynthesis of THC and CBD

Source: Modified from Small, 2015; Thomas, & Elsohly, 2015

4. Some bioactivities of cannabinoids

4.1 Delta-9-Tetrahydrocannabinol (Δ9-THC)

Δ9-THC is a psychoactive phytocannabinoid that is found abundantly in drug-type plants. It is known to have many pharmacological properties. The pure synthetic Δ9-THC (dronabinol) is orally administered to HIV/AIDS patients to

mitigate cancer chemotherapy-induced nausea and vomiting and for appetite stimulation. More recently, a medication that contains plant-extracted Δ9-THC and CBD (Sativex) is also effective in the treatment of anorexia as well as nausea; and is used for the relief of neuropathic pain from cancer and multiple sclerosis (Hill, Williams, Whalley, & Stephens, 2012).

4.2 Cannabigerol (CBG)

CBG is a non-psychoactive substance and acts as the precursor of phytocannabinoids in its acid form. It has been shown to relieve intraocular pressure, which is potentially useful in the treatment of glaucoma. Additionally, it possesses antioxidant, anti-inflammatory properties, antimicrobial, as well as analgesic activities (Alexander, 2016; Brenneisen, 2007; Izzo, Borrelli, Capasso, Di Marzo, & Mechoulam, 2009). For the antibacterial properties, it has activity against gram-positive bacteria, mycobacteria, and fungi (Eisohly, Turner, Clark, & Eisohly, 1982). It also has the potential for the treatment of colon cancer (Ligresti et al., 2006).

4.3 Cannabidiolic acid (CBDA)

CBDA is the first biogenic cannabinoid formed in the plant (Shoyama, Yagi, Nishioka, & Yamauchi, 1975). It has antimicrobial and anti-nausea properties (Alexander, 2016; Brenneisen, 2007; Izzo et al., 2009). In addition, it is an anti-inflammatory that acts as a selective inhibitor of cyclooxygenase-2 (COX-2) (Takeda, Misawa, Yamamoto, & Watanabe, 2008)

4.4 Cannabidiol (CBD)

CBD is a major non-psychotropic constituent of cannabis that was isolated in 1940 (Adams, Hunt, & Clark, 1940), and its structure was elucidated in 1963 by Mechou and Shvo (R Mechoulam, & Shvo, 1963). The pharmacological activities of CBD were studied and reported at about that time. It was found to have anticonvulsant properties in rats (Consroe, & Wolkin, 1977). Subsequently, CBD treatment, in the range of 900–1,200 mg/kg daily, was shown to be an effective treatment for seizures in humans (Trembly, & Sherman, 1990). Moreover, CBD has a protective effect against psychological effects from THC, called THC antagonist (Niesink, & van Laar, 2013). Therapeutic studies of CBD into its anti-inflammatory properties were undertaken by (Burstein, 2015), (Pisanti et al., 2017) reviewed its antioxidant properties, and its analgesic properties were researched by (Costa et al.,

2004). In addition, CBD also acts as a strong neuroprotective antioxidant against glutamate toxicity, which is more effective than vitamin C and E (Hampson, Grimaldi, Axelrod, & Wink, 1998) and can reduce acne (Oláh et al., 2014).

5. Anti-inflammatory properties of CBD

Inflammation is the process occurring in the body which initiates harmful or abnormal stimuli to the body, including infection, injury, toxicity, and stress conditions. It contributes to the development and progression of many diseases and conditions. The highly effective anti-inflammatory cannabinoids are THC and CBD (Hartsel et al., 2016). CBD can suppress the production of many proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) (Ribeiro et al., 2015), prostaglandins (PGs) and interleukin-1beta (IL-1β) (Pan et al., 2009), and growth factors. Moreover, it also inhibits many processes of the immune cells including activation, proliferation, maturation, migration as well as antigen presentation (Burstein, 2015).

The anti-inflammatory properties of CBD have been widely studied. In rat models, oral administration of CBD (5–40 mg/kg) reduced prostaglandin E2 (PGE_2), nitric oxide (NO), and malondialdehyde production, as well as COX activity in a carrageenan-induced inflammation (Costa et al., 2004). It was found to completely inhibit the production of TNF-α in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells at a concentration of 8 μM (Ben-Shabat et al., 2006). In addition to synovial cells culture, mice were treated with CBD (5 mg/kg, daily, for 10 days). The results showed that the release of TNF-α was less in *in vitro* cultures than in synovial cells from control animals (A. M. Malfait et al., 2000). This finding suggested that the therapeutic action of CBD in arthritis was the suppression of TNF-α, a major mediator of this disease. Corresponding to the result after inducing arthritis in rats by Freund's adjuvant, different CBD doses (0.6, 3.1, 6.2, or 62.3 mg/day) were applied daily (transdermal administration) for 4 days. CBD reduced immune cell infiltration, joint swelling, and the thickness of the synovial membrane, as well as nociceptive sensitization, in a dose-dependent manner. Further, the TNF-α levels were also reduced in the dorsal root ganglia (Hammell et al., 2016). Results from mice models indicated that the intraperitoneal or subcutaneous injection of CBD at a concentration of 10 mg/kg inhibited LPS-induced serum TNF-α (Raphael Mechoulam et al., 2007).

The reduction of IL-1β and TNF-α levels had also been observed in mitogen-activated human peripheral blood mononuclear cells as well (Watzl, Scuderi, & Watson, 1991). Recently, CBD had been found to significantly reduce cytokines production in an allergic contact dermatitis model, using human keratinocyte cell lines (HaCaT) (Petrosino et al., 2018). Likewise, CBD suppressed TNF-α, IL-1β, and IL-6 release by reducing nuclear factor-kappa B (NF-*κ*B) phosphorylation along with COX and iNOS activation in a model of neuroinflammation using LPS stimulated rat microglia (Kozela et al., 2010). Recently, CBD had been found to inhibit inflammation of oral ulcers in rat models with an intraperitoneal injection of CBD (5 mg/kg and 10 mg/kg). However, it was unable to accelerate wound healing (Klein et al., 2018). The effect of CBD on collagen was reported by Qi Xin and coworkers (Qi et al., 2021). RT-PCT analysis represented the expression of COL I and COL III genes in human dental pulp cells (HDPCs) that were up-regulated after exposure to 1 μM of CBD.

6. Anti-microbial properties of CBD

CBD can inhibit Staphylococci and Streptococci bacteria with the minimum inhibiting concentrations (MIC) range of 1-5 μg/mL (Van Klingeren, & Ten Ham, 1976). It also exhibits the potential activity against methicillin-resistant *Staphylococcus aureus* (MRSA) with MIC of 0.5-1 μg/mL (Appendino et al., 2008). Recently, MIC of CBD at 1–4 μg/mL was reported to inhibit more than 20 strains of gram-positive bacteria, including MRSA, multi-drug resistant (MDR), and the anaerobic bacteria *Clostridioides difficile* and *Cutibacterium acnes.* Interestingly, CBD was firstly reported to inhibit gram-negative bacteria, namely *Neisseria gonorrhoeae*, with an MIC of 1 μg/mL (Blaskovich et al., 2021).

Oral cavity

The oral cavity or mouth is defined as the area extending from lips to the anterior of the tonsils, palate, cheeks, muscular floor, and tongue (Goel, & Long, 2018). It is the first part of the digestive system, which is responsible for food digestion, chewing, swallowing and taste perception. In addition to digestion, it aids in breathing and speaking (Jose, Rajagopal, & Thankam, 2021). Normally, the oral cavity maintains a pH around 6.7-7.3 due to saliva production. The pH of saliva is approximately 6.2-7.6 (Baliga, Muglikar, & Kale, 2013). The pH below 5.5 leads to

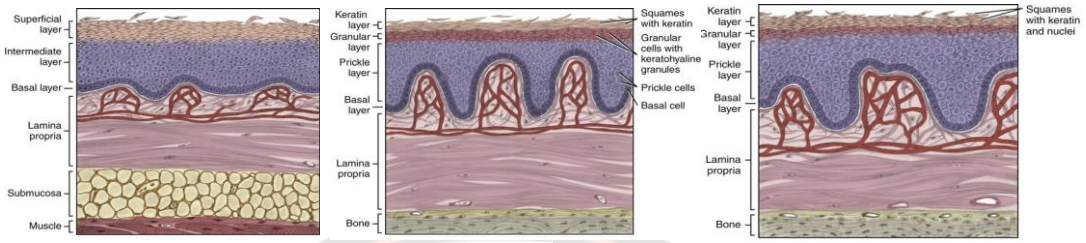
the demineralization of enamel and dental caries (Loke, Lee, Sander, Mei, & Farella, 2016).

Normal flora of the oral cavity

There are more than 700 types of bacteria or phylotypes, which are detected in the oral cavity. Streptococcus, Veillonella and dipheroids are the most found in the oral of healthy person (Vesna, 2018), depending on age. In breastfed babies, *Streptococcus salivarius* (*S. salivarius*), which is suggested as a probiotic, appears after the first breastfeeding to maintain the bacterial community structure (Wescombe, Hale, Heng, & Tagg, 2012). This then allows other anaerobic streptococci, including *S. mutans* and *S. sanguis*, to inhabit the mucosa surface and teeth. These various strains of beneficial bacteria are responsible for immune defense against invading pathogens by releasing fatty acids, bacteriocins, and peroxides. In addition to bacteria, fungi, which included Candida, Cladosporium, Aureobasidium, Saccharomycetales, Aspergillus, Fusarium, and Cryptococcus are also found in the oral cavity (Ghannoum et al., 2010). However, they can cause the diseases, such as dental caries, gingivitis, and periodontal disease. *S. mutans*, which are gram-positive facultative bacteria, are a significant contributor to dental caries and survive in low pH conditions. *C. albicans*, which are detected in gastrointestinal and genital tracts, are also opportunistic pathogenic yeasts in immunocompromised patients.

The structure of oral mucosa

The oral mucosa is composed of 3 layers; the epithelial layer, the laminar propria or subepithelial layer, and the submucosa. (Brizuela, & Winters, 2021)



(A) **(B)** **(C)**

Figure 6 The structure of oral mucosa (A) non-keratinized, (B) parakeratinized, (C) orthokeratinized epithelium

Source: https://pocketdentistry.com/9-oral-mucosa/#s0015

1. **Epithelial layer**

It is a stratified squamous with keratinized (para- and ortho-) and non-keratinized epithelial lining on the most upper area. This layer also acts as a barrier that protects the inner tissues from pathogens or hazardous conditions. The epithelial layer is composed of epithelial cells, which are linked together by desmosome, as well as melanocyte, langerhans cells, and merkel cells.

Non-keratinized epithelium is found on the labial mucosa, lateral buccal mucosa, soft palate, and ventral tongue. The difference between orthokeratinized and parakeratinized is prickle nuclei of the cells in the keratin layer, which is found only in the parakeratinized epithelium of the gingiva, and hard palate which overlay the bone. In addition, the epithelial lining of the tongue can be either keratinized or non-keratized.

2. **Lamina propria**

The lamina propria is a loose connective tissue layer located beneath the epithelium. It is separated from the epithelium by the basement membrane. This layer is composed of many cell types such as fibroblasts, endothelial cells, Schwann cells

and immune cells. Lamina propria contains collagen and elastic fiber which are produced by fibroblast.

3. Submucosa

This is the deepest layer of the mucosa. It contains adipose tissue, glands, and nerves and is supported by dense connective tissue.

Gingival fibroblast

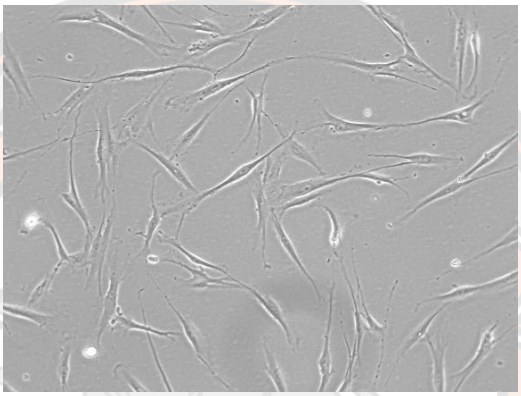


Figure 7 The morphology of human gingival fibroblast cells (100x magnification)

Gingiva is composed of fibrous tissue and is covered by stratified squamous epithelium. There are many cell types found in the connective tissue. Gingival fibroblast is usually found in and plays an important role in the synthesis of collagen, which is the major component of the extracellular matrix. Type-I collagen is a major protein that serves as a structural framework in many tissues. The formation of type I collagen is associated with fibrosis and fibrogenesis. In particular, it is involved in the wound healing, maintenance, and regeneration of the integrity of periodontal connective tissue (Pitaru, McCulloch, & Narayanan, 1994). Gingival fibroblast is generated from neural crest-derived ectomesenchyme during the development.

Normally, fibroblasts are inactive cell form but, when it receives stimuli, it becomes active. Once, tissue is damaged or is subject to bacterial infection, fibroblasts are the first cells that respond to these situations. Tissue injury releases the chemokines and growth factors that trigger the fibroblast to produce further

chemokines such as interleukin (IL); IL-6 and IL-8 as well as other chemokines. The release of IL leads to neutrophil recruitment to the site of injury (Bordin, Flemmig, Habil, & Verardi, 2009). It further promotes inflammation control and tissue repair. Moreover, it produces many cytokines, such as IL-1β, IL-6, IL-8, TNF-α, monocyte chemotactic protein (MCP)-1, osteoprotegerin (OPG), macrophage inflammatory protein-1α (MIP-1α) and receptor activator for nuclear factor-kB ligand (RANKL).

Chemotherapy and radiotherapy-induced oral mucositis

Chemotherapy (CT) and radiotherapy (RT) are commonly used as the primary treatment for head and neck cancers. They are the effective treatment of neoplasm but adversely affect the oral mucosa. These treatments destroy not only the rapidly dividing cells or cancer cells but also the normal cells in the oral cavity. The head and neck cancer patients may suffer from severe inflammation, ulceration and bleeding in their mouths after receiving these treatments (Duncan, & Grant, 2003). They are absorbed by the buccal and oral tissue, especially in rapidly dividing cells such as fibroblasts that cause the inflammation. The important factors involved in mucositis are the radiation dose, type of chemotherapy administration, the administration plan, location and size of the tumor, as well as the patient's immune system (Porock, 2002). The mucosal injury induced by CT can develop within 1-2 weeks of drug administration or occur concomitantly with radiotherapy (Lalla, Sonis, & Peterson, 2008). Further, mucositis occurs after receiving the cumulative doses of radiation around 15 to over 30 Gray (Gy) (Vissink, Jansma, Spijkervet, Burlage, & Coppes, 2003). Erythema is the first sign of mucositis which occurs after receiving RT more than 20 Gy. Ulceration develops after a week of receiving the cumulative dose of RT more than 30 Gy (Rodríguez-Caballero et al., 2012).

Oral mucositis is an inflammation of the oral mucosa, characterized by the presence of edema, erythema, inflammation and ulceration as well as bleeding (Scully, Epstein, & Sonis, 2004). The cells in epithelial and subepithelial layers changed are directly influenced by RT or CT. Patients who have received CT may be at increased risk of neutropenia and local infection or septicemia, particularly involving the streptococci, *Candida* spp., as well as gram-negative bacteria (Scully et al., 2004). Oral levels of *S. mutans*, *Lactobacillus* spp. and *Candida* spp. increase

significantly after radiotherapy (Scully, Epstein, & Sonis, 2003). Also, the mRNA levels of TNF-α and IL-1β in oral mucosal tissue are correlated with the severity of mucosal injuries. (S. Sonis et al., 2000) demonstrated, in a hamster cheek pouch model, that following treatment with anti-inflammatory cytokine IL-11, the levels of mucosal injuries were significantly decreased as well as reduced mucosal injury. Similarly, patients treated with synchronous radiotherapy and chemotherapy for head and neck cancer had increased the levels of PGE_2 in plasma corresponding to the severity of oral mucosal injury (Tanner, Stamford, & Bennett, 1981).

Some inflammatory cytokines related to oral mucositis

1. **Tumor necrosis factor-α (TNF-α)**

TNF-α is a pleiotropic cytokine produced by activated macrophages T lymphocytes, natural killer (NK) and monocytes. It induces the inflammatory response by binding to types 1 and 2 TNF receptors (TNF-R1 and -R2) of the target cells (Horiuchi, Mitoma, Harashima, Tsukamoto, & Shimoda, 2010). It also alters physiological and immunological sequelae as well as being a mediator of the pathophysiological responses of various disease conditions (Dinarello, 1996; Pauli, Beutler, & Peterhans, 1989).

2. **Interleukin-1β (IL-1β)**

IL-1β is a pro-inflammatory cytokine produced by many immune cells. It is released as a response to infection and inflammation and acts directly on the fibroblasts at the site of inflammation, attracts and activates immune cells, as well as controls immunomodulatory genes expression (Dunne, & O'Neill, 2003; Tatakis, 1993; Walsh, Wade, Mapp, & Blake, 1998). In addition, it stimulates the production of matrix metalloproteinases (MMP) such as MMP-1 and MMP-3 in human periodontal ligament cells and gingival fibroblasts (Kida et al., 2005).

3. **Prostaglandins (PGs)**

Prostaglandins are increased in inflamed gingiva (Weinberg et al., 2009) and are also produced by the stimulation of TNF-α and IL-1β in gingival fibroblast (Chiquet, Katsaros, & Kletsas, 2015). In addition, PGE_2 is produced from the conversion of arachidonic acid into prostaglandin H_2 (PGH_2). Then, PGE synthase converts PGH_2 into PGE_2. All processes are mediated by cyclooxygenase (COX) in

responding to acute inflammation (Lalla et al., 2010). Moreover, it is the key mediator in periodontal inflammation by suppression of lymphocyte production, inhibition of collagen synthesis and inducing bone resorption. (Dewhirst, Moss, Offenbacher, & Goodson, 1983; Tipton, Flynn, Stein, & Dabbous, 2003)

The pathobiology of oral mucositis

The mechanisms of oral mucositis are divided into 5 phases; initiation, message generation, signaling and amplification, ulceration, and healing (as illustrated in Figure 8) (S. T. Sonis, 2004).

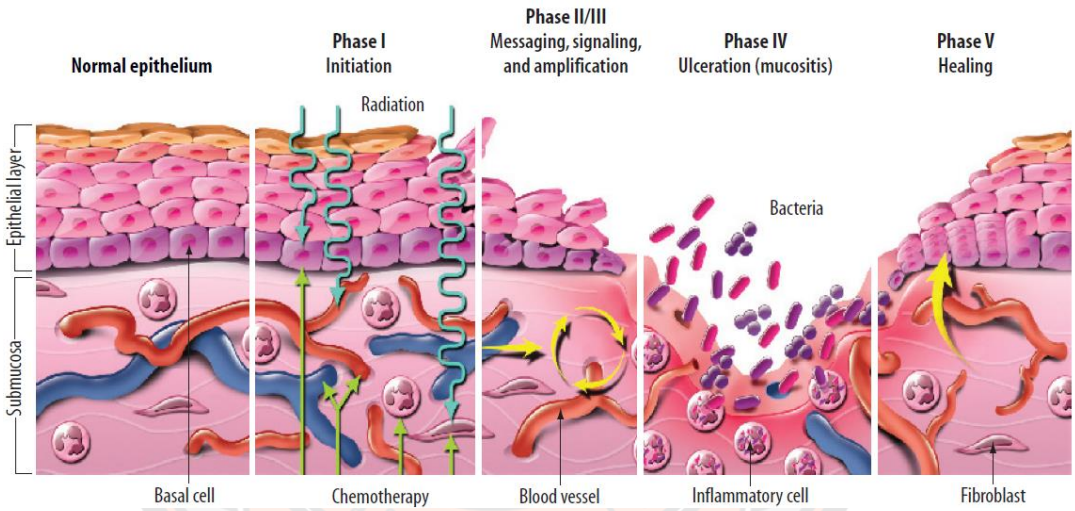


Figure 8 The pathobiology of oral mucositis

Source: S. T. Sonis, 2004

The normal epithelium of the oral mucosa is a stratified squamous epithelium with an intact basal membrane, intact blood vessels, and a normal cell population.

Phase 1 Initiation

Radiation and chemotherapy can directly damage tissue and the DNA of epithelial cells, endothelial cells, macrophages, and fibroblasts. The damaged cells generate reactive oxygen species (ROS). Further, the DNA strand break leads to cell death. (Redding, 2005; S. T. Sonis, 2004)

Phase 2 Message generation

RT, CT, and ROS further activate many transcription factors, particularly nuclear factor Kappa-B (NF-κB). The activation of NF-κB leads to gene expression and release of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6. In addition, the ceramide pathway is activated through sphingomyelinase and ceramide synthase, resulting in the cell death of the submucosal endothelial cells and fibroblasts which are destroyed when the fibronectins break, leading to the release of MMP. The increasing tissue injury causes apoptosis. (Maria, Eliopoulos, & Muanza, 2017; S. T. Sonis, 2004)

Phase 3 Signaling and amplification

This stage occurs from a positive feedback loop of the major pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6). They are mediated through the NF-κB and the ceramide and caspase pathways, resulting in the transcription of genes from the Mitogen-activated protein kinase (MAPK), Cyclooxygenase-2 (COX-2), and tyrosine kinase pathways. IL-1β and TNF-α can induce gingival tissue damage by stimulating the production of prostaglandins (PGs), MMP-1 and MMP-3. These activations lead to extracellular matrix degradation (Graves, & Cochran, 2003), including periodontal tissue. (Maria et al., 2017; S. T. Sonis, 2004)

Phase 4 Ulceration

Ulceration is a loss of epithelium and underlying connective tissue, which is caused by excessive cell death. Patients who are the most symptomatic suffer considerable pain during this phase. The secondary infection from opportunistic yeasts and cross-infection from bacteria, *Staphylococcus aureus* and *Candida albicans*, can occur. Their cell wall products, lipopolysaccharide (LPS), enter the submucosa and macrophages then infiltrate those areas and produce additional levels of inflammatory cytokines leading to severe inflammation and tissue damage. (Maria et al., 2017; S. T. Sonis, 2004)

Phase 5 Healing

Oral healing exhibits the same mechanism as dermal healing. Signaling molecules from the extracellular matrix, such as fibroblast growth factor-2 (FGF-2), Platelet-Derived Growth Factor (PDGF), insulin-like growth factor and transforming growth factors (TGF), control the migration, proliferation, and differentiation of

ulcerative areas. Epithelial cells continuously proliferate until the thickness of the mucosa returns to a normal or intact wound surface (S. T. Sonis, 2004). In addition, fibroblast plays an essential role in the regeneration of the connective tissue, particularly collagen fibers type I, III and V (Chiquet et al., 2015).

Basic treatment of oral mucositis

The treatment methods for oral mucositis focus on the emerging symptoms, such as providing pain relief, anti-inflammation, and subsequent improvement of oral hygiene (Lalla et al., 2008; McGuire et al., 2013). The therapeutic approaches, which are suitable for cancer treatment-induced oral mucositis, are based on the stage of mucositis. The use of several agents or methods, such as growth factors, cytokines, anti-inflammatory agents, natural agents, laser therapy, and cryotherapy, is recommended for the management of mucositis (Raber-Durlacher et al., 2013). Normally, there is no intervention, which is usually used to prevent and treat oral mucositis on its own. So, the use of combination treatments is better. However, good oral hygiene is the simplest and most effective method, which is recommended in the Multinational Association of Supportive Care in Cancer (MASCC) and the International Society of Oral Oncology (ISOO) guidelines. Basic oral care includes rinsing with a non-irritating solution, brushing with a soft toothbrush, etc. (Lalla, & Ashbury, 2013; Maria et al., 2017)

Some therapeutic approaches in the management of oral mucositis

Benzydamine is a nonsteroidal anti-inflammatory agent with antimicrobial and anesthetic properties. It moderates TNF-α production and inhibits NO production as well as ROS scavenging. (Müller-Peddinghaus, 1987)

Chlorhexidine is one of the most effective broad-spectrum antibacterial agents for controlling dental plaque but is known to have a bitter taste.

Low-energy laser therapy with the He-Ne laser technique is recently used to prevent and treat mucositis by accelerating tissue regeneration, healing wounds, as well as reducing pain and inflammation (Posten et al., 2005). It is reported to inhibit the expression of IL-1β and IFN-γ as well as to induce platelet-derived growth factor and TGF-β in gingival wounds in the rat (Safavi et al., 2008).

Vitamin E is a cytoprotective agent and acts as an antioxidant against peroxyl radicals in the cell membrane. It can reduce the symptoms of mucositis in head and neck cancer patients (Wadleigh et al., 1992).

Indigo woad (*Isatis indigotica* Fort.) root is a common herb used in China. It provides antiviral and anti-inflammatory properties and decreases mucosal damage and inflammation in patients who are receiving radiotherapy (You, Hsieh, & Huang, 2009).

Mouthwash

Oral rinses, mouth rinses or mouthwashes have been used by people for many years for the prevention of bad breath, elimination of bacteria and other oral microorganisms, as well as prevention of tooth decay and plaque. In addition, it is used for treating inflammatory conditions such as gingivitis, mucositis, and periodontitis.

Conventional mouthwashes contain 10%-30% by volume of ethyl alcohol. Alcohol is used as an anti-microbial agent and a solvent to dissolve and disperse other additives such as astringents, bactericidal actives, fluorides, essential oils, flavoring agents and color additives. Alcoholic mouthwashes cause a burning sensation, irritation of the mucosa, dry mouth, and pain in patients with existing soft tissue injuries. Especially, cancer patients are sensitive to the inflamed oral mucosa, due to chemotherapy and radiotherapy. Few types of mouthwash are suitable for this group of patients in terms of comfort and safety. Most types of mouthwash contain either a high concentration of chlorhexidine (0. 12%) or alcohol to promote a significant reduction in microbial load in the oral cavity (Shapiro, Giertsen, & Guggenheim, 2002). However, the MASCC/ISOO guidelines prohibit the use of chlorhexidine mouthwash that contains alcohol for oral mucositis patients undergoing RT or CT (Lalla et al., 2008). Therefore, the alcohol-free mouthwash with non-irritation is appropriate for mucositis patients.

CHAPTER III

RESEARCH METHODOLOGY

Materials

Extraction

1. 95% Ethanol (Liquor distillery organization, Chachoengsao, Thailand)
2. Ammonium formate (Sigma-Aldrich, Mumbai, India)
3. Acetonitrile (HPLC) (RCI Labscan, Bangkok, Thailand)

Bioactivity assays

4. Dulbecco's Modified Eagle Medium (D-MEM) powder (high glucose) (Gibco; Thermo Fisher Scientific, Life technologies, New York, USA)
5. Sodium Bicarbonate (Amresco, Ohio, USA**)**
6. Fetal Bovine Serum (FBS) (Gibco; Thermo Fisher Scientific, Life technologies, Paisley, UK)
7. Penicillin Streptomycin (Pen Strep) (Gibco; Thermo Fisher Scientific, Life technologies, New York, USA)
8. L-glutamine (Gibco; Thermo Fisher Scientific, Life technologies, Brazil)
9. 0.25% Trypsin-EDTA (Gibco; Thermo Fisher Scientific, Life technologies, Canada)
10. Dimethyl sulfoxide (DMSO) ≥ 99.5% (GC) (Sigma-Aldrich, St. Quentin, France)
11. Dimethyl sulfoxide (DMSO) (AR) (RCI Labscan, Bangkok, Thailand)
12. Phosphate buffered saline (tablet) (Sigma-Aldrich, St. Louis, USA)
13. L-ascorbic acid (Chem-supply, South Australia, Australia)
14. Sodium Chloride (Calbiochem; EMD Chemicals, New Jersey, USA)
15. Human Pro-Collagen I alpha 1 Matched Antibody Pair kit (ab216064, Abcam, Massachusetts, USA)
16. Mouse TNF alpha Uncoated ELISA Kit (Invitrogen; Thermo Fisher Scientific, Bender MedSystems GmbH, Vienna, Austria)
17. Mouse IL-1 beta Uncoated ELISA Kit (Invitrogen; Thermo Fisher Scientific, Bender MedSystems, Vienna, Austria)

18. Prostaglandin E2 ELISA Kit (ab133021, Abcam, Massachusetts, USA)
19. Hydrocortisone (Sigma-Aldrich, Beijing, China)
20. Lipopolysaccharides from Escherichia coli O26:B6 (Sigma-Aldrich, Rehovot, Israel)
21. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Darmstadt, Germany)
22. Ethanol (AR) (RCI Labscan, Bangkok, Thailand)
23. Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma Aldrich, St. Louis, USA)
24. Soybean Casein Digest Agar (Tryptone Soya Agar; TSA) (Himedia, Mumbai, India)
25. Soybean Casein Digest Medium (Tryptone Soya Broth; TSB) (Himedia, Mumbai, India)
26. Sabouraud Dextrose Agar (SDA) (Himedia, Mumbai, India)
27. Sabouraud Dextrose Broth (SDB) (Himedia, Mumbai, India)
28. Brain Heart Infusion Agar (Himedia, Mumbai, India)
29. Brain Heart Infusion Broth (Himedia, Mumbai, India)
30. Tetracycline 30 µg (Susceptibility test disc) (Oxoid, Basingstoke, UK)
31. Amphotericin-B 20 µg (Susceptibility test disc) (Himedia, Mumbai, India)
32. Tween 80 (PanReac Quimica, Becelona, Spain)
33. Lecithin (Thailand)
34. Cannabidiol (CBD) (THC Pharm, Frankfurt, Germany)

Formulation

35. Sodium benzoate (AR) (Fluka, Neu-Ulm, Germany)
36. Poloxamer 407 (BASF corporation, New Jersey, USA)
37. Xylitol (Krungthepchemi, Thailand)
38. Mannitol (Univar, Ajax Finechem, Australia)
39. PEG-40 hydrogenated castor oil (Cosmetic grade) (Phitsanuchemicals, Thailand)
40. Sorbitol 70% solution (PureChem, Manila, Philippines)
41. Tocopherol acetate (Eisai Co., Ltd, Tokyo, Japan)

42. Menthol (Anhui great nation essential oils Co., Ltd, Shanghai, China)
43. Thymol (Anhui great nation essential oils Co., Ltd, Shanghai, China)
44. Spearmint oil (Anhui great nation essential oils Co., Ltd, Shanghai, China)
45. Peppermint oil (Anhui great nation essential oils Co., Ltd, Shanghai, China)
46. Butylated hydroxytoluene (BHT) (Jiangsu Maida New Material Co., Ltd, China)
47. Fast green (CI 42053) (food grade) (Chemipan, Thailand)
48. Normal Saline Solution (0.9% NaCl w/v) (A.N.B. laboratories Co., Ltd, Thailand)

Apparatus

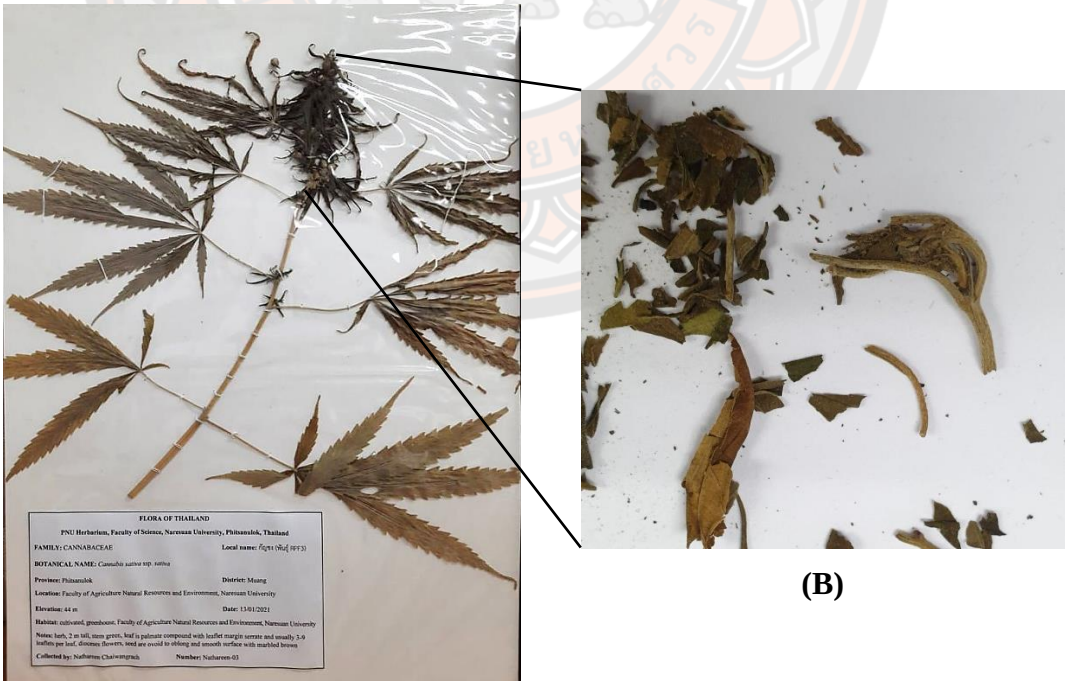
1. Rotary Evaporator (R-210, Buchi, Switzerland)
2. Water bath for evaporation (WNB14, Memmert, Schwabach, Germany)
3. High Performance Liquid Chromatography (1260 infinity II LC system, Agilent, California, United states)
4. Hot air oven (UN 55, Memmert, Schwabach, Germany)
5. Hot air oven (UE 400, Memmert, Schwabach, Germany)
6. Ultrasonic cleaner (1510E-DTH, Bransonic, Danbury, USA)
7. Plate shaker (CRP-412X, CAPP, Denmark)
8. Microplate reader (Eon, BioTek, Vermont, USA)
9. Centrifuge (Universal 320R, Hettich, Tuttlingen, Germany)
10. Laminar airflow cabinet (1300 Series A2, Thermo Scientific, Ohio, USA)
11. CO_2 incubator (Forma Steri-Cult, Thermo Scientific, Ohio, USA)
12. Inverted microscope (AE2000, Motic, Hong Kong)
13. Spectrophotometer (Genesys 20, Thermo Scientific, Ohio, USA)
14. Incubator (WiseCube, Witeg Labortechnik GmbH, Wertheim, Germany)
15. pH meter (pH/Ion S220, Mettler Toledo, Switzerland)
16. Viscometer (DV2T, Ametek Brookfield, Massachusetts, USA)

Methodology

Preparation of the hemp extract

Plant material

Hemp (*Cannabis sativa* L. subsp. *sativa*) female pistillate inflorescences (RPF3 strain; 2019) were received from Highland Research and Development Institute (HRDI) (Public Organization, Chiang Mai, Thailand) on 9 December 2019. The hemp materials were identified by Assistant Professor Dr. Pranee Nangngam with a voucher specimen number 05784 and has been collected at the PNU herbarium (Faculty of Science, Naresuan University). They were dried at 50°C for 24 hours, ground into a fine powder and then sieved through 250 µm/60 meshes. The dried hemp powder 200 g was extracted by using Ultrasonic Assisted Extraction with 95% denatured ethanol (1 L) in the ratio of 1:5 (w/v) for 30 min at 27°C/40 kHz. The ethanolic extract was then filtered through Whatman No.1 filter paper and subsequently, the filtrate was evaporated under a vacuum at 45°C using a rotary evaporator. The remaining hemp powder was extracted twice more. The crude extract was kept at -20°C before using in further experiments.



(A) **(B)**

Figure 9 The herbarium of hemp (RPF3) (A) and its inflorescences (B)

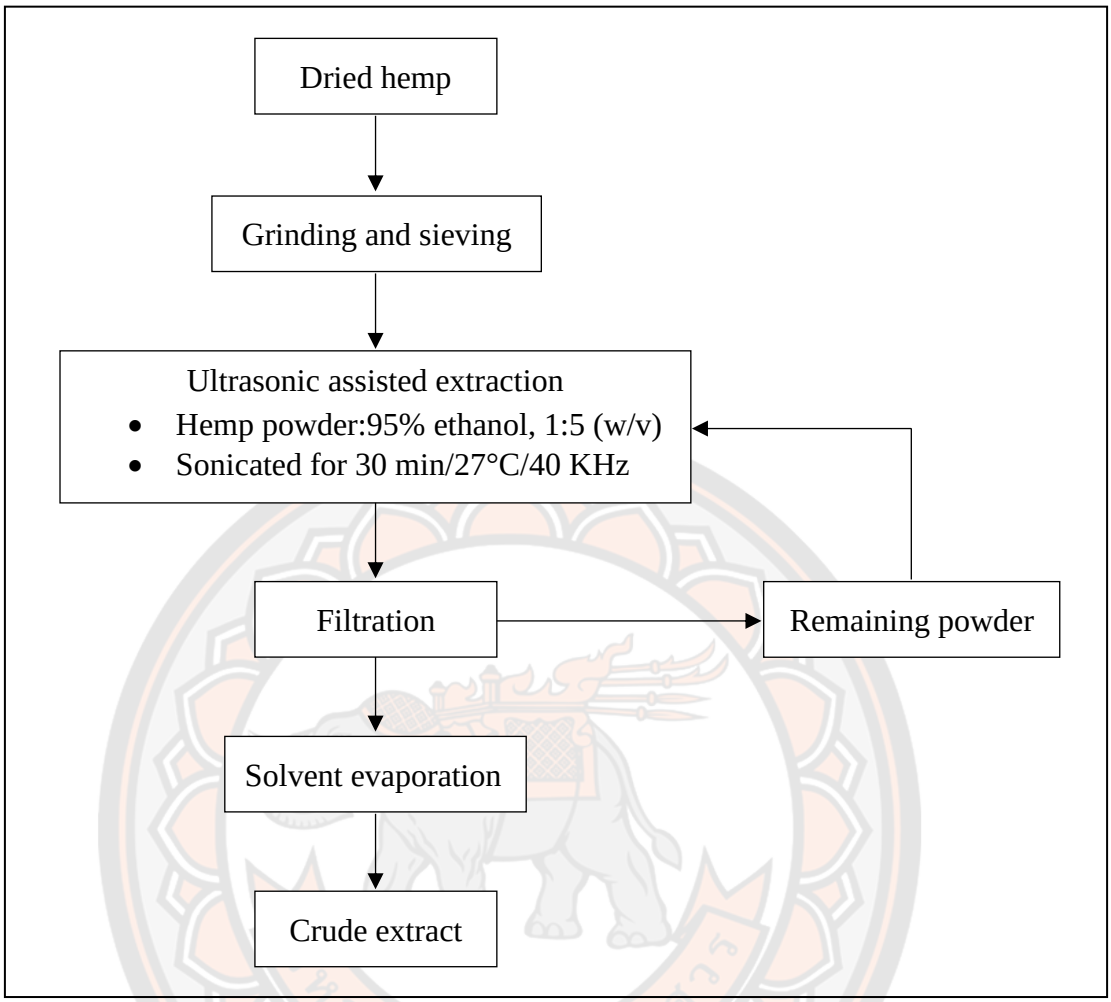


Figure 10 The hemp extraction method by Ultrasonic assisted extraction

Quality control of the hemp extract

1. Quantitative analysis of CBD and THC in the extract

The hemp extract was analyzed by High-Performance Liquid Chromatography (HPLC) to identify the cannabinoid content. CBD and THC were quantified using an Agilent 1260 HPLC system. The hemp ethanolic extract (5 mg) was dissolved in methanol 1 mL and filtered through a 0.45 µm Nylon Syringe Filter. HPLC analysis was carried out using a Phenomenex Kinetex C18 column (150 mm × 4.6 mm, 2.6 µm). Mobile phases of analysis were 20 mM ammonium formate (pH 3.6) and Acetonitrile (ACN) at the ratio of 25:75. The column temperature was maintained at 40°C, with a constant flow rate of 0.8 mL/min for 15 min, and 5 µL of injection volume. Absorbance was evaluated at 220 nm. The quantity of CBD and THC was calculated by comparing the peak area with the standard calibration curve. The percentage of yield was calculated by the following equation:

$$\% \text{ Extract yield} = \frac{\text{Weight of extract}}{\text{Weight of dried plant}} \times 100$$

2. Solubility study of the hemp extract

A 50 mg sample of hemp extract was weighed into plastic tubes to which 10 ml of distilled water was added. The tubes were gently shaken for 24 hours then centrifuged at 5000 rpm for 5 min and the supernatant fluid was discarded from the tube. The tubes were then dried at 50°C in a hot air oven and weighed again.

The solubility of hemp extract in water was calculated by the following equation:

$$\text{Solubility of hemp} = \text{Weight of hemp}_{\text{before}} - \text{Weight of hemp}_{\text{after dried}}$$

3. Bacterial contamination

The number of bacteria, mold and fungi contamination in the plant material was estimated by total plate count. One gram of the hemp extract was mixed with 10 mL of neutralizing diluent that was composed of 5% tween 80 and 0.5% lecithin in sterile water. One hundred microliters of the sample were spread on Tryptic soy agar (TSA) and incubated for 24-72 hours at 37°C for the bacterial count. One milliliter of the sample was spread on Sabouraud dextrose agar (SDA) and incubated

for 24-72 hours at 37°C for mold and fungi count. The colony formed by microorganisms was counted in units of CFU/g.

Determination of the antioxidant activity by DPPH radical scavenging assay

A DPPH radical scavenging assay was used to determine the ability of the hemp and CBD to neutralize the stable DPPH radicals and antioxidant activity. DPPH has an absorbance of about 517 nm and turns a deep violet color in the solution. It becomes colorless or pale yellow when neutralized by antioxidants (Molyneux, 2004).

Seventy-five microliters of each different concentration of the hemp extracts (0.001-10 mg/mL) or CBD (0.001-10 mg/mL) was added to 96 well plate. Then, 150 µL of DPPH solution (0.2 mM in ethanol) was added and incubated on a shaker (200 rpm) by avoiding the light. After 30 min, the absorbance of the preparations was taken at 517 nm using a spectrophotometer. The radical scavenging activity of L-ascorbic acid was used to compare with the concentrations of 0.001-5 mg/mL.

The percentage of DPPH scavenging was calculated by the following equation:

$$\%\ \text{DPPH scavenging} = \left(\frac{A_{sample} - B_{sample}}{A_{control} - B_{control}}\right) \times 100$$

Where; A_{sample} is the absorbance of the test compound mixed with the DPPH solution

B_{sample} is the absorbance of the color of the sample mixed with the solvent

$A_{control}$ is the absorbance of the DPPH solution mixed with the solvent

$B_{control}$ is the absorbance of the solvent

Results interpretation

The calibration curve was plotted from log concentrations of samples against %DPPH scavenging. The half-maximal inhibitory concentration (IC_{50}) was determined using GraphPad Prism version 5.00 for Windows (GraphPad Software, California, USA). The IC_{50} value denoted the concentration of sample required to scavenge 50% of the DPPH radicals. (Banothu, Neelagiri, Adepally, Lingam, & Bommareddy, 2017)

Determination of the cytotoxicity by MTT assay

The cell culture protocol was reviewed by the Naresuan University Institutional Review Board (IRB no. P10140/64).

Cell culture preparation

1. Human Gingival Fibroblast (HGF-1) cells were purchased from ATCC (Manassas, Virginia, USA). They were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% pen-strep, and 2 mM L-glutamine at 37°C, 95% humidity, and 5% CO_2 in an incubator.

2. Murine macrophage (RAW 264.7) cells were purchased from ATCC (Manassas, Virginia, USA). They were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% pen-strep at 37°C, 95% humidity, and 5% CO_2 in an incubator.

Each cell type was sub-cultured at around 80% confluence. The cell culture medium was discarded from the culture flask. Then, 2 mL of sterile PBS (pH 7.2-7.4) was used to rinse off the remaining medium which was done twice. After that, 2-3 mL of 0.25% trypsin-EDTA solution was added to detach the adhesion cells from the flask which were then incubated for 5 min. The same ratio of complete medium was added to stop the trypsin activity. The detached cells were transferred to the centrifuge tube and centrifuged at 1500-3000 rpm for 5 min. Finally, the cells were counted or seeded for the experiment.

Sample preparation

The hemp extract and CBD were solubilized with 100% DMSO. The concentrations of hemp extract and CBD were prepared by 2-fold serial dilution ranging from 3.9 to 500 µg/mL for the hemp extract and 0.08 to 10 µg/mL for the CBD. However, given that a high percentage amount of DMSO can damage the cells, the final percentage of DMSO should be less than 1% which was solubilized in a serum-free medium.

MTT assay

The MTT assay was used to assess the cytotoxicity, cell viability, or cellular metabolic activity from the measurement of mitochondrial activity. Viable cells which have normal mitochondrial activity can reduce a yellow tetrazolium salt or MTT (methyl-thiazolyl-tetrazolium; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide) to purple formazan crystals using mitochondrial succinate dehydrogenase. This activity is detected by the spectrophotometer at 500-600 nm. The dark purple color indicates higher cell viability.

RAW 264.7 and HGF-1 cells were seeded into a 96-well plate at the density of 1.5×10^4 for the RAW 264.7 and 1×10^4 cells/well for the HGF-1 cells. After 24 hours of incubation or when the density of the cells reached 80% confluence, cells were treated with 100 µL of prepared samples. Untreated cells were used as the control. The cells were incubated for 24 hours after which the supernatant fluids were removed and the cells were then incubated with 100 µL of MTT solution (1 mg/mL) for 3 hours in an incubator, avoiding the light. The MTT solution was discarded and 100 µL/well of pure DMSO was added to solubilize the violet formazan crystals on a shaker for 15 min.

Result interpretation

Absorbance was measured at 595 nm using a microtiter plate spectrophotometer.

The percentage of cell viability was calculated by the following equation:

$$\%\ \text{Cell viability} = \frac{\text{Optical density}_{595}\text{of treated cell}}{\text{Optical density}_{595}\text{of untreated cell}} \times 100$$

Determination of the anti-inflammatory activities by ELISA

Sample preparation

The hemp extract and CBD were first dissolved in 100% DMSO which produced the stock solution of 50 mg/mL of hemp extract and 2 mg/mL of CBD. The solutions were then further diluted with the serum-free medium before further treatment. The hemp extract samples were prepared at 2, 10, 20 and 100 µg/mL and CBD samples were prepared at 0.2, 0.5, 1 and 2 µg/mL. The final percentage of DMSO should be less than 1%.

Assay for TNF-α production by sandwich ELISA

RAW 264.7 cells were seeded into a 96-well plate around 1.5×10^4 cells/well with a complete medium and incubated for 24 hours. To induce inflammation, 100 µL/well of lipopolysaccharide (LPS) from *E. coli* (2 µg/mL) was added and incubated for 30 min, and the prepared samples 100 µL/well were then added to the cells.

Hydrocortisone 20 µg/mL was used as a positive control because it is a steroid drug for inflammatory treatment, and untreated cells were used as the negative control. The final concentration of each well was diluted by 50%. After 24 hours of incubation, the supernatant fluids were collected for determining the amount of TNF-α production using Mouse TNF-α Uncoated ELISA kit. (Ben-Shabat et al., 2006)

A 96-well high-binding microplate was coated with captured antibodies (1X) 100 µL/well, the plate was sealed and stored at 4°C overnight. The plate was then washed three times with 300 µL/well of washing buffer (1X). The 200 µL/well of ELISA/ELISPOT diluent (1X) was added to block the non-specific protein binding and incubated at room temperature on a shaker (at 200 rpm) for 1 hour. The plate was washed three times before adding 100 µL/well of TNF-α standard, sample or diluent and subsequently incubated at room temperature on a shaker for 2 hours or, alternatively, stored at 4°C overnight and the plate was then washed five times before adding 100 µL/well of the detection antibody (1X) and incubated on the shaker at room temperature for 1 hour. The plate was washed five times before adding 100 µL/well of Streptavidin/HRP (1X) and incubated at room temperature on a shaker. After 30 min of incubation, the plate was washed seven times, 100 µL/well of TMB was added and the solution was then incubated at room temperature on a shaker for 15 min, avoiding the light. After that, 100 µL/well of stop solution was quickly added to stop the enzymatic reaction. The absorbance was measured at 450 nm using a microtiter plate spectrophotometer. The amount of TNF-α concentration in the culture supernatant from the RAW 264.7 cells was calculated using the TNF-α calibration curve.

Assay for IL-1β production by sandwich ELISA

RAW 264.7 cells were seeded at the density of 5×10^5 cells/well into a 12-well plate with a complete medium and incubated for 24 hours. Similarly, the RAW 264.7 cells were incubated with 2 µg/mL of LPS 500 µL/well for 30 min. Then, 500 µL of the prepared sample of hemp extract and CBD was added. Hydrocortisone 10 µg/mL was used as a positive control, whereas untreated cells were used as a negative control. So, the final concentration of each well was diluted by 50%. The supernatant fluids were collected after 24 hours and assayed for IL-1β using Mouse IL-1β Uncoated ELISA Kit.

A 96-well high-binding microplate was coated with capture antibody (1X) 100 µL/well, the plate was sealed and stored at 4°C overnight. The plate was washed three times with 300 µL/well of washing buffer (1X). The 200 µL/well of ELISA/ELISPOT diluent (1X) was added to block the non-specific protein binding and incubated at room temperature on a shaker (at 200 rpm) for 1 hour. The plate was washed three times before adding 100 µL/well of IL-1β standard, sample or diluent and subsequently incubated at room temperature on a shaker for 2 hours or, alternatively, stored at 4°C overnight. The plate was washed five times before adding 100 µL/well of the detection antibody (1X) and incubated at room temperature on the shaker for 1 hour. The plate was again washed five times before adding 100 µL/well of Avidin/HRP (1X) and incubated on the shaker at room temperature. After 30 min of incubation, the plate was washed seven times, added 100 µL/well of TMB and then incubated at room temperature on a shaker for 15 min, avoiding the light. Then 50 µL/well of stop solution was quickly added to stop the enzymatic reaction. The absorbance was measured at 450 nm using a microtiter plate spectrophotometer. The amount of IL-1β concentration in culture supernatant from RAW 264.7 cells was calculated using the IL-1β calibration curve.

Assay for PGE_2 production by competitive ELISA

HGF-1 cells were seeded at the density of 2×10^4 cells/well into a 24-well plate with a complete medium and incubated until cells reached 80-90% confluence. The cells were then incubated with 100 µL/well of IL-1β standard 2 ng/mL for 20 min to induce the inflammation, following which, 100 µL/well of the hemp extract (2, 20 and 100 µg/mL) was added to the cell. Hydrocortisone 20 µg/mL was used as a positive control. So, the final concentration of each well was diluted by 50%. The supernatant fluids were collected after 24 hours and assayed for PGE_2 concentration using an ELISA kit.

One hundred microliters per well of PGE_2 standard or culture supernatant was added into a Goat anti-Mouse IgG microtiter plate. The 50 µL/well of PGE_2 conjugate was added before adding 50 µL/well of PGE_2 antibody. The plate was incubated at room temperature on a plate shaker (at 300 rpm) for 2 hours and the wells were then washed three times with 400 µL/well of wash solution. A 200 µL quantity of pNpp Substrate solution was added to every well which was then

incubated at room temperature for 45 min without shaking. A 50 µL/well of stop solution was then added and the absorbance was measured at 405 nm using a microtiter plate spectrophotometer. The amount of PGE_2 concentration in the supernatant was calculated using the PGE_2 calibration curve.

Determination of pro-collagen type I production by ELISA

HGF-1 cells were seeded into a 96-well plate at the density of 8×10^3 cells/well with a complete medium and incubated for 24-72 hours until they reached 80-90% confluence. The hemp extract was prepared in different non-toxic concentrations (1, 10, 25 and 50 µg/mL) in a serum-free medium. Two hundred microliters per well of the prepared samples were added to the cells. Untreated cells in a serum-free medium were used as negative control and 10 µg/mL of L-ascorbic acid-treated cells were used as a positive control. After 24 hours of incubation, the supernatant fluids were collected. The pro-collagen type I concentrations in culture supernatant were determined using Human Pro-Collagen I alpha 1 Matched Antibody Pair kit.

A 96-well high-binding microplate was coated with 50 µL/well of a capture antibody (2 µg/mL), the plate was sealed and stored at 4°C overnight or, alternatively, incubated at room temperature on a shaker (at 400 rpm) for 2 hours. The plate was washed three times with 350 µL/well of washing buffer (1X) and 200 µL/well of blocking buffer (1X) was added to block the non-specific protein binding, the plate was sealed and stored at 4°C overnight or, alternatively, incubated at room temperature on a shaker for 2 hours. The plate was washed three times before adding 50 µL/well of pro-collagen type I standard, sample or diluent, and subsequently incubated at room temperature on a shaker for 2 hours. The plate was again washed three times before adding 50 µL/well of a detection antibody (0.5 µg/mL) and incubated at room temperature on the shaker for 1 hour. The plate was then washed three times before adding 50 µL/well of Streptavidin/HRP (0.5 µg/mL) and incubated on the shaker at room temperature for 1 hour. A 100 µL/well of TMB was added and the plate was then incubated at room temperature on a shaker, avoiding the light. After 20 min of incubation, 100 µL/well of stop solution was quickly added to stop the enzymatic reaction. The absorbance was measured at 450 nm using a microtiter

plate spectrophotometer. The amount of pro-collagen type I concentration in culture supernatant from HGF-1 cells was calculated using a pro-collagen type I calibration curve.

Determination of the gingival wound healing by scratch wound healing assay

HGF-1 cells were seeded into a 6-well plate at the density of 1×10^5 cells/well with a complete medium and incubated for 24-48 hours or until they reached 70% confluence. To avoid cell proliferation, HGF-1 cells were subjected to serum starvation for at least 8 hours before making artificial wounds by creating a vertical scratch with a tip of a 200 µL plastic pipette on the cell. The cells were then short-term rinsed with 1.5 mL of the hemp extract (5, 10 and 25 µg/mL), CBD (0.5 µg/mL) or NaCl 0.9% w/v in a serum-free medium for 3 min with 3 times/day (every 5-6 hours) for 48 hours. The short-term rinsing method was modified from the method of Huynh NC and coworker (Huynh, Everts, Leethanakul, Pavasant, & Ampornaramveth, 2016). Images of scratch wounds were captured for 8 continuous fields using the inverted microscope at 0, 12, 24, 36, and 48 hours after wounding.

Results interpretation

The wound closure areas were measured with the ImageJ software and Adobe Photoshop CS6 using the pixel area measurement. (Grada, Otero-Vinas, Prieto-Castrillo, Obagi, & Falanga, 2017; Ramenzoni, Attin, & Schmidlin, 2019)
The percentage of wound closure area was calculated by the following equation:

$$\%\ \text{wound closure} = \frac{A_{t=0h} - A_{t=xh}}{A_{t=0h}} \times 100$$

Where; $A_{t=0h}$ is the area (pixel) of the wound that was measured after scratching.

$A_{t=xh}$ is the area (pixel) of the wound that was measured x hours after scratching.

Determination of the antimicrobial activities

The protocol for determination of antimicrobial activities was reviewed and approved by Naresuan University Institutional Biosafety Committee (NUIBC MI 64-03-09). *Staphylococcus aureus* (DMST 8840), *Streptococcus mutans* (DMST 18777) and *Candida albicans* (DMST 5815) were obtained from the Department of Medical Sciences Ministry of Public Health, Thailand.

The disk diffusion method

Microorganisms preparation

S. aureus, *S. mutans,* and *C. albicans* were grown on Tryptic Soy Agar (TSA), Brain Heart Infusion agar (BHI) and Sabouraud dextrose agar (SDA), respectively. Bacteria were grown at 37 ºC incubator for 24-48 hours with O_2 supply, whereas *S. mutans* did not need to have an O_2 supply (anaerobic bacteria). *C. albicans* were grown either at 25°C for 48 hours or in an incubator at 37°C for 24 hours. The tested organisms were prepared by the direct colony suspension technique. Their colonies were distributed in 0.85% normal saline solution (NSS). This inoculum had a turbidity standard equivalent to an 0.5 McFarland standard or was measured the optical density at 600 nm which was equal to 0.08-0.10. So, the number of microorganisms was 1×10^8 CFU/mL. (Kirby, 2009)

Sample preparation

The hemp extract and CBD were solubilized with DMSO at the concentration of 200 mg/mL and 27 mg/mL, respectively, as the stock solution.

Method

Inoculum suspension in 0.85% normal saline (OD_{600} = 0.08-0.10) was swabbed uniformly in 3 planes on their agar plates and allowed to dry for 5 min. The prepared samples were loaded with the volume of 20 µL/disk on 6 mm sterile individual disks and dried at 40-50°C for 1-2 hours in a hot air oven. The loaded disks were placed on the surface of the agar and allowed to diffuse for 5 min. The plates were further incubated at each suitable condition, described above, for 24-48 hours. The solvents or DMSO were used interchangeably as a negative control. The 30 µg/disk of tetracycline was used as a positive control for bacteria and a 20 µg/disk of amphotericin B was used as a positive control for fungi. Patients with oral ulceration or mucositis were prescribed 0.12% w/v chlorhexidine gluconate commercial

mouthwash to reduce the oral inflammation and, at the end of incubation period of 24 h or 48 h, the diameter of the inhibition zones (mm) that formed around the disk was measured using Vernier calipers. (CLSI, 2018; Thirumurugan, 2010)

Results interpretation

Following the Clinical and Laboratory Standards Institute (CLSI) guideline, each antimicrobial agent has its disk diffusion breakpoint against individual microorganisms.

Table 1 Zone diameter breakpoint of antimicrobial agents against organisms

Microorganisms	**Antimicrobial agents**	**Interpretive categories and Zone Diameter Breakpoints (mm)**		
		Susceptible (S)	**Intermediate (I)**	**Resistant (R)**
S. aureus (CLSI, 2018)	Tetracycline (30 ug/disk)	≥19	15-18	≤14
S. mutans (CLSI, 2018)	Tetracycline (30 ug/disk)	≥23	19-22	≤18
C. albicans	Amphotericin B (20 ug/disk)	no established interpretive criteria (Jabeen et al., 2016)		

Where Susceptible (S) is the infection from microorganism is inhibited by the usage of the concentrations of antimicrobial agent recommended for infection treatment, Intermediate (I) is the antimicrobial agent that may be appropriate for infection treatment, depending on some conditions, and Resistant (R) is the usage of the antimicrobial agent that did not inhibit the infection.

The broth dilution test

The broth dilution method was used to prove the efficacy of antimicrobial agents against microorganisms by the disk diffusion (Kirby–Bauer) method. There are 2 types of dilution procedures: macro and micro broth dilution.

In this study, the macro broth dilution method was used. The results arising from this method were shown in terms of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The MIC is the lowest concentration of antimicrobial agent that inhibits the growth of the microorganisms. The lowest concentration of an antimicrobial agent used to kill microorganisms is MBC. (EUCAST, 2003)

Microorganisms preparation

S. aureus, *S.mutans*, and *C.albicans* were grown in Tryptic Soy Broth (TSB), Brain Heart Infusion broth (BHI), and Sabouraud dextrose broth (SDB), respectively. First, an individual microorganism was prepared into 1×10^8 CFU/mL, as previously described. Before the experiment, they were diluted with their sterile broth to 1×10^6 CFU/mL.

Sample preparation

The hemp extract and CBD were solubilized with 10% tween 80 in their broth which produced a 20 mg/mL concentration of the hemp extract and 2.7 mg/mL concentration of the CBD.

Method for determination of MIC

The prepared samples were subjected to 2-fold serial dilutions for 10 concentrations and then added to sterile 13×100 mm tubes for 500 µL. The individual microorganism was loaded to each tube for 500 µL, in accordance with the CLSI guideline (CLSI, 2012), which produced the cell density of 5×10^5 CFU/mL giving the final volume of 1 mL/tube, and the final concentration of each tube was halved. Broth with 5% tween 80 was used as the negative control whereas broth with microorganisms was used as a positive control. Finally, they were incubated at the individual conditions.

Results interpretation

The concentrations which did not change the optical density and turbidity, or the least microbial growth were the MIC.

Method for determination of MBC

After the MIC test, the concentrations which did not change the optical density and turbidity, or which showed the least microbial growth were transferred to suitable agar plates using the simple streak plate technique. The microorganisms were then incubated in their conditions for 24-48 hours.

Results interpretation

The lowest concentration of sample which did not have any microorganism growth from the streak plate was the MBC.

Formulation of the mouthwash containing hemp extract

The mouthwash formulation for the stability study was prepared by mixing these ingredients (as shown in Table 2) together. According to Table 2, the differences between Rx1 and Rx2 were the amount of sodium benzoate (preservative), BHT (antioxidant) and fast green (coloring agent). This mouthwash was prepared on a laboratory scale by the beaker method following Figure 11. The appearance of the finished product was observed, the pH and the initial viscosity were measured, according to the finished product specification. Finally, the mouthwash formulation was kept in a closed plastic container at the critical temperature of 50°C for 3 months during which time the stability of the formulation was studied.

Table 2 The ingredients of mouthwash containing hemp extract

Phases	Ingredients	Master formulation (%w/w)		Uses
		Rx1	Rx2	
A	Normal saline solution (0.9% NSS)	Qs to 100	Qs to 100	Solvent
	Sorbitol 70% solution	5.00	5.00	Humectant, flavoring agent
	Xylitol	2.00	2.00	Sweetening agent
	Mannitol	2.00	2.00	Sweetening agent
	Poloxamer 407	0.40	0.40	Wetting agent
	Sodium benzoate	0.50	0.20	Preservative
B	PEG-40 hydrogenated castor oil	5.00	5.00	Solubilizer
	Tocopherol acetate (Vitamin E)	0.50	0.50	Antioxidant
	Spearmint oil	0.30	0.30	Flavoring agent
	Hemp extract	0.20	0.20	Active
	Menthol	0.10	0.10	Anti-microbial agent
	Thymol	0.08	0.08	Anti-microbial agent
	Peppermint oil	0.02	0.02	Flavoring agent
	Butylated hydroxytoluene (BHT)	-	0.02	Antioxidant
C	Fast green (CI 42053)	-	0.003	Coloring agent

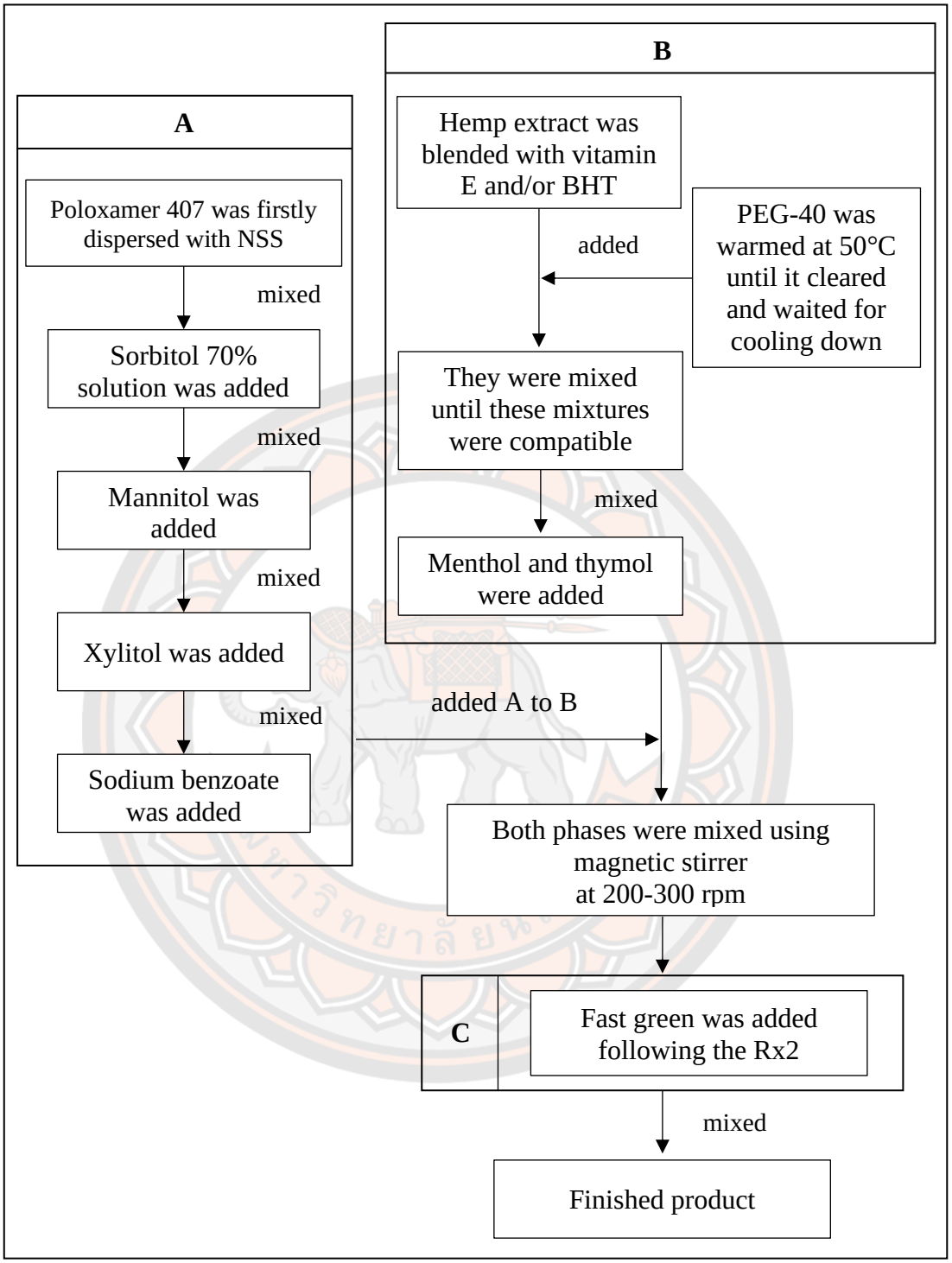


Figure 11 Preparation method of the mouthwash formulation

Stability study of the finished product

The mouthwash containing hemp extract was stored at 50°C, which is the stress condition, for 3 months, according to the World Health Organization technical report series on the topic of stability testing of active pharmaceutical ingredients and finished pharmaceutical products (WHO, 2018). Stress testing can help identify the degradation of the active ingredient in the product. The mouthwash was sampled at 0, 1, 2 and 3 months for further stability testing.

Chemical stability

Sample preparation

The formulated mouthwash was diluted in the ratio of 1:1 with ultrapure water (18.2 MΩ.cm at 25°C). This solution was filtrated through a 0.45 µm pore size nylon syringe filter before HPLC analysis.

HPLC analysis

The percentage of CBD remaining in the mouthwash at each time point was quantified by HPLC, using the Agilent 1260 HPLC system. HPLC analyses were carried out on a Phenomenex Kinetex C18 column (150 mm × 4.6 mm, 2.6 µm). The mobile phases of analysis were 20 mM ammonium formate (pH 3.6) and ACN at the ratio of 25:75, freshly prepared and filtered before use. The column temperature was maintained at 40°C, with a constant flow rate of 0.8 mL/min for 15 minutes. The injection volume was 5 µL. An absorbance was evaluated at 220 nm.

Physical stability

The pH of mouthwash was measured by a pH meter at each time point. The acceptance value from Thai industrial standard (TISI, 2007) guidelines for oral rinse was in the range of 3.0-10.5. The color was observed visually.

Determination of the efficacy of preservative in the mouthwash

Preservative efficacy test (PET) or challenge test was conducted to observe the microbial contamination in cosmetics during the time of use, storage or manufacturing by following the United States Pharmacopeia standard (USP 35, chapter 51) in the topic of antimicrobial effectiveness testing that utilized 5 microorganisms (3 bacteria and 2 fungi) for challenge testing.

Microorganisms preparation

The following bacterial strains, known as pathogen species for humans, were used in the challenge test: *Pseudomonas aeruginosa* (ATCC 25783), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (TISTR 1466), *Aspergillus niger* (ATCC 16404) and *Candida albicans* (ATCC 10231). They were obtained from the Department of Medical Sciences Ministry of Public Health, Thailand. The inoculum was prepared by direct colony suspension to produce 10^8 CFU/mL of microorganisms count, as previously described. Each 1 mL of inoculum was diluted with 9 mL of 0.85% NSS which produced a concentration of about 10^7 CFU/mL. In the case of *A. niger*, their spores were counted to produce a concentration of about 10^6 spore/mL.

Method

The mouthwash and 0.85% NSS were divided into 5 bottles with a volume of 10 mL/bottle. The 0.85% NSS was used as the control for each microorganism. One hundred microliters of inoculum were added to each bottle. At the baseline, each bottle of 0.85% NSS was 10-folded diluted 3 times to 10^{-1}, 10^{-2}, and 10^{-3}. Each 100 µL of dilution was transferred to an agar plate (TSA for bacteria and SDA for yeast and mold growth) and subsequently spread the plate. The bacteria were incubated at 37°C for 24-48 hours. Mold was incubated at 25°C for 48-72 hours. The yeast was incubated at 25°C for 5-7 days. The remaining bottles of mouthwash were determined after 7, 14, and 28 days. At the date of the evaluation, 1 mL of mouthwash from each bottle was mixed with 9 mL of neutralizing diluent (10^{-1} dilution) which contained 5% tween 80 and 0.5% lecithin, and subsequently 10-folded diluted to 10^{-2} and 10^{-3}. Similarly, 100 µL of the mouthwash was transferred to a suitable agar plate and spread over the plate. The forming colonies were counted, calculated CFU/mL, and log CFU/mL. (Giorgio et al., 2018; Siegert, 2012)

Result interpretation

Criteria for category 2 products was used to evaluate the results.

Criteria for bacteria: not less than 2.0 log reduction from the initial count at 14 days, no increase from 14-day count at 28 days (Chapter, 2014; Sutton, & Porter, 2002)

Criteria for yeast and mold: no increase at days 7, 14 and 28 relative to initial count (Chapter, 2014; Sutton, & Porter, 2002)

Statistical Analysis

The experimental results were shown the mean value with standard deviation or standard error of the mean (mean ± SD or mean ± SEM). One-way analysis of variance (ANOVA) followed by Dunnett's test was used to calculate the differences of data using GraphPad Prism version 5.00 software. A *p*-value of less than or equal to 0.05 ($p < 0.05$) indicated a statistically significant result.

CHAPTER IV

RESULTS AND DISCUSSION

Specification of the hemp extract

HPLC analysis of CBD and THC in the hemp extract

Dry female hemp pistillate inflorescences were extracted by ultrasonic-assisted extraction (UAE). The percentage of yield from this hemp extract was 5.30%. The quantitative analysis of CBD and THC in the hemp extract compared with standard was determined by HPLC analysis. HPLC method was used to analyze non-volatile organic compounds or semi-volatile organic compounds in the solution. The substance which was high compatibility with the mobile phase was eluted first. In this study, CBD and THC were eluted at 5.10 ± 0.20 and 8.50 ± 0.20 min, respectively. The chromatogram of CBD standard 200 µg/mL is shown in Figure 12, and hemp extract 1 mg/mL is shown in Figure 13. The calculated amount of CBD in hemp extract was 13.45 ± 0.05% w/w or 134.52 ± 0.51 µg/mL following the calculation from linearity of analyzing a series of CBD standard ($y = 26.506x - 1.2595$; $R^2 = 1$). In addition, hemp extract was composed of THC at the concentration of 0.68 ± 0.00% w/w or 6.80 ± 0.00 µg/mL. Legally, *Cannabis sativa* L. subsp. *sativa* or hemp, as the class 5 Narcotic, has a limit on the amount of THC content. THC should not exceed 1% per dried weight of the inflorescence and leaves (Ratchakitchanubeksa, 2019) due to its psychoactive dose. Following the declaration of the Ministry of Public Health (Ratchakitchanubeksa, 2020), the inflorescence part of hemp with a higher than 0.2% of THC by weight has been being classified as a narcotic. The percentage of yield and cannabinoids in the extract is affected by different growing conditions and methods of extraction.

The extraction method was modified from (Brighenti et al., 2017) who demonstrated that the simple dynamic maceration (DM) with ethanol, at room temperature for an overall time of 45 min, gave the highest percentage of cannabinoids yield. The small amount of sample volume that they used indicated the significant difference between DM and UAE in cannabinoids yield. On the contrary,

higher amounts of hemp powder used in our study may take several days to complete the maceration process because using a magnetic stirrer was not appropriate in stirring a large sample volume. However, it is probable that the UAE with ethanol at room temperature for an overall time of 90 min accelerates the extraction. Because of the ultrasound-wave property, it can travel through the container to vibrate all parts of the sample.

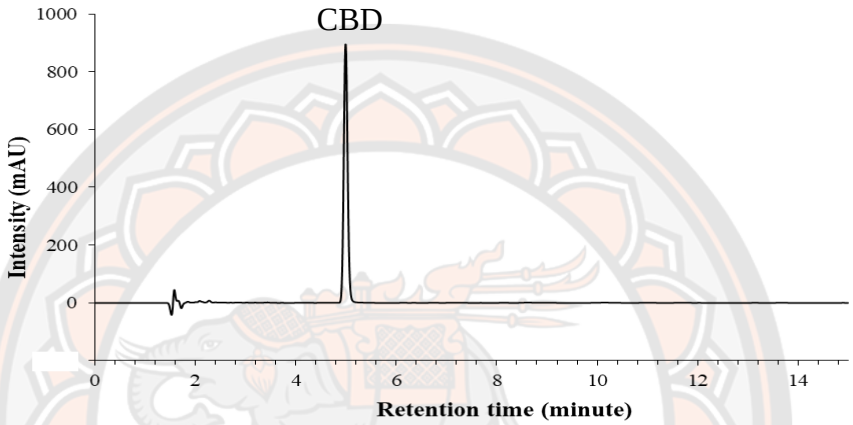


Figure 12 HPLC chromatograms of CBD standard 200 µg/mL

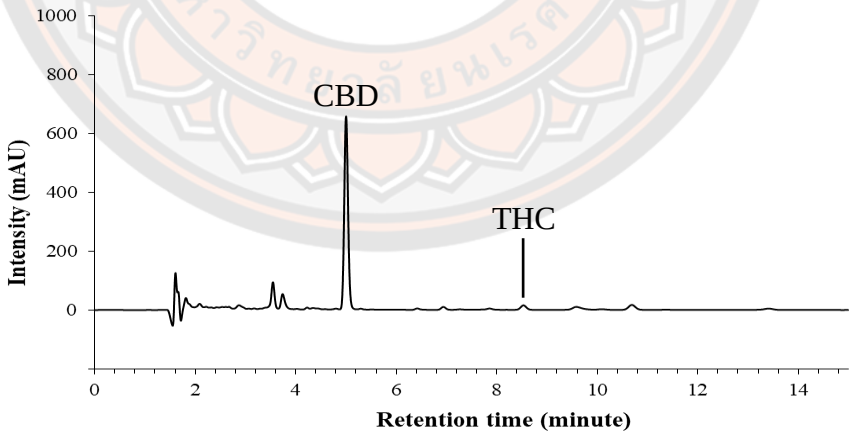


Figure 13 HPLC chromatograms of the hemp extract 1 mg/mL

Note: The Y-axis shows the number of absorbance units (milli-absorbance unit; mAU) or intensity from the UV detector while the X-axis shows the time of the run or retention time.

Solubility of the hemp extract

Hemp extract was obtained by ethanolic extraction. The solubility of this extract in water was 0.55 ± 0.05 mg/mL.

Bacterial contamination

The number of bacteria, mold and fungi contaminating the plant material was estimated by total plate count (TPC) or aerobic plate count (APC). The contamination from bacteria and fungi occurs due to various factors including environmental conditions and the storage conditions for the raw material, which are the major sources of contamination. The results showed that hemp ethanolic extract did not have any microorganisms contamination.

Antioxidant activity of the hemp extract and CBD

Radiotherapy and chemotherapy in head and neck cancer patients lead to oral mucositis. The radiation induces the generation of reactive oxygen species (ROS) resulting in oxidative stress response. The oral epithelium and subepithelial mucosa damage, as well as DNA damage (initiation phase), can occur. So, because the hemp extract can scavenge free radicals, it will be protected against cell damage. Vitamin C or ascorbic acid is a good natural antioxidant substance by acting as an electron donor and a reducing agent, scavenging radicals such as superoxide radicals ($O_2^{\bullet-}$) and hydroxyl radicals ($OH^{\bullet}$) (Kishimoto et al., 2013). To protect against oxidative stress, an antioxidant substance is often used.

A DPPH scavenging assay is used to predict the ability of antioxidants in quenching ROS. DPPH is a stable free radical that reacts with antioxidant molecules and the antioxidant reaction converts 1,1-diphenyl-2-picrylhydrazyl (deep violet color) to 1,1-diphenyl-2-picrylhydrazine (discoloration or yellow color) (Blois, 1958).

In the present study, hemp extract, CBD standard and L-ascorbic acid were used to determine the antioxidant activity using DPPH scavenging assays. The dose response curve is shown in Figure 14 which illustrates the concentration-dependent scavenging effect against DPPH radicals. The hemp extract was able to reduce the stable DPPH radicals with an IC_{50} of 216.23 ± 5.80 µg/mL. The maximum percentage inhibition of the hemp extract was 88.23 ± 0.51%. However, the CBD standard showed more potent antioxidant activity than the hemp extract with an IC_{50} of 107.67

± 5.58 µg/mL. The maximum percentage inhibition of CBD was 92.11 ± 0.21%. The L-ascorbic acid, which was used as the positive control, showed very high efficacy in radical scavenging with an IC_{50} of 4.66 ± 0.20 µg/mL. A lower IC_{50} value indicates higher antioxidant activity.

Previously, the mode of action of CBD in reducing the production of ROS was chelating transition metal ions. This reaction is the Fenton reaction which forms strongly reactive hydroxyl radicals (Hamelink, Hampson, Wink, Eiden, & Eskay, 2005). Recently, the IC_{50} values of hemp leaves were reported as having 2.73 ± 0.42 mg/mL and IC_{50} values of the seeds were 14.39 ± 2.27 mg/mL (Manosroi et al., 2019). The hemp seed is composed of protein, PUFA fatty acid and carbohydrates, which is less than the CBD content of the leaf. However, the inflorescences were a major part of CBD deposition. So, Manosroi et al.'s results showed lower antioxidant activity than ours. This finding also supports the previous study by (Hacke et al., 2019) who found that the antioxidant activity of hemp extract was influenced by CBD. The IC_{50} of hemp and CBD were much higher than in the non-cytotoxic concentration. It is suggested that these concentrations should not be used.

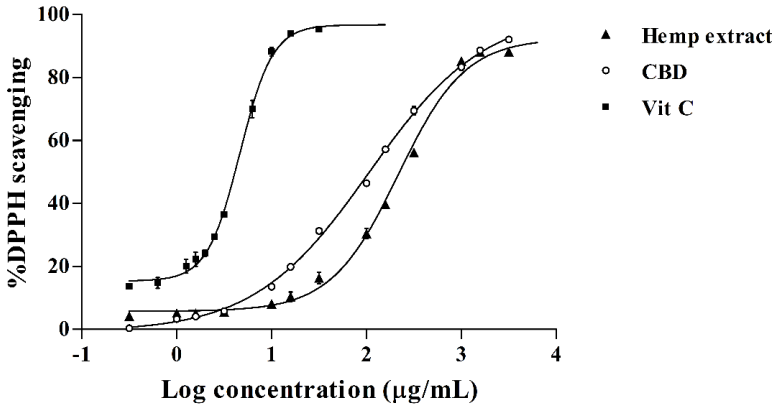


Figure 14 Dose-response curve of hemp extract, CBD, and L-ascorbic acid against DPPH radical

Note: The data are presented as mean ± SD (n=3, triplicate).

Table 3 The inhibitory concentration of 50% of DPPH radical

Sample	Maximum percentage inhibition	IC_{50} (µg/mL)
Hemp extract	88.23 ± 0.51	216.23 ± 5.80
CBD	92.11 ± 0.21	107.67 ± 5.58
L-ascorbic acid	95.85 ± 0.24	4.66 ± 0.20

Note: The experiments were done in triplicate and data are presented as mean ± SD.

Cytotoxicity study of the hemp extract and CBD

Effects of the hemp extract and CBD on the viability of RAW 264.7 cells

To determine the abilities of the hemp extract and CBD against cell viability, RAW 264.7 macrophage cells were exposed to the hemp extract with concentrations ranging from 3.9 to 500 µg/mL and CBD with concentrations ranging from 0.08 to 10 µg/mL, for 24 hours. The viabilities of the cells were then measured by an MTT assay.

The non-cytotoxic concentration of hemp extract was 3.91-100 µg/mL and the CBD was 0.08-1 µg/mL, but the cellular morphology started to change at 100 µg/mL after being exposed to the hemp extract. The viability of the RAW 264.7 cells significantly decreased after being treated with hemp extract at a concentration higher than 125 µg/mL and CBD at a concentration higher than 1.25 µg/mL ($p < 0.05$). At low concentrations of the hemp extract exposure group significantly increased cell viability ($p < 0.05$), whereas the CBD exposure group significantly decreased cell viability ($p < 0.05$). It is possible that some compounds in the hemp extract can stimulate RAW 264.7 cells proliferation. Therefore, the maximum concentrations of hemp extract and CBD were 50 and 0.5 µg/mL which were chosen for subsequent study.

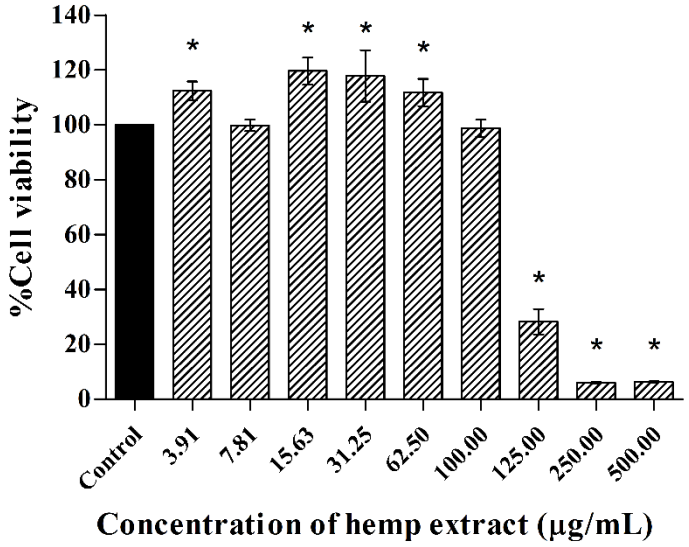


Figure 15 Viability of RAW 264.7 cells after exposure to the hemp extract

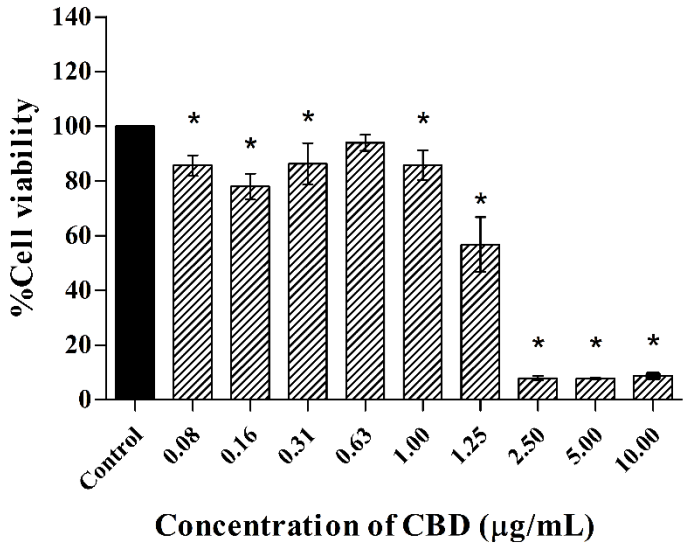


Figure 16 Viability of RAW 264.7 cells after exposure to CBD

Note: The data are presented as mean ± SD (n=3, triplicate). *$p < 0.05$.

Effects of the hemp extract and CBD on the viability of HGF-1 cells

To evaluate the cytotoxicity effect of hemp extract and CBD on HGF-1 cells viability, cells were exposed to different concentrations of the hemp extract ranging from 3.9 to 500 µg/mL and CBD ranging from 0.08 to 10 µg/mL, for 24 hours. The cytotoxicity of the HGF-1 cells was detected using an MTT assay.

The results showed that 3.91-125 µg/mL of the hemp extract and 0.08-0.63 µg/mL of CBD were not cytotoxic concentrations. Following International Organization for Standardization guidelines (ISO, 2009), a percentage of cell viability higher than 70% indicates non-cytotoxicity. The percentage of cell viability significantly reduced at 125-500 µg/mL of the hemp extract exposure group, as well as 1.25-10 µg/mL of CBD exposure group ($p < 0.05$). On the other hand, hemp extract at the concentration of 31.25-62.50 µg/mL and CBD at 0.16 µg/mL significantly increased the viability of HGF-1 cells. However, the cell morphology after being treated with the hemp extract started to change at 100 µg/mL. Thus, neither the hemp extract nor the CBD at a concentration less than 100 and 0.63 µg/mL exerted a significant cytotoxicity on HGF-1 cells. Consequently, the maximum non-cytotoxic concentrations used for the further biological experiments of the hemp extract and CBD were 50 and 0.5 µg/mL, respectively.

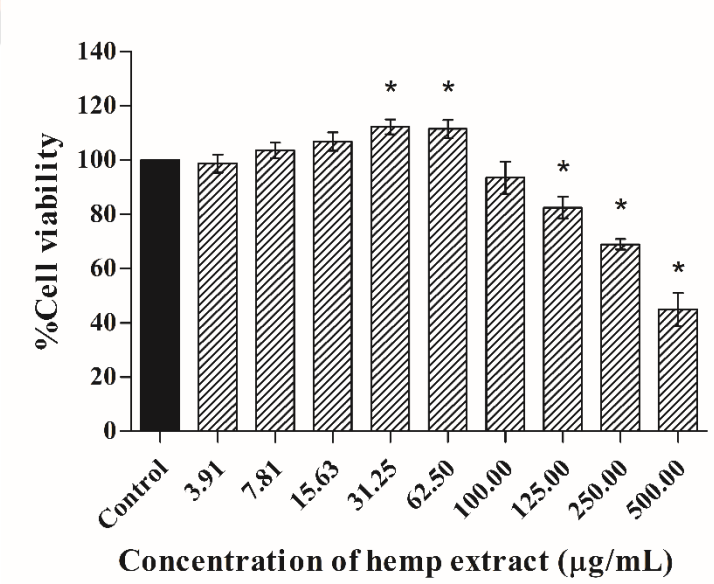


Figure 17 Viability of HGF-1 cells after exposure to the hemp extract

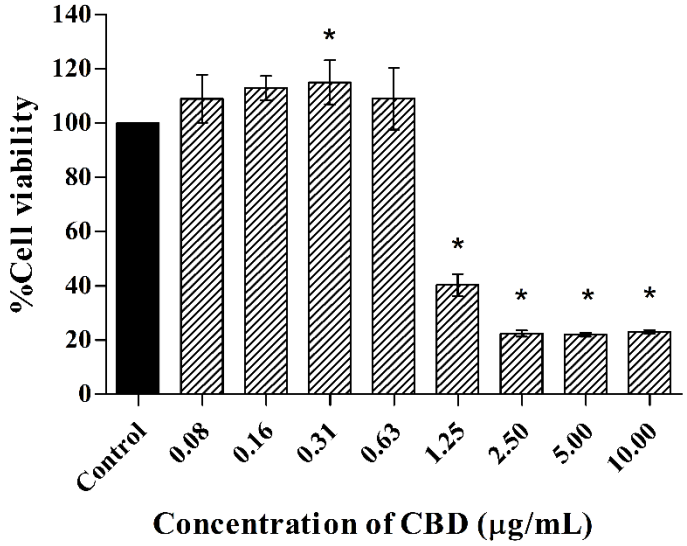


Figure 18 Viability of HGF-1 cells after exposure to CBD

Note: The data are presented as mean ± SD (n=3, triplicate). *$p < 0.05$.

Anti-inflammatory activities

Radiation-induced inflammatory response leads to damage of the DNA of epithelial cells and ROS generation from epithelial, endothelial, fibroblast and macrophage. The injured cells further secrete proinflammatory cytokines, such as TNF-α, IL-1β and IL-6 in response to the inflammation. In addition, IL-1β indirectly stimulates PGE_2 production via COX-2 in human gingival fibroblast via tyrosine kinase pathways (Iwasaki, Noguchi, & Ishikawa, 1999; Yucel-Lindberg, Ahola, Carlstedt-Duke, & Modéer, 1999).

So, the effectiveness of hemp extract against TNF-α, IL-1β, and PGE_2 was determined after the onset of inflammation in RAW 264.7 and HGF-1 cells.

Effects of the hemp extract and CBD on LPS-induced TNF-α production

The production of TNF-α in the culture supernatants was assessed using an ELISA kit. RAW 264.7 cells were pretreated with LPS 2 µg/mL to induce cell inflammation. The results, as shown in Figures 19-21, indicate that the LPS treatment increased the production of TNF-α, compared with the untreated cells (control group).

The hemp extract treatment significantly decreased TNF-α production at concentrations ranging from 5-50 μg/mL ($p < 0.05$). However, no significant difference in the inhibition activity between the hemp extract concentrations 5 μg/mL and 10 μg/mL was observed while 50 μg/mL of the hemp extract obviously presented an inhibition effect on TNF-α synthesis. In addition, the release of TNF-α was significantly reduced by the CBD treatments (0.25-1 μg/mL) ($p < 0.05$). One microgram per milliliter of CBD presented the highest inhibition activity while the lowest concentration significantly inhibiting the production of TNF-α was 0. 25 μg/ mL. These results from this assay showed dose-dependent anti-inflammatory activity. The percentage inhibition of TNF-α is displayed in Table 4. The ability of hemp extract in inhibiting 50% of TNF-α activity was 23.05 ± 0.08 and for CBD, 0.79 ± 0.06 μg/mL. Hydrocortisone (HC) 10 μg/mL, as a positive control, significantly decreased TNF-α production ($p < 0.05$).

In addition, the results also revealed that CBD is the influenced bioactive component of hemp extract because CBD was the potent anti-inflammatory agent (Nagarkatti, Pandey, Rieder, Hegde, & Nagarkatti, 2009). The concentration of CBD which was composed in 1 μg/mL of the hemp extract was approximately equivalent to 0.135 μg/mL; they did not show significant effectiveness on TNF-α inhibition when compared to the negative control. However, significant activity was obtained when the hemp extract was 5 μg/mL which was equivalent to CBD 0.673 μg/mL. The mechanism of CBD on TNF-α production has not been fully understood; previous reports suggested that CBD may inhibit the formation of reactive oxygen species that cause cells damage and induce the release of TNF-α (El-Remessy et al., 2008; Mukhopadhyay et al., 2011; Muthumalage, & Rahman, 2019). Otherwise, CBD was capable of blocking the lipopolysaccharide-induced rise in TNF-α (Ben-Shabat et al., 2006; A. M. Malfait et al., 2000; Rajan et al., 2016; Silva et al., 2019).

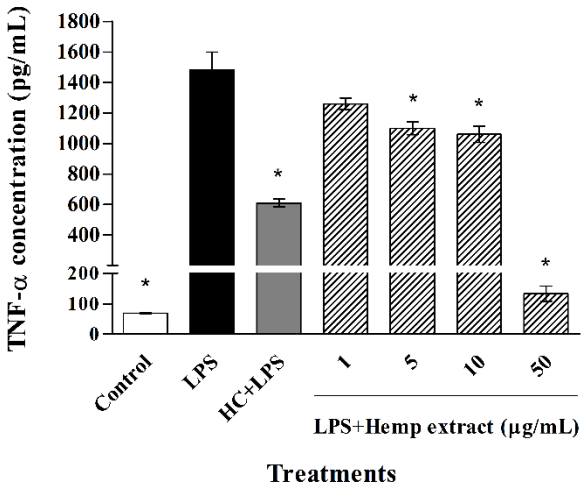


Figure 19 The inhibitory effects on TNF-α release of the hemp extract in LPS-induced inflammatory response in RAW 264.7 cells

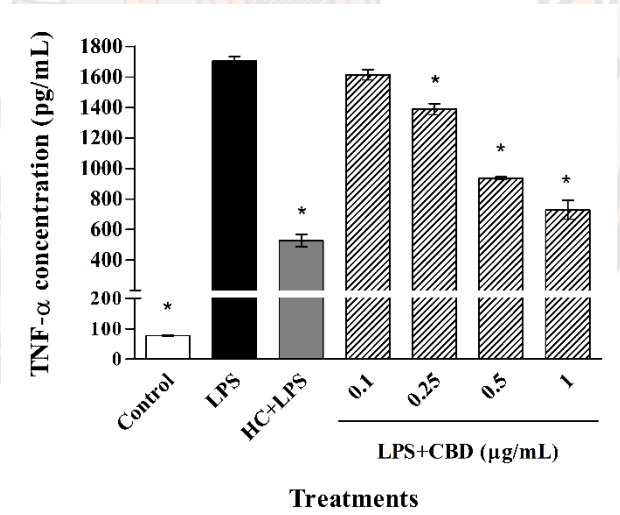


Figure 20 The inhibitory effects on TNF-α release of CBD in LPS-induced inflammatory response in RAW 264.7 cells

Note: The values are presented as the mean ± SEM (n=3, triplicate). Compared with the LPS group, *$p < 0.05$.

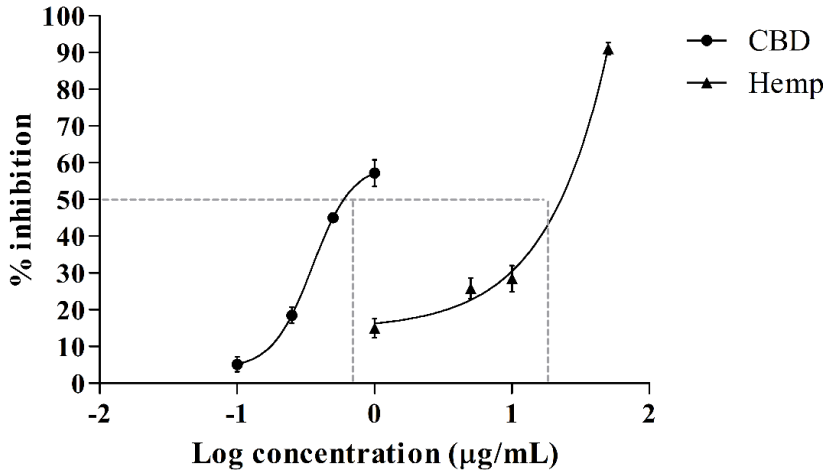


Figure 21 The TNF-α inhibition curve of the hemp extract and CBD in RAW 264.7 cells

Note: The values are presented as the mean ± SD.

Table 4 The percentage inhibition of TNF-α

Treatments	Concentration (µg/mL)	%TNF-α inhibition (mean ± SD)
Hemp extract	1	14.96 ± 4.51
	5	25.81 ± 4.88
	10	28.46 ± 6.20
	50	91.05 ± 2.91
Hydrocortisone	10	58.86 ± 3.00
CBD	0.1	5.16 ± 3.50
	0.25	18.48 ± 3.80
	0.5	45.01 ± 0.89
	1	57.18 ± 6.27
Hydrocortisone	10	69.09 ± 4.06

Effects of the hemp extract and CBD on LPS-induced IL-1β production

The IL-1β production in the culture supernatants was detected with an ELISA kit. RAW 264.7 cells were pretreated with LPS 2 μg/mL to induce cell inflammation. The results demonstrated that the LPS induced the production of IL-1β in RAW 264.7 cells, compared with the control group. The treatment of hemp extract and CBD in LPS-induced RAW 264.7 cells at these followed concentrations showed significantly decreased IL-1β production in a dose-dependent manner ($p < 0.05$), with the IC_{50} of 26.97 ± 1.50 and 0.55 ± 0.02 μg/mL, respectively (Figures 22-24), which was approximate to TNF-α values. The percentage of IL-1β inhibition is presented in Table 5. Hydrocortisone 5 μg/mL significantly reduced IL-1β ($p < 0.05$) in LPS-induced RAW 264.7 cells. The hemp extract exposure exhibited the IL-1β inhibition activity consistent with the TNF-α inhibition activity. Although, there were few reports of CBD on IL-1β production in LPS-induced models while it was found to suppress IL-1β gene expression in other inflammatory models (Lee et al., 2016; Pan et al., 2009; Wang et al., 2017). The possible mechanism indicated that CBD attenuates the activity of the NF-κB in LPS-induced cells inflammatory models leading to inhibit TNF-α (Muthumalage, & Rahman, 2019) and IL-1β (Kozela et al., 2010) as well. Also, CBD at the concentration of at least 0.1 μg/mL indicated significant IL-1β inhibition ($p < 0.05$). These results demonstrate that the treatment with pure CBD is more effective in the inhibition of TNF-α and IL-1β secretions than the hemp extract treatment. Probably, some chemical components in the hemp extract other than cannabinoids might interfere with some mechanisms of inflammation. These results showed the correlation between hemp extract and CBD in a dose-dependent manner. It is likely that the results from the hemp extract treatment can predict the ability of CBD.

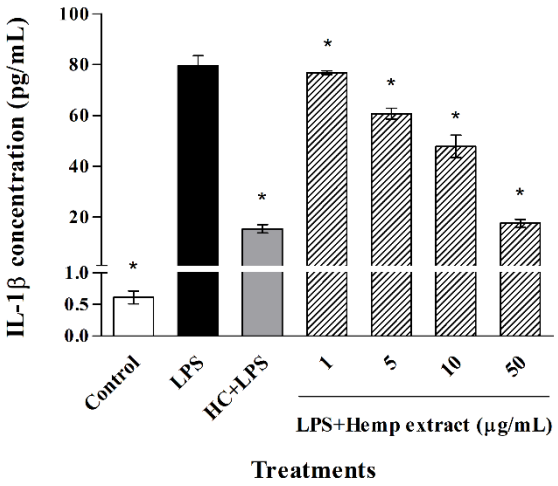


Figure 22 The inhibitory effects on IL-1β release of the hemp extract in LPS-induced inflammatory response in RAW 264.7 cells

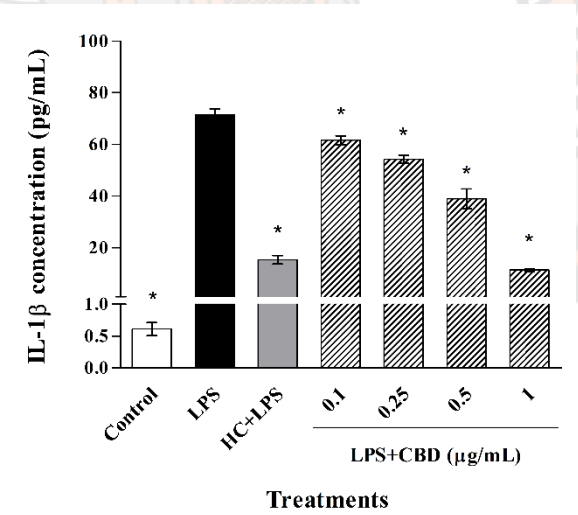


Figure 23 The inhibitory effects on IL-1β release of CBD in LPS-induced inflammatory response in RAW 264.7 cells

Note: The values are presented as the mean ± SEM (n=3, triplicate). Compared with the LPS group, *$p < 0.05$.

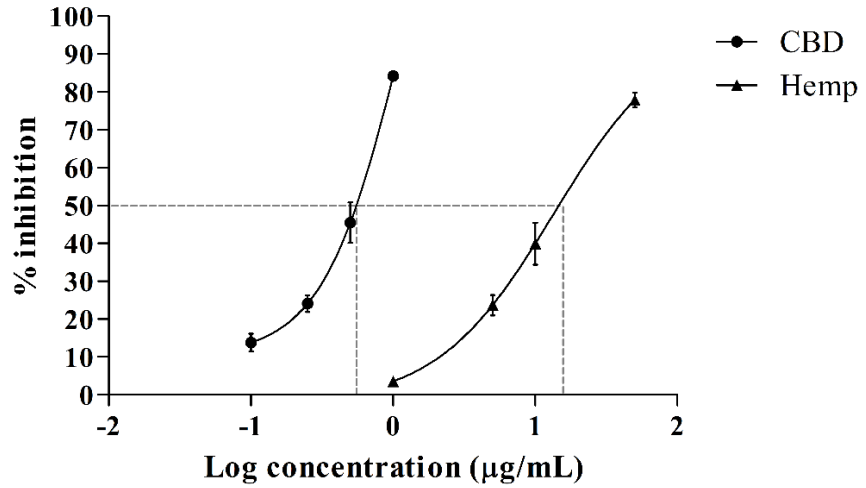


Figure 24 The IL-1β inhibition curve of the hemp extract and CBD in RAW 264.7 cells

Note: The values are presented as the mean ± SD.

Table 5 The percentage inhibition of IL-1β

Treatments	Concentration (µg/mL)	% IL-1β inhibition (mean ± SD)
Hemp extract	1	3.53 ± 1.62
	5	23.68 ± 4.68
	10	39.94 ± 9.60
	50	77.90 ± 3.36
CBD	0.1	13.80 ± 4.00
	0.25	24.09 ± 3.84
	0.5	45.47 ± 9.33
	1	84.17 ± 1.24
Hydrocortisone	5	78.50 ± 3.97

Effects of the hemp extract on IL-1β induced PGE_2 production

The production of PGE_2 was induced by IL-1β (Iwasaki et al., 1999; Yucel-Lindberg et al., 1999). Hence, 2 ng/mL of IL-1β was used to induce HGF-1 cells inflammation. The inflamed cells were treated with hemp extract (1, 10, 50 µg/mL) for 24 hours. The levels of PGE_2 in the cell culture supernatant were determined by ELISA. The hemp extracts significantly reduced PGE_2 levels ($p < 0.05$) (Figure 25). The percentage inhibition of PGE_2 is illustrated in Table 6. Hydrocortisone 10 µg/mL significantly inhibited PGE_2 release ($p < 0.05$). This finding also supports the previous study by (Duangnin et al., 2017) who showed that the hemp extract and CBD reduce the PGE_2 level during inflammation. They found that hemp leaf extracts (1, 5, 10 µg/mL) reduced the production of NO and PGE_2 in a dose-dependent manner.

The level of PGE_2 at 50 µg/mL was higher than 10 µg/mL. If 5 µg/mL of the hemp extract is used, it will exhibit the dose dependent activity but it did not show the concentration dependent manner when concentration increased to 50 µg/mL. It is possible that the hemp extract at this concentration enhanced the viability of cells, as indicated by the MTT results. A larger number of cells led to larger production of PGE_2 than other concentrations. Another possibility is that some compounds in this extract, other than CBD, may increase PGE_2 levels in gingival fibroblast.

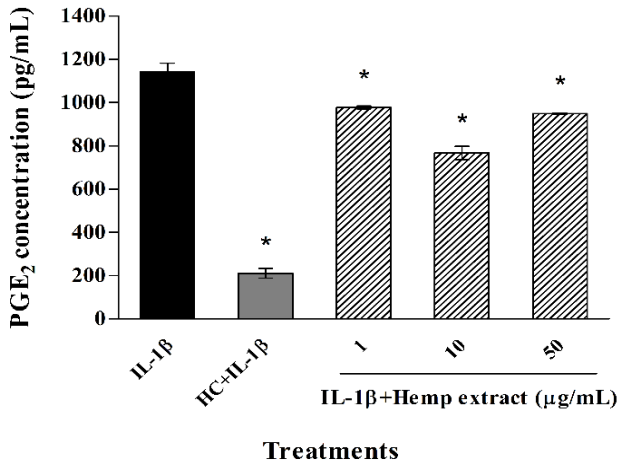


Figure 25 The inhibitory effects on PGE_2 released of the hemp extract on IL-1β induced inflammatory response in HGF-1 cells

Note: The values are presented as the mean ± SEM (n=3, triplicate). Compared with IL-1β group, $*p < 0.05$.

Table 6 The percentage inhibition of PGE_2

Treatments	Concentration (μg/mL)	% PGE_2 inhibition (mean ± SD)
Hemp extract	1	14.40 ± 0.97
	10	32.83 ± 4.59
	50	16.88 ± 0.57
Hydrocortisone	10	81.56 ± 3.36

Effects of the hemp extract on pro-collagen type I production

Type I collagen is the most component found in connective tissues, produced by fibroblast. It is a fibril form of heterotrimer molecule which is composed of a triple helix with two α1 and one α2 chains. The collagen structure is divided into 3 domains: Amino acid (N)-terminal, a central triple helix which is composed of glycine, proline and hydroxyproline (G-X-Y) repeated and Carboxy (C)-terminal. In the experiment, N and C-terminal propeptide are used as the biomarker of collagen synthesis. The biosynthesis of collagen needs a cofactor for proline and lysine hydroxylation. Vitamin C or ascorbic acid helps form hydroxyproline and hydroxylysine, as well as stabilizes the collagen structure. (Henriksen, & Karsdal, 2016; Kishimoto et al., 2013)

The pro-collagen type I α1 produced by human gingival fibroblast was measured in the cell culture medium using the ELISA technique. The treatments with hemp extract at concentrations of 1, 10, 25 and 50 µg/mL significantly increased pro-collagen type I production after 24 hours of incubation and to a greater extent than the increase in the control or untreated cell. However, fluctuations in the cell seeding density in the cell culture experiment occurred, as seen from the error bar. It is probable that the 50 µg/mL of the hemp extract is not a suitable concentration for collagen synthesis. However, L-ascorbic acid 10 µg/mL was used as the positive control and indicated the highest collagen type I production (Figure 26). Previously, (Mohammed et al., 2016) demonstrated that administering vitamin C to mice increased collagen synthesis in the wound to a greater extent than in the control group. (Chaitrakoonthong, Ampornaramveth, & Kamolratanakul, 2020) demonstrated that rinsing with vitamin C increased the expression of COL1 gene which is involved in wound healing in HGF-1 cells. Recently, RT-PCT analysis showed that COL I and COL III expression in Human dental pulp cells (HDPCs) were up-regulated after exposure to CBD (1 µM or 0.31 µg/mL) (Qi et al., 2021). This finding is in agreement with (Jin, & Lee, 2018), who reported that hemp seed extract induced collagen production in the human fibroblast cell line (Hs68) and exhibited the ability to heal wounds.

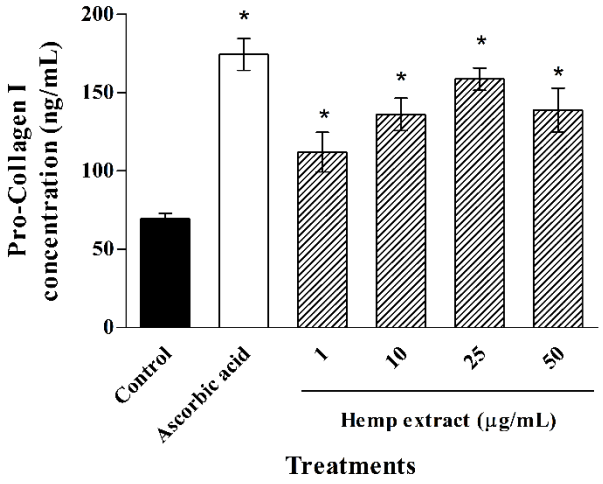


Figure 26 Effects of the hemp extract on pro-collagen type I production in HGF-1 cells

Note: The data are the mean ± SEM (n=3, triplicate). Compared with the control group, $^{*}p < 0.05$.

Effects of the hemp extract and CBD on wound healing

Wound healing is a process that occurs after tissue injury. Fibroblasts play an essential role in wound healing by migrating to the wound area where it proliferates and synthesizes cytokines and extracellular matrix (ECM) for wound remodeling.

The wound healing assay was performed on HGF-1 cells using a pipette tip to scratch the cell monolayer. To avoid cell proliferation during the assay, serum starvation is necessarily performed at least 8 hours before wound making. The rinsing protocol was conducted to mimic a routine oral rinse. The short-term rinsing protocol was modified from (Huynh et al., 2016). A previous study reported that rinsing with 0.9 and 1.8% NaCl solution significantly enhanced wound closure as compared to the untreated group in human gingival fibroblast (Huynh et al., 2016). Therefore, 0.9% NaCl in a serum-free medium was used as a positive control. The HGF-1 cells were rinsed either with hemp extract (5, 10 and 25 µg/mL) or CBD (0.5 µg/mL) for 3 min, 3 times daily at 5-6 h intervals over 2 days. At each time point, images of the cells were captured under a light inverted microscope. The percentage of wound closure is

indicated in Figure 27. The wound closure area after rinsed with 5 µg/mL of hemp extract significantly increased at 24 and 36 hours as compared with the control group. Unfortunately, the morphology of the cells had changed from spindle cells to more shrinkage cells after 24 hours of being rinsed with 10 µg/mL hemp extract or after 6 hours being rinsed with 25 µg/mL hemp extract, probably because of the hyperosmolarity of treatment. The HGF-1 cells would have usually died, but in this experiment, some of the cells were still alive after 48 hours. According to (Solinas et al., 2012), human umbilical vein endothelial cells (HUVECs) treated with 9 µM of CBD (2.83 µg/mL), displayed significantly inhibited cell migration at 16 hours and persisted to 24 hours after wounding with cytostasis. Our results indicated that the concentration of CBD in 10 µg/mL hemp extract was about 1.35 µg/mL and in 25 µg/mL hemp extract was about 3.36 µg/mL. It is probable that the cells were in cytostasis or showing growth inhibition under intermittent periods of exposure. Another possibility is that mechanical stress (shear stress) caused by shaking method together with hemp or CBD exposure induces cell morphology change and cytostasis through mechanosensitive pathway. In addition, after being rinsed with CBD, the HGF-1 cells showed significantly enhanced wound closure at 24, 36 and 48 hours. However, 0.9% NaCl solution used to rinse cells significantly increased wound closure and trended to complete the wound healing. NaCl stimulates hGF cell migration and cytoskeleton re-organization via the up-regulation of Focal Adhesion Kinase (FAK) and F-actin, and the enhancement of COL1 and fibronectin (Fn) gene expression (Huynh et al., 2016).

After tissue injury, the actions of cannabinoids in periodontal tissue during wound healing were reported to involve the cannabinoid receptors (CBs). Especially, CB2 was expressed on gingival connective tissue and promoted cell migration (Liu, Qi, Alhabeil, Lu, & Zhou, 2019). However, the mechanism of CBD on gingival wound healing *in vitro* has been unclear. This finding is not consistent with a previous study of wound healing in an *in vivo* model where it was demonstrated that intraperitoneal injection of CBD did not induce faster healing at ventral tongue of Wistar rats (Klein et al., 2018). It is possible that the healing process in that study was influenced by uncontrollable factors (ex. complex mechanisms, stress conditions, pain, feeding). These findings are the first experiment that indicated short-term

rinsing with the hemp extract and CBD for 2 days promotes human gingival fibroblasts wound healing.

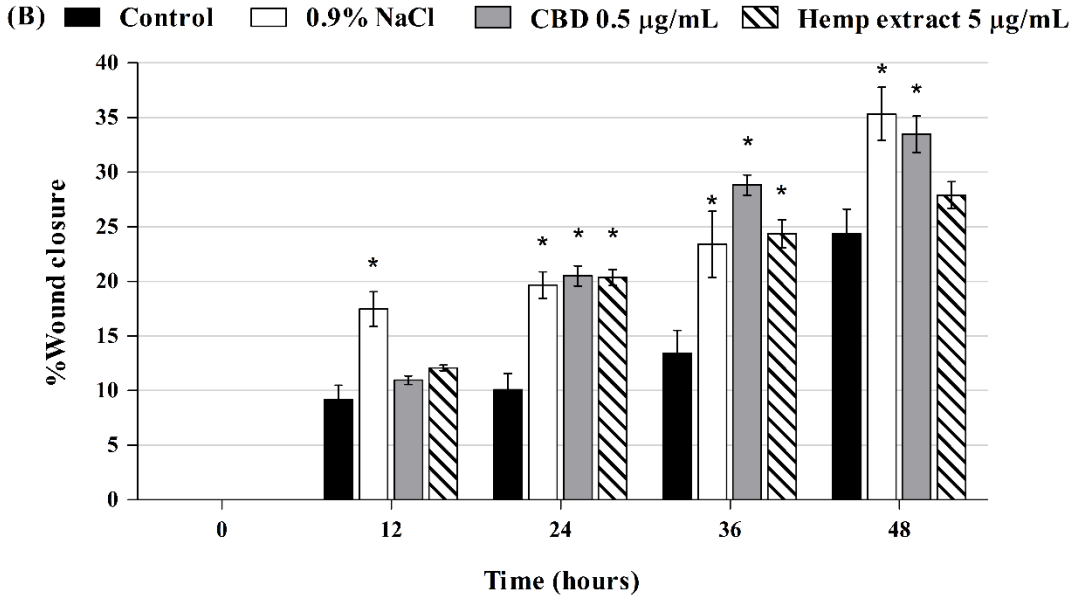


Figure 27 The percentage of wound closure area at indicated time points in HGF-1 cells

Note: The data are the mean ± SEM (n=3, duplicate). Compared with control, *$p < 0.05$, within time.

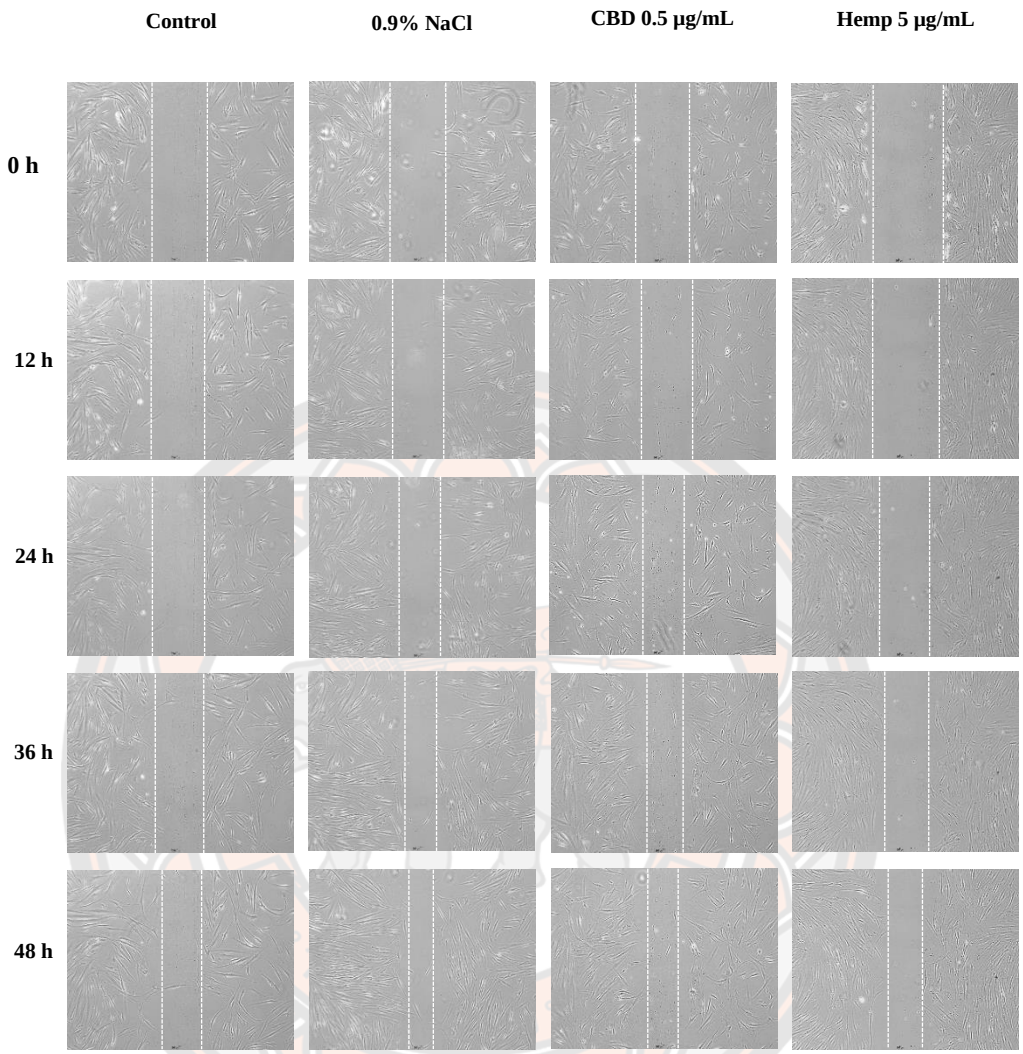


Figure 28 The representative images of the wound area at indicated time points during the scratch test assay

Antimicrobial activities of the hemp extract and CBD

Antimicrobial susceptibility test by disk diffusion method

The disk diffusion method was used to evaluate the antimicrobial susceptibility by measuring the inhibition zone against the test microorganisms.

Our results indicated that the concentrations of hemp extract and CBD were interrelated. The hemp extract 4.00 mg/disk composed of CBD around 0.54 mg/disk. Each sample was loaded on a disk for 20 µL and allowed to dry. After the end of incubation, the inhibition zones that developed around the disks were measured using a ruler and vernier caliper. The results of the antimicrobial activity of hemp extract and CBD tested against microorganisms by disk diffusion method are shown in Table 7. The hemp extract was resistant to *S. aureus*, whereas it showed intermediate antibacterial activity against *S. mutans* compared with tetracycline. Following the zone diameter breakpoints of tetracycline, CBD was resistant to both strains of bacteria. In antifungal activity, neither the hemp extract nor the CBD showed any activities against *C. albicans.*

However, patients with head and neck cancer undergoing chemo- and radiotherapy are recommended to use chlorhexidine gluconate mouthwash to reduce the inflammation and ulceration of oral mucositis (Rodríguez-Caballero et al., 2012). Similarly, 0.12% chlorhexidine (CHX) mouthwash can also be used because it demonstrates antimicrobial activity. *S. mutans* has been shown to be susceptible to 0.12% CHX mouthwash, which consisted of chlorhexidine gluconate (CHG) equivalent to 0.12 g or 1.2 mg/mL. In addition, the inhibition zones of hemp extract and CHX against *S.aureus* and *S.mutans* were almost the same. Probably, the hemp extract has possibility to inhibit bacterial growth when compared to the activity of CHG.

A previous study reported that the inhibition zone of *Cannabis sativa* L. extracts (leaves and stem) against *S. aureus* was 25 mm (Borchardt et al., 2008). However, their results were quite unclear because they did not state the concentration used but just indicated that 10 g of plant leaf and stem material was extracted using 10 mL of ethanol and a 50 µL sample was loaded onto a disk, whereas in our study it was the inflorescences only that were extracted. It is, therefore, probable that the chemical

compounds from the different parts of plant and at a high concentration of sample, may have exhibited the antimicrobial activities.

Table 7 The mean diameter of the growth inhibition zone of the hemp extract and CBD

Organisms	Test	Concentration (mg/disc)	Mean diameter of growth inhibition zone (mm ± SD)		
			results	positive	negative
S. aureus	**Hemp**	4.00	13.91 ± 0.06 (Resistant)	27.22 ± 0.10 (Susceptible)	NA
	CBD	0.54	8.09 ± 0.17 (Resistant)		
	CHG	0.024	13.53 ± 0.35 (Resistant)		
S. mutans	**Hemp**	4.00	22.39 ± 0.35 (Intermediate)	36.56 ± 0.19 (Susceptible)	NA
	CBD	0.54	11.89 ± 0.10 (Resistant)		
	CHG	0.024	27.17 ± 0.00 (Susceptible)		
C. albicans	**Hemp**	4.00	NA	10.33 ± 0.29	NA
	CBD	0.54			
	CHG	0.024			

Note: The 30 µg/disk of tetracycline and 20 µg/disk of amphotericin B were used as positive for antibacterial, and antifungal, respectively. The negative was DMSO.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by macro broth dilution method

The results from the disk diffusion method were insufficient to allow us to conclude the effectiveness of the antimicrobial activity of the sample. Hence, the macrobroth dilution method was used to investigate the MIC and MBC of hemp extract and CBD against *S. aureus*, *S. mutans* and *C. albicans*.

Because of the limitation of the solubility of hemp extract, the maximum concentration of hemp extract which used in the experiment was 10 mg/mL. The concentrations of the hemp extract and the CBD were interrelated and came from the solubilization trial. The concentration was higher than the non-cytotoxic concentration from the MTT assay. Since the low concentration did not show antimicrobial activity, the higher concentration was applied. The results showed that the hemp extract and CBD at these tested concentrations were not the MIC and MBC of all microorganisms. We know that chlorhexidine is an antiseptic in mouthwash, and the low concentration showed high sensitivity to all organisms. The results of MIC and MBC are shown in Table 8. A previous study by (Zengin et al., 2018) showed that the MIC and MBC of hemp essential oils against *S. aureus* were 8 mg/mL and 16 mg/mL, respectively, using microbroth dilution method. There are many factors that affect the results, such as which part of the plants is extracted, the extraction method, the environmental growing conditions, and the testing method, and, especially, the solubility of the extract is a significant concern.

In 1976, Van Klingeren and Ten Ham (Van Klingeren, & Ten Ham, 1976) reported MIC of CBD against *S. aureus* at 1-5 µg/mL in nutrient broth agar (pH 7.4). It is not surprising that their results showed a very low concentration of CBD used against bacteria, because they used 1000 cells, whereas, according to the 0.5 McFarland standard, the number of bacteria has approximately 1.5×10^8 cells/mL. The number of bacteria used in this study was 5×10^5 CFU/mL or cells/mL which is well diluted from the 0.5 McFarland standard. (Blaskovich et al., 2021) reported MIC of CBD against *S. aureus* and *C. albicans* were 1-2 µg/mL and more than 64 µg/mL, respectively. In our preliminary experiment, the method was almost the same as theirs but with the difference of using a 96-well plate and different growth medium. However, the results exhibited no activity.

Table 8 The evaluation of the MIC and MBC

Organisms	Treatments	MIC	MBC
S. aureus	Hemp extract	> 10 mg/mL	> 10 mg/mL
	CBD	> 1.35 mg/mL	> 1.35 mg/mL
	0.12% CHX mouthwash	0.04 mg/mL of chlorhexidine gluconate	0.15 mg/mL of chlorhexidine gluconate
S. mutans	Hemp extract	> 10 mg/mL	> 10 mg/mL
	CBD	> 1.35 mg/mL	> 1.35 mg/mL
	0.12% CHX mouthwash	0.01 mg/mL of chlorhexidine gluconate	0.02 mg/mL of chlorhexidine gluconate
C. albicans	Hemp extract	> 10 mg/mL	> 10 mg/mL
	CBD	> 1.35 mg/mL	> 1.35 mg/mL
	0.12% CHX mouthwash	0.30 mg/mL of chlorhexidine gluconate	0.30 mg/mL of chlorhexidine gluconate

The formulation of mouthwash

The mouthwash containing hemp extract was developed to reduce the inflammation and healing of oral ulcers. The concentration of hemp extract which we recommend to use is 5 µg/mL, based on our results, as it inhibited TNF-α and IL-1β release as well as performing gingival fibroblast healing. Additionally, this mouthwash was an alcohol-free formulation. The 0.9% NSS was used as a solvent instead of alcohol and was the positive control in the wound healing assay. Menthol has been reported to have a skin wound healing activity by modulating the antioxidant system of the cells and the inflammatory response, in addition to stimulating epithelialization (Rozza et al., 2021). Probably, this formulation accelerates oral healing and non-irritation. The concentration of hemp extract used for the stability

testing was 0.2% or around 2 mg/mL. Also, the color stability and %CBD were clearly observed when using a higher concentration.

Stability study of the mouthwash

The mouthwash containing hemp extract was kept under stress conditions at 50°C. The stability results after 3 months storage are shown in Tables 9-10. Both formulas trended to decrease in pH value or turned to be more acidic at each time point. This situation may occur because of the buffer system. The amount of sodium benzoate in Rx1 was higher than Rx2, it was more effective in controlling the pH system than Rx2. Hence, the pH of Rx2 was dramatically shifted to acid. According to the Thai Industrial Standard for oral rinse (TISI, 2007), the pH of formulas was in the acceptance values. It should be considered that pH < 7 was suitable for oral use.

Cannabidiol (CBD) was the major component in this hemp extract. Jaidee W et al. (Jaidee et al., 2021) reported about the stability of CBD in various conditions, CBD was stable at pH between 4 and 6. The Rx1 had pH around 6.48 at baseline, but Rx2 had pH 6.75 at baseline. It would be possible that CBD in the Rx1 was more stable than the Rx2. However, the percentage of CBD remaining in mouthwash was determined by HPLC analysis. The percentage of CBD reduction did not exceed 10%. It indicated the chemical stability. The data from HPLC at each time point showed a bit of variation, depending on solvent preparation and column.

Both formulas had problems with color stability. The color of the mouthwash had turned to brownish-green. Because of heat, the chlorophyll was degraded by heat, light, acid conditions and oxygen. The heat caused the conversion of chlorophyll to pheophytins (olive brown) (Erge, Karadeniz, Koca, & Soyer, 2008). Moreover, many antioxidants were restricted to oral use. All ingredients should be solubilized in water. The use of surfactants could help to solubilize hydrophobic substances, especially the hemp extract and essential oil. Furthermore, the color must be improved in future formulations.

Table 9 Stability of Rx1 mouthwash at 50°C

Parameters	Time (weeks)				
	0	**4**	**5**	**6**	**12**
pH	6.48 ± 0.04	6.29 ± 0.01	6.30 ± 0.00	6.25 ± 0.03	6.20 ± 0.02
% CBD remaining	100	94.27 ± 0.44	94.61 ± 0.52	93.01 ± 0.38	96.23 ± 0.34
Color					

Table 10 Stability of Rx2 mouthwash at 50°C

Parameters	Time (months)			
	0	**1**	**2**	**3**
pH	6.75 ± 0.00	6.26 ± 0.01	6.12 ± 0.00	6.05 ± 0.02
%CBD remaining	100	91.09 ± 0.73	90.12 ± 0.84	89.29 ± 0.09
Color				

Note: The data are the mean ± SD.

Table 11 Product specification

Appearance	Olive green solution
pH at 25°C	6.0-6.5
Initial viscosity at 25°C	2.82 ± 0.03 cP

The efficacy of preservatives in the mouthwash containing hemp extract

This mouthwash which is composed of water as the main component was risky for microbial growth. So, the preservative efficacy test (PET) or challenge test was conducted to monitor the microbial contamination in cosmetics during the manufacturing and the time of use or storage by following the United States Pharmacopeia standard (USP 35, chapter 51). The contamination of microorganisms in personal care products may cause spoilage because it contains a lot of ingredients, particularly water. Moreover, the storage temperature in Thailand is warm and rather humid which is optimal conditions for microbial growth. The tested microorganisms were commonly found in the environment and hand contamination.

Because of the stability, the Rx1 was selected to test. The results from the Rx1 formula showed that this formulation passed the acceptant criteria of antimicrobial effectiveness for category 2 product; topically used products made with aqueous bases or vehicles, nonsterile nasal products, and emulsions, including those applied to mucous membranes. The log reduction of bacteria at the time 14-day decreased more than 2.0 log and did not increase after 28 days. Also, the fungi did not increase at all during the incubation times (Table 12).

Table 12 The log reduction of microorganisms

Microorganisms	Initial count	Log reduction (CFU/mL)			Results
		7 d	14 d	28 d	
S. aureus	4.8	2.2	2.1	2.9	Pass
Ps. aeruginosa	4.6	2.3	2.2	2.9	Pass
E. coli	4.5	2.2	2.0	2.8	Pass
C. albicans	4.2	1.2	1.1	2.2	Pass
A. niger	4.0	0.2	1.1	1.5	Pass

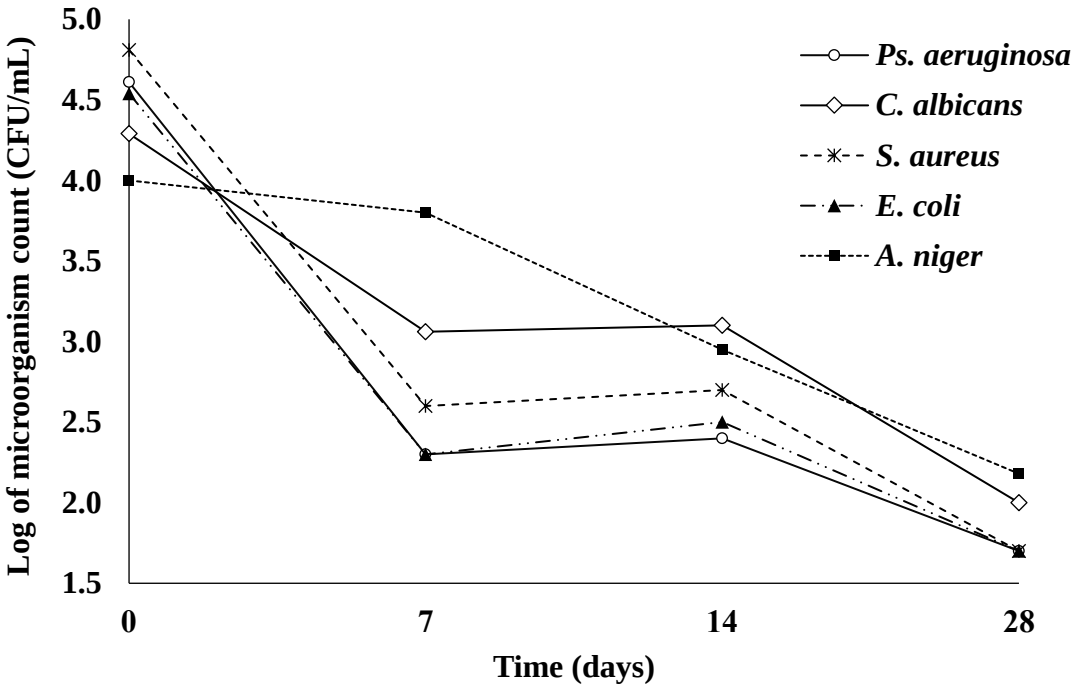


Figure 29 The growth of microorganisms in the mouthwash

CHAPTER V

CONCLUSION

In recent years, many hemp containing products have been available. The main compound with the non-psychoactive activity of hemp is CBD. Due to the pharmaceutical properties of CBD, it possessed an antioxidant, an anti-inflammatory and antimicrobial activities that allow it to be potentially used as an effective wound healing treatment.

This study demonstrated the cytotoxicity, antioxidation, anti-inflammation, pro-collagen type I production, wound healing and antimicrobial activities of the hemp extract and CBD. The finished product was also developed and evaluated for its stability over time. The hemp ethanolic extract that was obtained by ultrasonic-assisted extraction was composed of 13.45 ± 0.05% w/w of CBD which was the highest component. The hemp extract 216.23 ± 5.80 µg/mL inhibited 50% of DPPH activity but the CBD standard which had IC_{50} value of 107.67 ± 5.58 µg/mL showed more potency as an antioxidant than the hemp extract. It can be concluded that the antioxidant activity of hemp extract is influenced by CBD. A cytotoxicity study was performed prior to testing the cellular biological activities of the hemp extract and CBD. The non-cytotoxic concentrations of hemp extract at 50 µg/mL and CBD at 1 µg/mL were selected to determine further experiments. In addition, the significant inhibition of TNF-α and IL-1β release by LPS-induced RAW 264.7 cells inflammation occurred at the 5-50 µg/mL of the hemp extract while 0.25-1 µg/mL and 0.1-1 µg/mL of CBD significantly inhibited the production of TNF-α and IL-1β, respectively, in concentration-dependent activities ($p < 0.05$). The concentrations of CBD standard that exhibited the most anti-inflammatory activities were consistent with the concentration of CBD in the hemp extract. The hemp extract 50 µg/mL inhibited 91.05 ± 2.91% of TNF-α activity and 77.90 ± 3.36% of IL-1β activity. CBD 1 µg/mL inhibited 57.18 ± 6.27% of TNF-α activity and 84.17 ± 1.24% of IL-1β activity. Additionally, the hemp extract 10 µg/mL which was the most effective concentration significantly inhibited the PGE_2 production of 32.83 ± 4.59% in IL-1β-

induced HGF-1 cells inflammation ($p < 0.05$) which indicates that hemp extract also possesses anti-inflammatory activity. It can be inferred, therefore, that the anti-inflammatory activity of hemp extract is affected by CBD. Further, the hemp extracts 1, 10, 25 and 50 µg/mL significantly enhanced the pro-collagen type I synthesis ($p < 0.05$), especially at 25 µg/mL. It is probable that the hemp extract has the capability of connective tissue remodeling. To confirm the ability of hemp in oral wound healing, a scratch test assay was performed which showed that hemp extract 5 µg/mL and CBD 0.5 µg/mL increased wound closure by 27.92 ± 1.21% and 33.49 ± 1.67% at 48 hours, respectively. Unfortunately, neither the hemp extract nor CBD showed any antimicrobial activity.

The cellular biological activity tests identified the recommended dose of the hemp extract to develop the mouthwash should be 5 µg/mL. A non-ionic surfactant, PEG-40 hydrogenated castor oil, was used to solubilize the hemp extract in water. Further, the mouthwash consisted of 0.9% normal saline solution as a solvent, vitamin E as a potent antioxidant, menthol and thymol as antimicrobial agents. Probably, it will help to improve wound healing, antioxidant and antimicrobial activities. The stability study of the mouthwash at 50°C over 3 months indicated that the active compound and pH of Rx1 are stable. However, the color of the mouthwash was changed because of chlorophyll in the hemp. In addition, we have shown that the preservative in this mouthwash was effective in protecting the mouthwash against microbial growth during the manufacturing and storage time. Notably, this mouthwash is only used to study the stability of formulation. It is not a finished product. Nonetheless, the color of mouthwash must be improved, and the stability of finished product should be test.

It can be concluded, from the results of this study, that *Cannabis sativa* L. subsp. *sativa* inflorescence extract has the ability to protect against oxidants, can inhibit inflammation, induce collagen production, as well as healing the wound. Therefore, the mouthwash containing hemp extract is a possible alternative treatment to reduce inflammation and accelerate oral wound healing for oral ulcer patients, but a further clinical study is necessary.

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APPENDIX

Specification of *Cannabis sativa* L. subs. *sativa*

Source:	Female inflorescences of *Cannabis sativa* L. subs. *sativa*
Type:	Ethanolic extract
Active substance:	Cannabinoids
Activities:	- Antioxidant
	- Anti-inflammation
	- Wound healing
	- Pro-collagen type I production inducer
Physical property:	Dark green color and semi-solid crude extract
Solubility:	0.55 ± 0.05 mg/mL in water
Total plate count (TPC):	not detectable
Storage:	- Protect from light
	- Keep in tight container
	- Storage in cool place

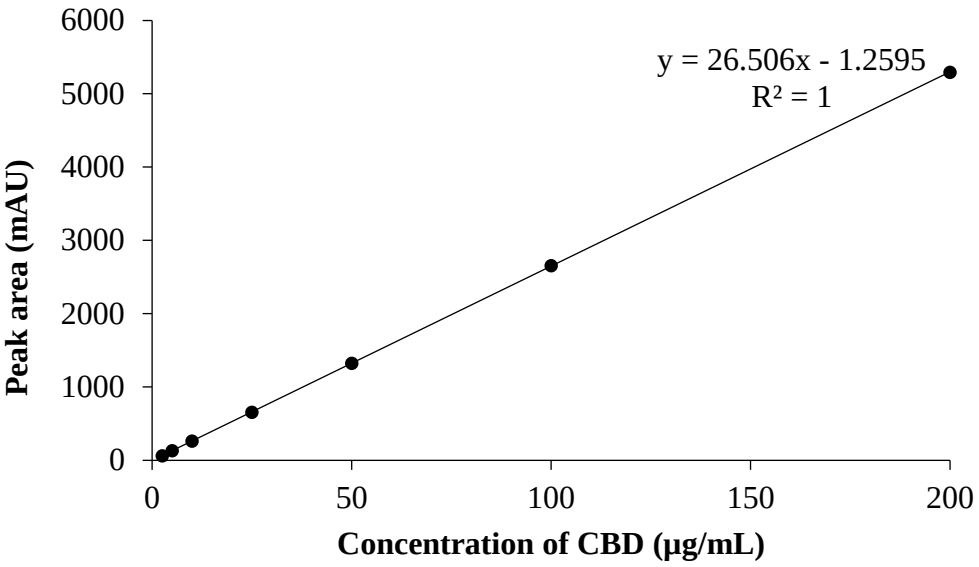

Figure A1 The calibration curve of CBD standard

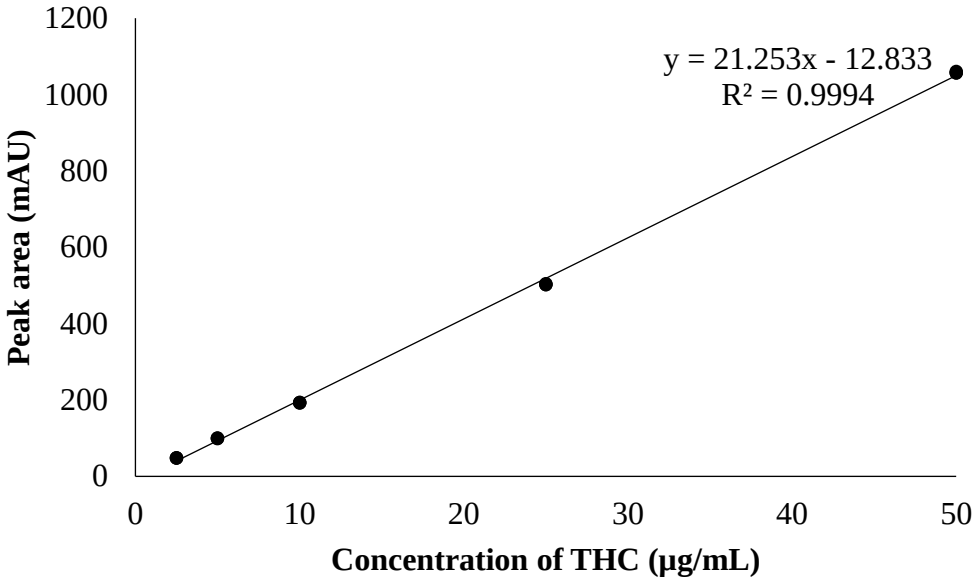

Figure A2 The calibration curve of THC standard

Note: The calibration curve of THC standard was supported by Ms. Nathareen Chaiwangrach, Faculty of Pharmeceutical Sciences, Naresuan University.

Table A1 Relative cell viability of HGF-1 cells after treatment with hemp extract

Concentration (µg/mL)	Cell viability (% of control)			
	N1	N2	N3	Mean ± SD
3.91	96.39	102.56	97.30	98.75 ± 3.33
7.81	104.69	105.70	100.34	103.57 ± 2.85
15.63	105.39	110.65	104.28	106.78 ± 3.40
31.25	113.08	114.50	109.14	112.24 ± 2.78
62.50	107.62	113.69	112.98	111.43 ± 3.32
100.00	86.75	98.08	95.66	93.50 ± 5.97
125.00	77.98	84.36	85.06	82.47 ± 3.90
250.00	71.11	67.46	68.27	68.95 ± 1.91
500.00	50.17	46.53	38.23	44.98 ± 6.12

Table A2 Relative cell viability of HGF-1 cells after treatment with CBD

Concentration (µg/mL)	Cell viability (% of control)			
	N1	N2	N3	Mean ± SD
0.08	98.74	115.06	112.83	108.88 ± 8.85
0.16	117.88	109.62	110.98	112.83 ± 4.43
0.31	121.28	105.83	117.98	115.03 ± 8.14
0.63	106.61	121.38	99.03	109.01 ± 11.37
1.25	37.32	44.90	38.68	40.30 ± 4.04
2.50	23.62	22.55	21.28	22.48 ± 1.17
5.00	22.74	21.67	21.48	21.96 ± 0.68
10.00	23.81	22.64	22.84	23.10 ± 0.62

Table A3 Relative cell viability of RAW 264.7 cells after treatment with the hemp extract

Concentration (µg/mL)	Cell viability (% of control)			
	N1	N2	N3	Mean ± SD
3.90	108.86	112.71	115.61	112.39 ± 3.39
7.81	97.56	101.80	100.05	99.80 ± 2.13
15.63	125.06	118.40	115.32	119.60 ± 4.98
31.25	107.16	121.31	124.97	117.82 ± 9.40
62.50	112.83	106.41	116.07	111.77 ± 4.92
100.00	95.87	102.14	98.41	98.91 ± 3.15
125.00	32.26	23.17	29.29	28.24 ± 4.63
250.00	6.24	5.94	5.79	5.99 ± 0.23
500.00	6.24	6.45	6.18	6.29 ± 0.14

Table A4 Relative cell viability of RAW 264.7 cells after treatment with CBD

Concentration (µg/mL)	Cell viability (% of control)			
	N1	N2	N3	Mean ± SD
0.08	81.60	86.31	89.07	85.66 ± 3.78
0.16	72.68	79.36	81.78	77.94 ± 4.71
0.31	78.36	93.37	87.11	86.28 ± 7.54
0.63	90.57	96.02	95.33	93.97 ± 2.97
1.00	80.16	86.04	91.11	85.77 ± 5.48
1.25	55.82	67.26	47.26	56.78 ± 10.04
2.50	8.87	7.76	7.10	7.91 ± 0.89
5.00	7.87	7.91	7.45	7.74 ± 0.26
10.00	8.14	9.37	8.64	8.72 ± 0.62

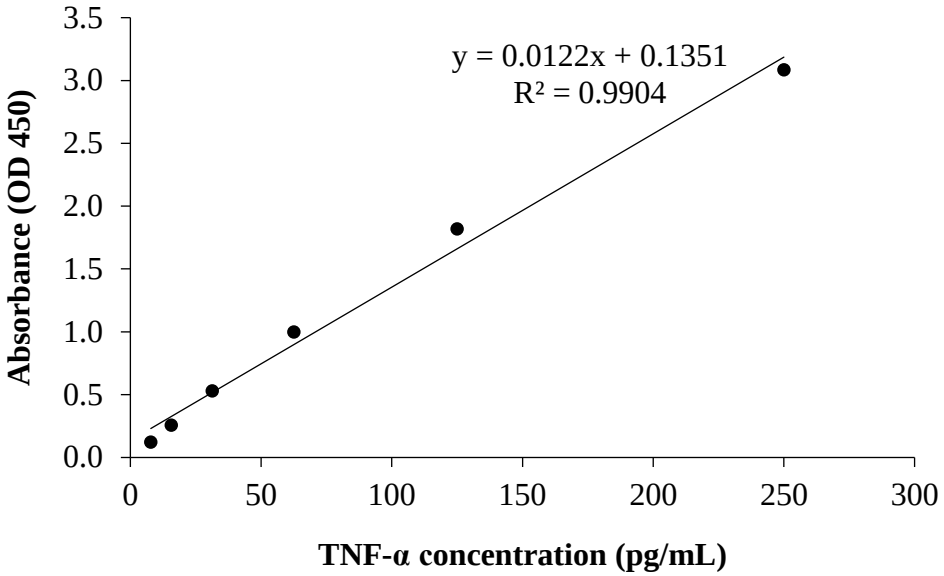

Figure A3 The calibration curve of TNF-α standard

Table A5 The TNF-α concentration in RAW 264.7 cells culture supernatant

Treatments	TNF-α concentration (pg/mL)			
	N1	N2	N3	Mean ± SEM
Control	67.05	65.41	73.61	68.69 ± 2.17
LPS	1715.77	1333.52	1394.73	1481.34 ± 91.82
Hydrocortisone	567.05	605.57	655.57	609.40 ± 25.63
Hemp 1 µg/mL	1251.42	1330.16	1197.46	1259.68 ± 38.53
Hemp 5 µg/mL	1180.16	1075.25	1041.72	1099.04 ± 41.70
Hemp 10 µg/mL	981.07	1037.54	1160.57	1059.73 ± 52.99
Hemp 50 µg/mL	92.73	178.33	126.69	132.59 ± 24.89

Table A6 The TNF-α concentration in RAW 264.7 cells culture supernatant (Cont.)

Treatments	TNF-α concentration (pg/mL)			
	N1	N2	N3	Mean ± SEM
Control	80.98	73.61	76.89	77.16 ± 2.13
LPS	1658.39	1758.31	1691.64	1702.78 ± 29.38
Hydrocortisone	477.70	495.74	605.57	526.34 ± 39.96
CBD 0.1 µg/mL	1583.25	1683.52	1577.79	1614.85 ± 34.37
CBD 0.25 µg/mL	1386.53	1453.47	1324.23	1388.08 ± 37.31
CBD 0.5 µg/mL	946.64	919.04	943.36	936.35 ± 8.70
CBD 1 µg/mL	605.93	791.64	790.00	729.19 ± 61.13

Table A7 The percentage inhibition of TNF-α

Treatments	Concentration (µg/mL)	% Inhibition vs LPS			
		N1	N2	N3	Mean ± SD
Hemp extract	1	15.52	10.21	19.16	14.96 ± 4.51
	5	20.33	27.41	29.68	25.81 ± 4.88
	10	33.77	29.96	21.65	28.46 ± 6.20
	50	93.74	87.96	91.45	91.05 ± 2.91
Hydrocortisone	10	61.72	59.12	55.74	58.86 ± 3.00
CBD	0.1	7.02	1.13	7.34	5.16 ± 3.50
	0.25	18.57	14.64	22.23	18.48 ± 3.80
	0.5	44.41	46.03	44.60	45.01 ± 0.89
	1	64.42	53.51	53.61	57.18 ± 6.27
Hydrocortisone	10	71.95	70.89	64.44	69.09 ± 4.06

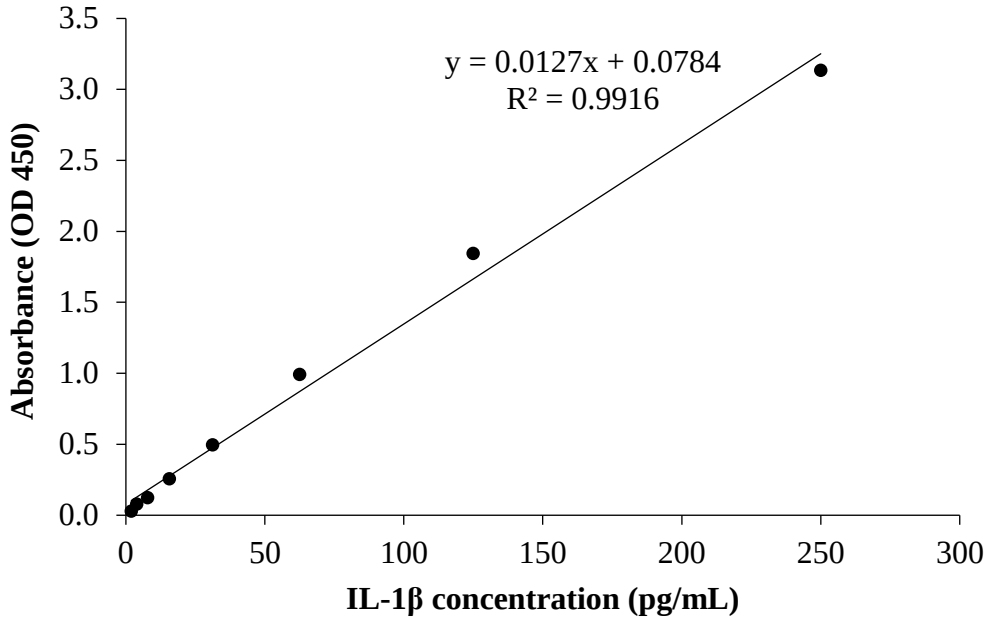

Figure A5 The calibration curve of IL-1β standard

Table A8 The IL-1β concentration in RAW 264.7 cells culture supernatant

Treatments	IL-1β concentration (pg/mL)			
	N1	N2	N3	Mean ± SEM
Control	0.60	0.44	0.80	0.61 ± 0.10
LPS	74.54	87.29	76.98	79.60 ± 3.91
Hydrocortisone	12.20	16.22	17.69	15.37 ± 1.64
Hemp 1 µg/mL	76.11	78.28	75.98	76.79 ± 0.74
Hemp 5 µg/mL	58.24	58.98	65.03	60.75 ± 2.15
Hemp 10 µg/mL	56.62	43.04	43.77	47.81 ± 4.41
Hemp 50 µg/mL	18.28	19.85	14.64	17.59 ± 1.54

Table A9 The IL-1β concentration in RAW 264.7 cells culture supernatant (Cont.)

Treatments	IL-1β concentration (pg/mL)			
	N1	N2	N3	Mean ± SEM
Control	0.60	0.44	0.80	0.61 ± 0.10
LPS	68.84	69.71	75.90	71.48 ± 2.22
Hydrocortisone	12.20	16.22	17.69	15.37 ± 1.64
CBD 0.1 µg/mL	64.73	59.10	61.02	61.62 ± 1.65
CBD 0.25 µg/mL	52.84	57.42	52.51	54.26 ± 1.58
CBD 0.5 µg/mL	41.54	31.41	43.98	38.98 ± 3.85
CBD 1 µg/mL	10.68	10.94	12.33	11.32 ± 0.51

Table A10 The percentage inhibition of IL-1β

Treatments	Concentration (µg/mL)	% Inhibition vs LPS			
		N1	N2	N3	Mean ± SD
Hemp extract	1	4.39	1.67	4.55	3.53 ± 1.62
	5	26.84	25.90	18.30	23.68 ± 4.68
	10	28.87	45.93	45.01	39.94 ± 9.60
	50	77.04	75.06	81.61	77.90 ± 3.36
CBD	0.1	9.44	17.32	14.64	13.80 ± 4.00
	0.25	26.08	19.67	26.53	24.09 ± 3.84
	0.5	41.88	56.06	38.47	45.47 ± 9.33
	1	85.06	84.70	82.75	84.17 ± 1.24
Hydrocortisone	5	82.93	77.32	75.26	78.50 ± 3.97

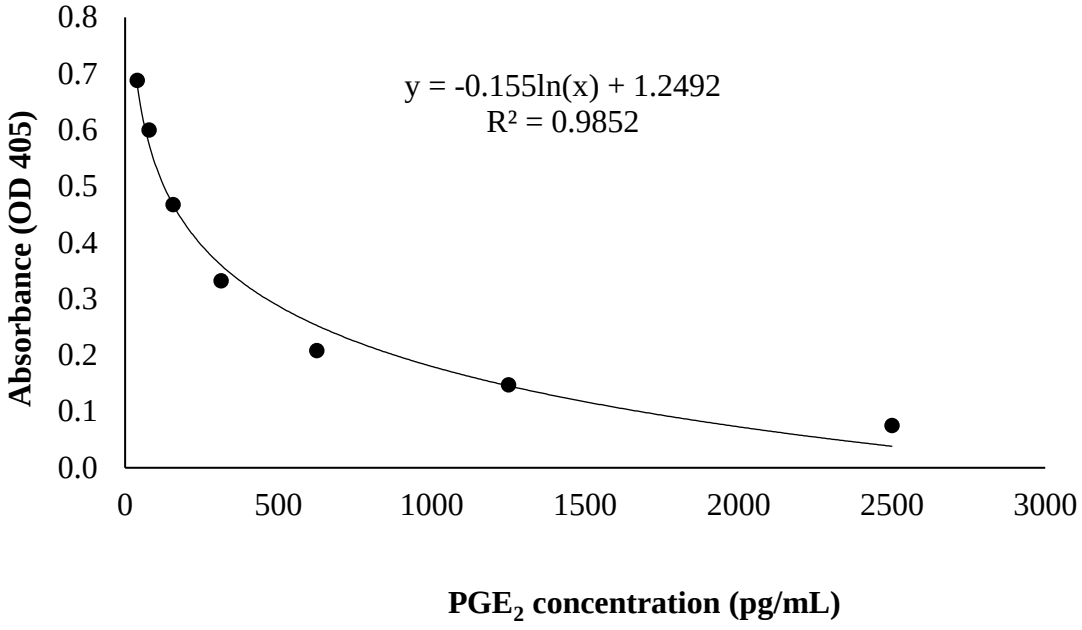

Figure A7 The calibration curve of PGE_2 standard

Table A11 The PGE_2 concentration in HGF-1 cells culture supernatant

Treatments	PGE_2 concentration (pg/mL)			
	N1	N2	N3	Mean ± SEM
IL-1β	1102.31	1221.39	1105.16	1142.95 ± 39.23
Hydrocortisone	223.13	241.09	167.44	210.55 ± 22.17
Hemp 1 µg/mL	988.12	977.66	966.06	977.28 ± 6.37
Hemp 10 µg/mL	772.54	711.76	816.08	766.79 ± 30.25
Hemp 50 µg/mL	946.93	943.58	956.14	948.88 ± 3.76

Note: The results of PGE_2 inhibition were supported by Dr. Wudthichai Wisuittiprot, and Dr. Vanuchawan Wisuittiprot, Naresuan University.

Table A12 The percentage inhibition of PGE_2

Treatments	Concentration (µg/mL)	% Inhibition vs IL-1β			
		N1	N2	N3	Mean ± SD
Hemp extract	1	13.45	14.36	15.38	14.40 ± 0.97
	10	32.33	37.65	28.52	32.83 ± 4.59
	50	17.05	17.35	16.25	16.88 ± 0.57
Hydrocortisone	10	80.46	78.88	85.33	81.56 ± 3.36

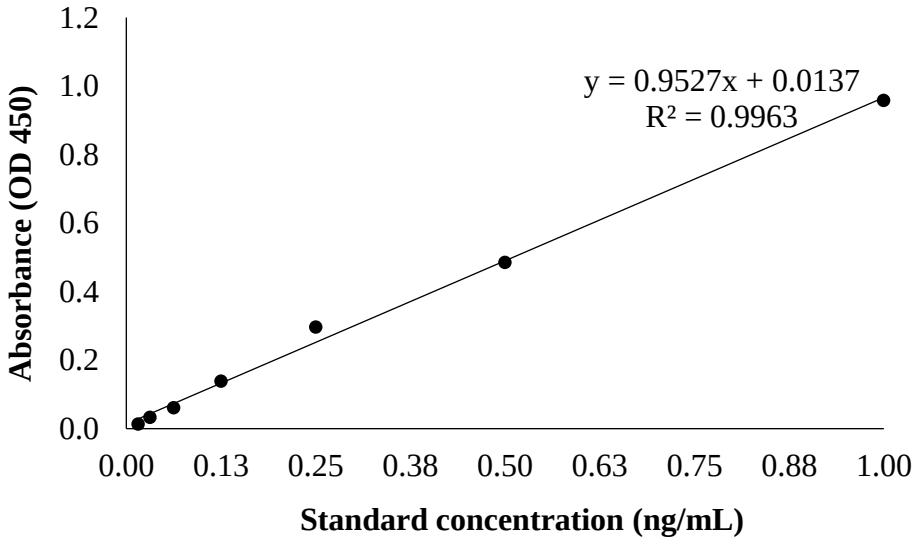

Figure A8 The calibration curve of pro-collagen type I standard

Table A13 The pro-collagen type I concentration in HGF-1 cells culture supernatant

Treatments	Pro-col I concentration (ng/mL)			
	N1	N2	N3	Mean ± SEM
Control	76.18	66.99	65.68	69.62 ± 3.30
L-ascorbic acid	157.92	172.22	183.77	171.30 ± 7.48
Hemp 1 µg/mL	137.19	102.29	96.65	112.04 ± 12.68
Hemp 10 µg/mL	116.98	152.80	138.63	136.14 ± 10.41
Hemp 25 µg/mL	172.22	155.56	148.34	158.71 ± 7.07
Hemp 50 µg/mL	123.02	126.95	166.84	138.94 ± 14.00

Table A14 The percentage of the wound closure area

Time (hour)	% Wound closure area vs control							
	Control				0.9% NaCl			
	N1	N2	N3	mean ± SEM	N1	N2	N3	mean ± SEM
12	11.74	8.39	7.38	9.17 ± 1.32	14.37	19.58	18.45	17.47 ± 1.58
24	12.85	9.58	7.86	10.10 ± 1.46	17.79	21.96	19.28	19.68 ± 1.22
36	17.60	11.66	11.07	13.44 ± 2.09	18.97	29.19	22.03	23.40 ± 3.03
48	26.25	26.98	19.78	24.34 ± 2.29	37.66	37.89	30.51	35.35 ± 2.42
Time (hour)	**Hemp extract**				**CBD**			
	N1	N2	N3	mean ± SEM	N1	N2	N3	mean ± SEM
12	12.30	11.60	12.40	12.10 ± 0.25	11.74	10.39	10.74	10.96 ± 0.40
24	21.63	19.08	20.38	20.36 ± 0.74	20.47	18.95	22.12	20.51 ± 0.92
36	26.73	22.35	24.01	24.36 ± 1.28	30.63	27.53	28.37	28.84 ± 0.93
48	29.98	25.80	27.97	27.92 ± 1.21	33.50	30.59	36.38	33.49 ± 1.67

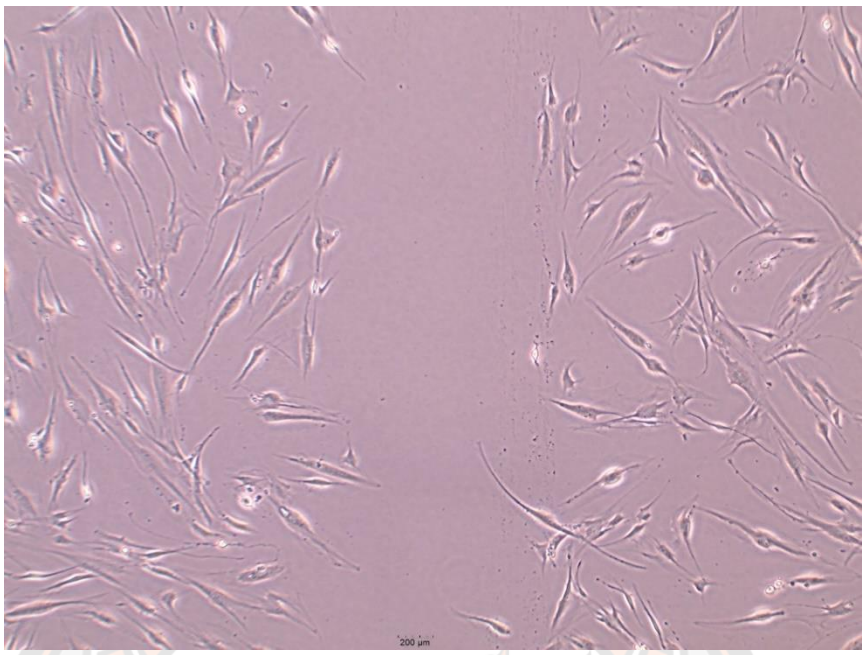

Figure A9 The changed morphology of HGF-1 cells at 36 hours after rinsing with 10 µg/mL of hemp extract

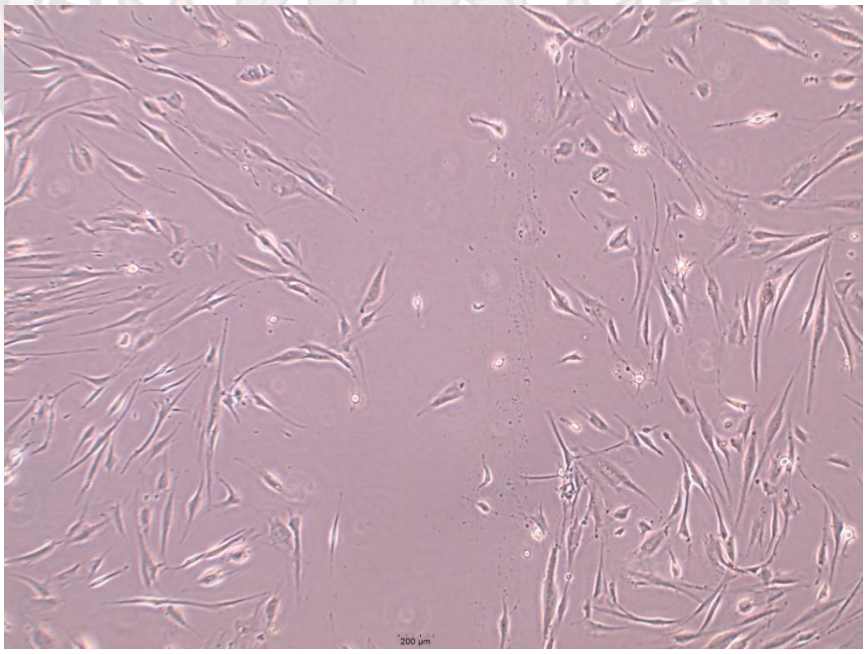

Figure A10 The changed morphology of HGF-1 cells at 48 hours after rinsing with 10 µg/mL of hemp extract

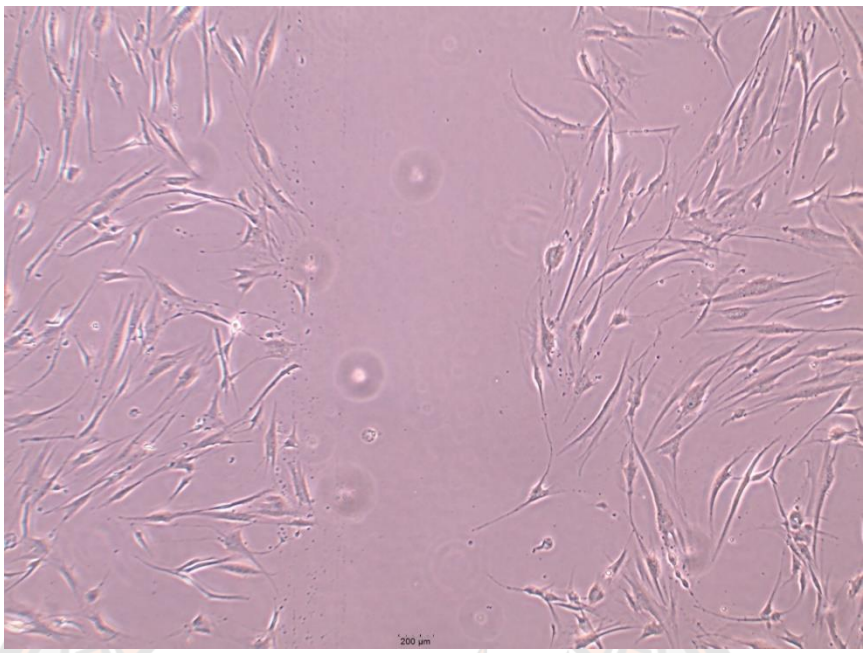

Figure A11 The changed morphology of HGF-1 cells at 12 hours after rinsing with 25 µg/mL of hemp extract

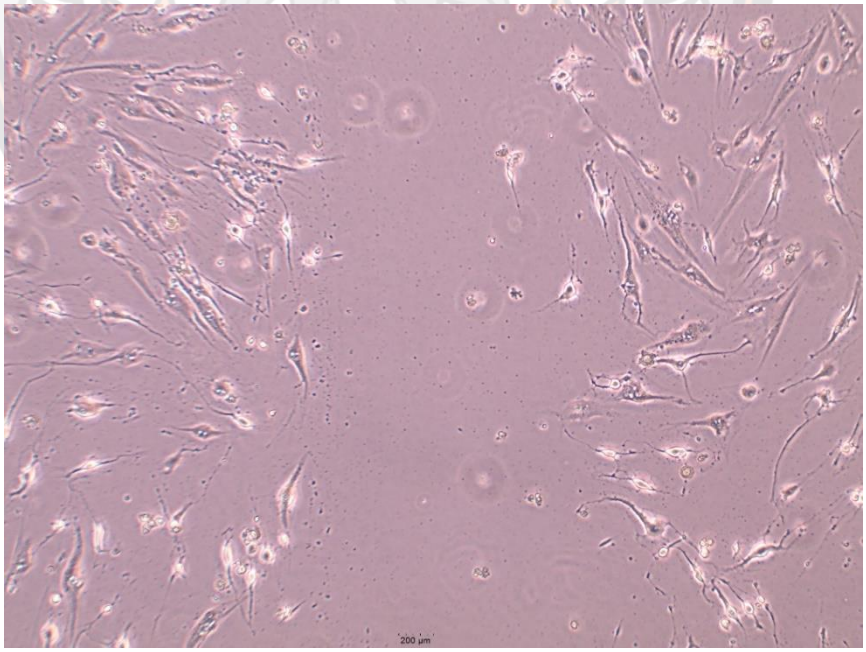

Figure A12 The changed morphology of HGF-1 cells at 24 hours after rinsing with 25 µg/mL of hemp extract

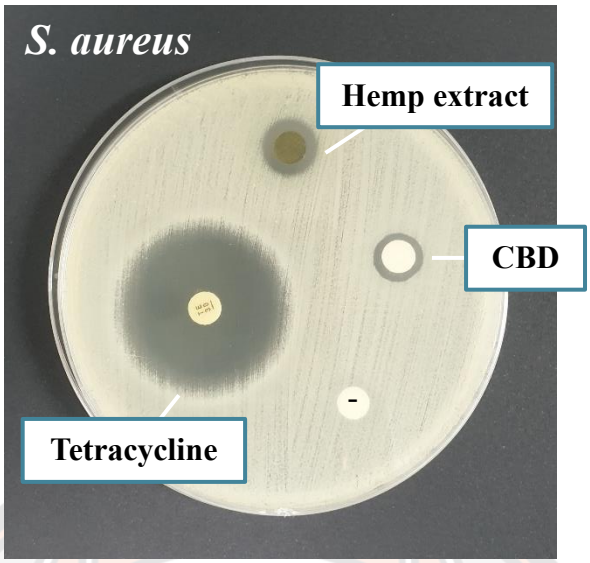

Figure A13 The inhibition zone of hemp extract, CBD, tetracycline and DMSO against *S. aureus*

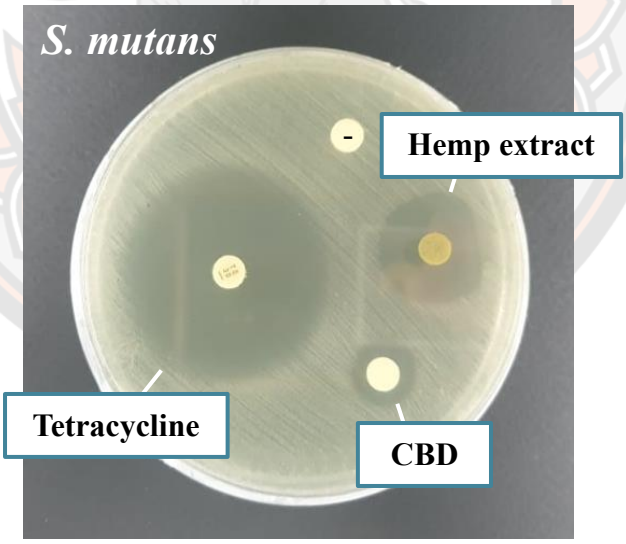

Figure A14 The inhibition zone of hemp extract, CBD, tetracycline and DMSO against *S. mutans*

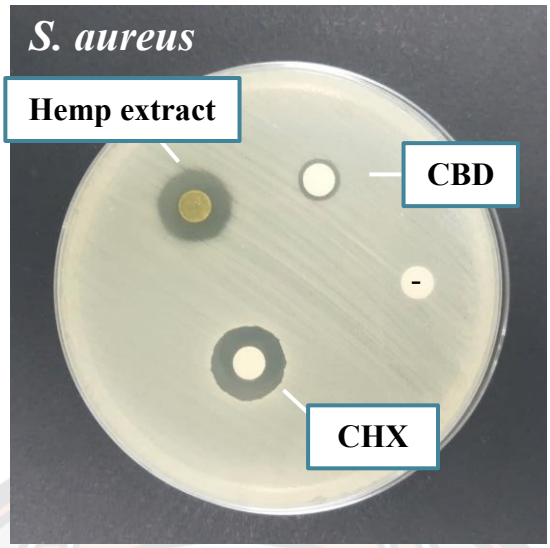

Figure A15 The inhibition zone of hemp extract, CBD, CHX and DMSO against *S. aureus*

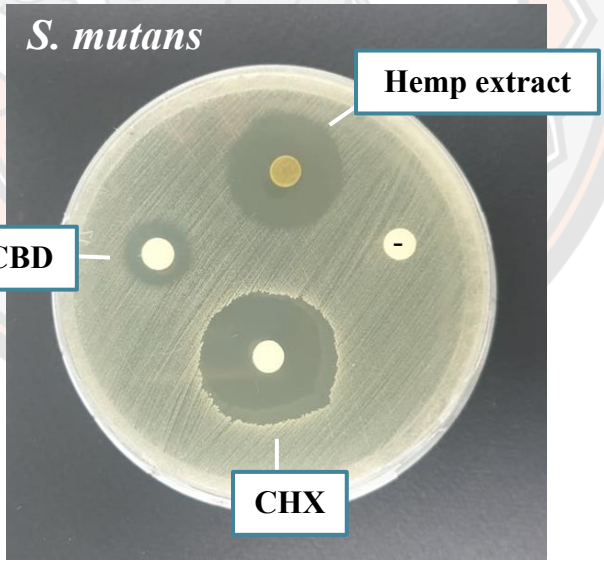

Figure A16 The inhibition zone of hemp extract, CBD, CHX and DMSO against *S. mutans*