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AN IN VITRO INVESTIGATION OF THE STABILITY AND PERMEABILITY OF PHYTOCANNABINOIDS FOR SKINCARE FORMULATIONS

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AN IN VITRO INVESTIGATION OF THE STABILITY AND PERMEABILITY OF PHYTOCANNABINOIDS FOR SKINCARE FORMULATIONS

BY

TOYOSI AKANJI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

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MASTER OF SCIENCE IN CHEMISTRY THESIS

OF

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2021

ABSTRACT

The endocannabinoid system (ECS) is comprised of ligands, enzymes, and receptors, which are involved in the regulation of various biological functions including cell growth, sensory phenomena, and immune and inflammatory responses. Recent studies have shown that human skin cells contain components of the ECS including cannabinoid receptor 1 and 2 (CB1, CB2) and transient receptor potential channel subfamily V (TRPV1) receptors. The ECS is critical for in maintaining homeostatic conditions and overall skin health, and disruption of ECS may cause pathological disorders including allergic dermatitis, cutaneous itch and pain, and neoplastic cell growth. Published studies also support that cannabinoids from the plant *Cannabis sativa* are ligands of several receptors in the ECS; thereby they may exert various ECS-mediated biological functions including beneficial skin effects. However, the lack of fundamental understanding of phytocannabinoids' physico-chemical properties and their skin protective effects limit the use of phytocannabinoids as bioactive ingredients for dermatological and/or cosmeceutical applications. Notably, the skin permeability of phytocannabinoids including cannabidiol (CBD), cannabigerol (CBG), delta-8-tetrahydrocannabinol (Δ-8-THC), cannabinol (CBN), and cannabidivarin (CBDV) has not been reported. Herein, the current study aims to evaluate the skin permeability of CBD, CBG, Δ-8-THC, CBN, and CBDV using the parallel artificial membrane permeability assay (PAMPA) as well as their biological effects, including tyrosinase-modulating activity.

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Finally, I thank my family and friends. Without their prayer, encouraging words, and support I would not be where I am today. Thank you, mom and dad, for the laughs, the food and for cheering me on every step of the way.

DEDICATION

This is dedicated to my family and friends who have supported me throughout this journey. In addition, this is dedicated to the Black women in STEM who came before me, and to those who will come after.

"They tried to bury us, but they didn't know we were seeds"

– Dinos Christianopoulos.

PREFACE

This thesis has been prepared in manuscript format to meet the criteria set by the Graduate School of the University of Rhode Island. The first manuscript is formatted based on the journal *"Cannabis and Cannabinoid Research".*

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CHAPTER 1. INTRODUCTION

1. BACKGROUND

1.1 The role of the skin

The skin acts as an environmental barrier, regulates homeostasis, and provides an alternative route for drug delivery. Both its health and appearance can affect one's personal perception and quality of life (Green, 2010). As the largest organ in the body, it serves as a protective layer from the harsh external environment, exogenous chemicals, and from injury induced mechanically or through radiation (Fox et al., 2011; Barry, 1991). The skin also controls homeostatic conditions in the body through regulation of blood pressure and temperature, excretion of waste, and the mediation of pain sensations (Río et al., 2018; Fox et al., 2011; Barry, 1991). Appendages such as sweat glands maintain the pH of the skin and regulate the secretion of waste, antibodies, protein and drugs through sweat (Barry, 1991). The innermost layer of the skin, the dermis, contains connective tissues, nerve endings and critical anatomical structures (Figure 1). The major function of the dermis is to maintain thermoregulation, sensation and protection for deeper anatomical structures (Brown and Krishnamurthy, 2021; Barry, 1991). This is followed by the epidermis, which provides a hydrophilic barrier to the skin as well as UV protection through melanin formed by melanocytes (Yousef, Alhajj, and Sharma, 2021). The outermost layer of the skin, the stratum corneum (SC), is the skin's outermost barrier and limits the passage of chemicals and pathogens to the skin's other layers (Menon et al., 2012 ; Chen et al., 2013). The health of the skin is imperative to the maintenance of

homeostasis throughout the body. Diseases and disorders such as atopic dermatitis (AD), psoriasis, and post inflammatory hyperpigmentation (PIH) can affect both the function and the appearance of the skin. Many of these diseases are linked to autoimmune issues and inflammation (Baumgarth and Bevins, 2007). Studying permeability and pharmacology may be beneficial in understanding how to better choose and formulate active ingredients to treat diseased skin thereby helping its appearance and homeostatic function.

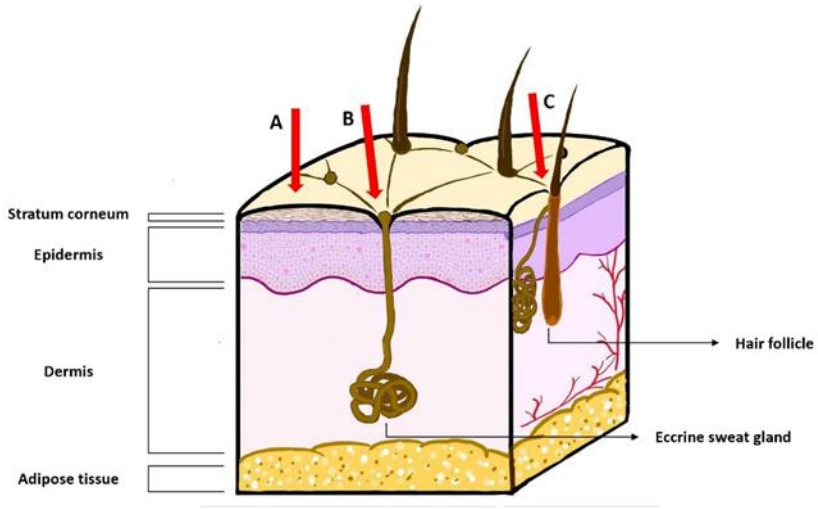

Figure 1: Diagram of the human skin and routes of permeability. The human skin is comprised of 3 major layers, the epidermis, dermis, and subcutaneous adipose tissue. Within the epidermis, the outermost layer of the skin called the stratum corneum is the rate limiting step in skin permeability. Transdermal drugs delivery can occur through permeation through skin (A) or through appendages such as eccrine sweat glands (B) and hair follicles (C). Figure modified from Barry 1991.

1.2 Benefits and Challenges of Transdermal Drug Delivery

Benefits of transdermal route:

Transdermal drug delivery describes the pharmacokinetics of an active ingredient or drug through the skin. Compared to the traditional oral route, this drug delivery method can be advantageous in the treatment of both systemic diseases and localized skin disorders. Notably, the largest advantage in transdermal drug delivery is the bypass of first-pass metabolism of the liver. The skin is considered a zero-order release route and therefore the stability of the drug is maintained for longer amounts of time (Fox et al., 2011). A drug that is delivered through the skin will require less frequent dosing and therefore improved patient compliance (Fox et al., 2011; Barry, 1991). Transdermal drug delivery also avoids the extreme changes in the pH of the stomach to intestines while also avoiding the effects that food and enzymes have on absorption. Finally, this method minimizes pulse entry into the blood stream and any side effects related to peak plasma levels (Barry, 1991).

Challenges of transdermal route:

Although the transdermal route is desirable for the administration of drugs to either the skin or body, there are many challenges regarding this approach. The first and least harmful being an aesthetic issue. Many skin disorders are generally non-life threatening. However, quality of life is greatly affected when the appearance of the skin is not ideal (Green, 2010). The next challenge is the complexity of the more systemic skin diseases and disorders. Chronic diseases, like psoriasis, which affects millions of Americans, are the result of an auto-immune response (Baumgarth and

Bevins, 2007). Transdermal drug formulations may be beneficial in the treatment of psoriasis related symptoms and skin appearance, but more research is needed to fully cure this disease. The lipophilicity of drugs used as active ingredients also poses a challenge to transdermal drug delivery. Highly lipophilic drugs may be able to effectively pass through the stratum corneum but may experience difficulties diffusing into the aqueous tissue of the epidermis. If the drug partitions from its vehicle in the SC or if the vehicle is not able to penetrate the epidermis, this diffusion may become the rate limiting step in transdermal drug delivery (Barry, 1991). Finally, the permeability of the stratum corneum presents another significant challenge as it is the rate limiting step of drug permeability through the skin (Chen, Han, and Lian, 2013; Barry, 1991; Fox et al., 2011). Although this function is beneficial for the protection against exogenous chemical toxins and trans epidermal water loss (TEWL), it presents a large barrier for drug permeability (Barry, 1991) .

Formulations

Understanding the benefits and challenges of transdermal delivery of a drug is valuable when trying to understand how the active ingredient of a topical is applied and then permeates. Depending on the active ingredient or drug, formulations can be adjusted according to their specific use or target. There are five targets to consider regarding transdermal drug delivery: the skin surface; the stratum corneum; epidermis and upper dermis; skin glands; and the entire body, also referred to as systemic circulation (Barry, 1991). For example, a drug needed to reach the bloodstream may be formulated to have better permeability, whereas this may not be the case for a drug

that is meant solely to illicit a physiological change to the skin. Topicals can also be formulated to sit directly on the skin surface if no physiological change is needed but aesthetic change is desired. Aside from permeability, formulations must also account for the drug's stability and maintain its chemical potential (Barry, 1991; Fox et al., 2011). The possibility of drug degradation must also be considered. Degradation may occur through light, oxygen and bacterial metabolism that occur on the skin's surface (Barry, 1991). The vehicle used for transdermal drug delivery is equally as important as the drug itself and can be the main influence in a drug's permeability through the skin.

1.3 Skin Permeability

As previously stated, skin permeability is generally the rate limiting step in transdermal drug delivery (Chen, Han, and Lian, 2013). Knowing if and how a drug permeates the skin gives insight to the ideal formulation environment for that drug to reach its ideal target. Regarding the drug itself, there are four main influences that affect its ability to permeate the layers of the skin. Most commonly, drugs permeate the skin through molecular diffusion caused by a concentration gradient. The natural force for a solution to be at equilibrium drives the passive diffusion of particular chemicals into the skin (Brodin et al.; Barry, 1991; Naegel et al., 2013). This will generally allow substances to enter the stratum corneum but partitioning generally needs to occur for a substance to reach the epidermis. Formulations are commonly a mixture of two or more immiscible phases: aqueous and lipophilic. The partitioning coefficient of a substance, K, can be used to calculate the concentration of drug in

each phase based on the drugs lipophilicity or hydrophilicity. For the drug to enter the aqueous filled epidermis, the concentration of the drug in each phase of the formulation must be at equilibrium, and a natural separation of the two phases must occur. Drugs may also permeate the skin through phase changes or metabolic activity of the skin. Phase changes are generally seen in evaporation or dissolution of drug in the vehicle (Naegel et al., 2013). Metabolism occurs through the biotransformation of drugs by enzymes present in the skin or even clearance into the body through systematic circulation (Pyo and Maibach, 2019; Naegel et al., 2013). Finally, the drug may enter the stratum corneum through binding or adsorption. This generally occurs when hydrophilic drugs are bound to proteins or other substances to enter the SC (Naegel et al., 2013). Regarding the most common permeability influence, concentration gradient, the permeability pathway of a drug through the stratum corneum can be described as either intercellular or transcellular (Figure 2). It should also be noted that skin appendages such as shunts, pores, hair follicles and sweat glands also greatly influence permeability (Mitragotri, 2003; Barry, 1991). Often, multiple processes of permeability may be valid for one drug and therefore investigation of an active ingredient is needed to understand its permeability profile.

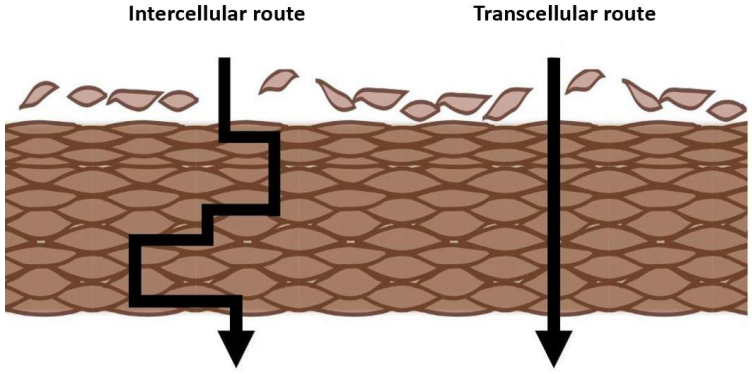

Figure 2: Routes of permeation through the stratum corneum. The stratum corneum is the rate limiting step in drug permeability of the skin. Passive permeability of a drug influenced by concentration gradients can be either intracellular or transcellular. Figure adapted from Barry 1991.

1.4 Evaluation & Application

in silico & in vivo methods

The permeability of a drug through the skin can be evaluated *in silico*, *in vivo* and *in vitro*. *In silico* models utilize computer programming to predict the permeability of a drug through the skin. Various models are multi-dimensional, meaning they can account for multiple parameters that may affect permeability. For example, some *in silico* methods can calculate both 2D and 3D models of the stratum corneum which gives valuable permeability insight such as corneocyte permeability, alignment, diffusion, partition coefficients and protein binding (Naegel et al., 2013). However, Quantitative Structure Activity Relationships (QSAR) *in silico* models are used more often because they compare a drug's physiochemical and/or structural properties to measured skin permeability data (Chen et al., 2013). A QSAR *in silico* model, the Assessment of Absorption, Distribution, Metabolism and Excretion (SwissADME) can be used to predict the skin permeability coefficient (K_p). This *in silico* tool utilizes

the Potts and Guy equation for determining K_p which accounts for lipophilicity and size. Compounds that easily permeate the skin are represented by positive, higher values of Log K_p while more negative and lower values represent less skin permeability (Chen et al., 2013; Daina et al., 2017; Potts and Guy, 1992). Although this method is useful, it should be noted that Log K_p values of hydrophilic compounds are usually underestimated (Chen et al., 2013; Mitragotri, 2003).

In vivo models such as animal or human studies are beneficial because they provide a more comprehensive view of permeability. Animal models are generally easier and more cost efficient than clinical trials but are more expensive and labor intense than *in vitro* experiments (Sinkó et al., 2012) . Mice, which are commonly used in animal studies, overestimate the permeability rate of a compound. These animal models do not give an accurate representation of human skin permeability since compounds can potentially reach the body through hair follicle permeation (Sugino et al., 2009; Sinkó et al., 2012). Human studies can give nuanced and unique information that animal, *in silico* and *in vitro* models cannot provide such as side effects, patient compliance and unique side effects. However, clinical trials are usually avoided due to practicality and ethical issues (Abd et al., 2016). It should be noted that there is no one parameter for measurement in clinical trials for skin diseases (Spuls et al., 2017). The permeability profile and pharmacokinetics of a drug could potentially be assessed through blood serum levels.

In vitro Methods

In vitro methods provide a more cost effective less time-consuming alternative for the evaluation of a drug's skin permeability (Sinkó et al., 2012). *In vitro* methods generally involve a biomimetic membrane that simulates either the stratum corneum or the lipid bilayer of the epidermis. *In vitro* methods such as the Parallel Artificial Permeability Membrane Assay (PAMPA) can serve as a preliminary screening evaluation of a drugs permeability through a synthetic membrane (Figure 3). This high throughput technique utilizes an artificial lipid membrane included on a 96-well plate (Luo et al., 2016; Sinkó et al., 2012). This skin PAMPA plate is then attached to another 96-well plate, thereby "sandwiching" the biomimetic membrane in between the two plates. This method can be adjusted for the evaluation of compounds of semi-solid dosage forms (formulations), by adding the compound solutions to the bottom well or by adding the formulation to the top well. Sinko et al. found that the permeability of analyzed compounds were statistically similar to a study that measured the permeability of the same compounds using full thickness human skin at 37˚C (Sinkó et al., 2012). In the PAMPA model, a permeability constant, Log P_e value is given for a tested compound. As with the Log K_p values of *in silico* models, a more negative Log P_e value corresponds to a less permeable compound.

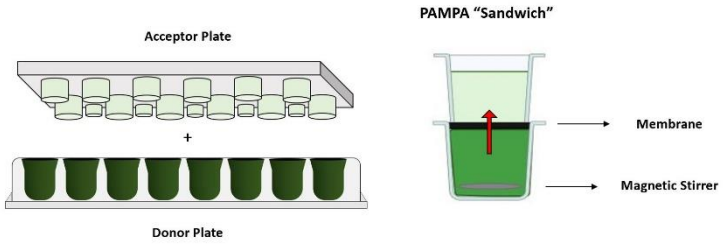

Figure 3: The parallel artificial permeability membrane assay (PAMPA). The PAMPA assay is an *in vitro* method that consists of two 96-well plates "sandwiched" together. The concentration of drug that permeates from the donor well (bottom) to the acceptor well (top), is presented as -Log P_e values.

The standard for *in vitro* skin permeability techniques is the Franz cell diffusion assay (Figure 4). A 2010 study showed that validation of this assay decreased variations of results dramatically, therefore showing that this is a precise skin permeability model (Ng et al., 2010). In a Franz cell diffusion assay, a membrane is placed in between two cells, the acceptor and donor. Drug formulations are loaded onto a membrane in the donor cell, while an aqueous buffer is used to fill the acceptor cell. Samples of permeated drug are taken through the acceptor cell at set time points, and the drug concentration is then evaluated using analytical instrumentation (Chen et al., 2013). Assay temperatures are set to the temperature of the skin (32˚C), thereby essentially emulating how a drug will permeate the skin when it is applied. The Franz cell diffusion assay can be classified as either an *in vitro* Release Test (IVRT) or an *in vitro* Permeability Test (IVPT). These studies differ only by type of membrane used. An IVRT study uses only synthetic biomimetic membranes while IVPT studies use human or animal skin (Zsikó et al., 2019). An IVRT study can be considered a

precursor to the more physiologically pertinent IVPT. In an IVRT study, the permeability profile of a drug is defined by its release rate or the concentration of drug that is released over a given period of time (Tiffner et al., 2018). The permeability profile of a drug in an IVPT study is defined by its flux, J. Flux values are given as the amount of drug permeating through a cross sectional area in a given period of time; units are written as mol cm^2 min^1 or mg cm^2 h^1 (Brodin et al.).

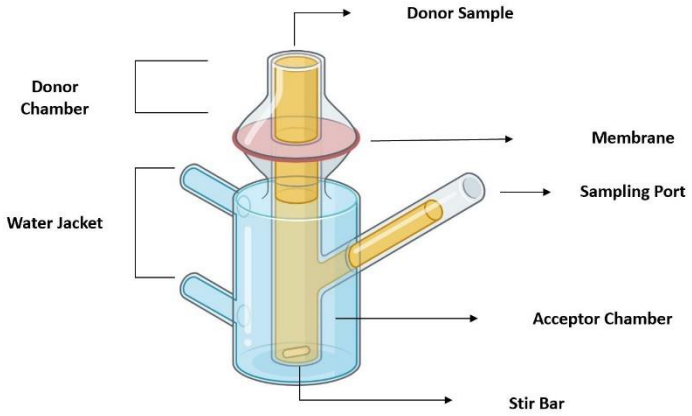

Figure 4. The Franz cell apparatus. The Franz cell assay is the golden standard of *in vitro* permeability evaluations. Samples are loaded into the donor chamber and passive permeability of a drug through a biomimetic skin membrane is evaluated. The concentration of drug that permeated through is presented as the release rate. This image was created with BioRender.com.

Enzymatic assays

The skin is saturated with enzymes, each one serving a specific purpose that aids in the skin's health and physical appearance. However, these enzymes can cause serious side effects if subjected to extreme environmental influence. Active ingredients in topicals that permeate the skin can be metabolized by these enzymes and possibly inhibit or amplify their function. Enzymes are an ideal cosmeceutical

drug target because of their specificity, which reduces the likelihood of side effects and, and their large impact on the skin's appearance.

Aged skin appears wrinkled, sagging and is not at its full homeostatic potential (Ye et al., 2002). Two enzymatic targets that can directly impact the appearance of aging skin are the collagenase and elastase enzymes. Collagen is responsible for the skin's tensile strength and youthful appearance. The collagenase enzyme initiates the digestion of collagen within the skin. Similarly, the enzyme elastase metabolizes elastin, which is responsible for the plumpness and recoil (Jiratchayamaethasakul et al., 2020; Choi et al., 2015). Compounds that inhibit collagenase and elastase enzymes will directly reduce the appearance of aging skin. Aging skin can also be subjected to melasma, also known as age/sun spots. These hyperpigmentated spots are due to excessive exposure to UV radiation over time (Ma et al., 2017; Smit et al.,2009). Another more severe form of hyperpigmentation is post inflammatory hyperpigmentation (PIH). Inflammation due to abrasion or blows to the skin can illicit the over production of melanin in the dermis. Over-accumulation may result in melanin migrating to the epidermis where it becomes trapped, creating a chronic dark spot. The tyrosinase enzyme (TYR) is the rate limiting step in the production of melanin (Pillaiyar et al., 2017; Roh et al., 2017). Compounds that inhibit TYR mitigate the production of melanin and therefore treat hyperpigmentation disorders.

2. CANNABINOIDS AND THE ENDOCANNABINOID SYSTEM

The Endocannabinoid System

The ECS is responsible for various biological functions including ingestion, pain perception, learning and memory, neuroprotection, and immune response (Río et al., 2018). The ECS is comprised of cannabinoid receptors, endocannabinoids, and the enzymes responsible for their production and degradation (Nikan et al., 2016; Eagleston et al., 2018; Maccarrone et al., 2003). It has recently been discovered that the Endocannabinoid System (ECS) also resides in the skin (Río et al., 2018; Bíró et al., 2009). ECS ligands, receptors and enzymes are found within both healthy and diseased skin (Eagleston et al., 2018; Jeong et al., 2019). The ECS of the skin functions to regulate epidermal homeostasis, pain sensation, inflammation, and the skin's appendages (Caterina, 2014). It is also responsible for cutaneous function through the regulation of cell growth differentiation, immune and inflammatory response, and proliferation (Río et al., 2018; Bíró et al., 2009). Disruption of the ECS has been linked to many skin diseases, including atopic dermatitis (Río et al., 2018).

Endocannabinoid Receptors

Cannabinoid receptor one (CB1) and Cannabinoid receptor two (CB2) are the major receptors associated with the endocannabinoid system (Franco et al., 2020). Both CB1 and CB2 are expressed within keratinocytes, sebaceous glands, hair follicles, and immune cells in the skin (Río et al., 2018; Bíró et al., 2009; Jeong et al., 2019). These receptors have also been found in the myoepithelial cells of eccrine sweat glands, melanocytes and dermal fibroblasts. Cell cultures of Normal Human

Epidermal Keratinocytes (NHEK) and HaCaT keratinocytes have also been shown to possess these receptors (Pucci et al., 2012; Ständer et al., 2005; Garcia-Gonzalez et al., 2009; Río et al., 2018; Bíró et al., 2009). The expression of these receptors in many types of skin cells and appendages, suggest that the ECS is a viable drug target for the maintenance of homeostasis as well as the treatment of many skin diseases. The function of the CB1 receptor is presumably associated with protective activity related to the disturbance of the skin barrier or inflammation (Gaffal et al., 2013). CB2 has been associated with the positive regulation of sebum levels and cell death (Bíró et al., 2009; Dobrosi et al., 2008). A study done by Karsak et al. exhibits how ECS receptors are vital in the attenuation of inflammatory response. The animal study showed that the depletion or blockade of cannabinoid receptors increased instances of contact allergic inflammation that was greatly decreased with the stimulation of these same receptors (Karsak et al., 2007). It has also been suggested that CB receptor agonists with low efficacy can provide sufficient therapy using entourage compounds (Di MARZO et al., 2001). Although it is assumed that the stimulation of these receptors is generally positive, it is suggested that antagonism of these receptors may also be beneficial in specific instances, such as the treatment of cutaneous contact hypersensitivity (CHS). A study found that the antagonism of the CB2 was initially effective in treating CHS symptoms; however it should be noted that chronic blockade of this receptor is detrimental (Ueda et al., 2005; Oka et al., 2006), thus showing a potential wide range of applications where these receptors may be pursued.

Endocannabinoids

Endocannabinoids are a class of endogenously synthesized ligands of the endocannabinoid system. The most comprehensively studied endocannabinoids are anandamide (*N*-arachidonoylethanolamine, AEA), a low efficacy agonist for both the cannabinoid receptors CB1 and CB2, and 2-arachidonoylglycerol (2-AG), a high efficacy agonist of CB1 and CB2 (Gonsiorek et al., 2000; Lu and Mackie, 2016; Bíró et al., 2009). However, *N*-acyl-ethanolamides (NAEs) and monoacylglycerols (MAGs) are two families of endocannabinoids that are equally important (Piscitelli and Bradshaw, 2017; Jeong et al., 2019). These endocannabinoids are generated within mammalian tissue when required (Río et al., 2018). Their chemical precursors reside within lipid membranes and synthesis occurs upon depolarization or activation of specific G protein-coupled receptors (GPCRs). Structurally, endocannabinoids are identified through two functional groups: the presence of saturated or unsaturated long chain fatty acids and hydrophilic moieties such as glycerol, dopamine or ethanolamine (Lu et al., 2016). It is inferred that the hydrophobic moieties are a result of amino acids being conjugated to the long fatty acid chains (Jeong et al., 2019). This conjugation is said to occur through one or two enzymatic steps before being released into the extracellular space (Lu and Mackie, 2016).

Phytocannabinoids

The popularity of the plant *Cannabis sativa* has included decades of research to discover its medicinal purpose. This plant is a prolific producer of a class of cannabinoids, referred to as phytocannabinoids, that act as ligands to the ECS (Hanuš

et al., 2016). *Cannabis sativa* produces over 100 phytocannabinoids, all differing slightly in structure and pharmacology (Hanuš et al., 2016; Sampson, 2021). Structural differences in the isoprenyl residue, resorcinyl core, or side-chain moieties may be the biproduct of degradation through auto-oxidation (Hanuš et al., 2016). The major phytocannabinoids from this plant, Δ9-Tetrahidrocannabinol (Δ9-THC) and cannabidiol (CBD), have been extensively studied for their pharmacological impacts (Franco et al., 2020). However, this research focus limits the full potential of medical *Cannabis*, especially for patients looking to avoid Δ9-THC and its psychoactive effects. Minor phytocannabinoids such as Δ8-Tetrahydrocannabinol (Δ8-THC) , Cannabidivarin (CBDV), Cannabigerol (CBG), and Cannabinol (CBN) also exhibit similar pharmacological properties to CBD and Δ9-THC (Sampson, 2021). Further investigation is needed to study the medicinal benefits of the minor phytocannabinoids individually or together in *Cannabis* extracts. Although often studied individually, the synergism of multiple phytocannabinoids, known as the entourage effect, may have a larger medicinal impact (Russo, 2011; Di Marzo et al,. 2001)

3. CANNABINOIDS AND THE SKIN

Current Applications

Cannabinoids can be used in the treatment of various skin diseases through the modulation of cannabinoid receptors in the ECS (Petrosino et al., 2010; Jeong et al., 2019). One of the most extensively studies skin diseases connected to the ECS is eczema. Eczema is considered an "umbrella term" diagnosis to describe dry, inflamed, and itchy skin. Variations of eczema, such as atopic dermatitis (AD), have been connected to the ECS. One study using RNAseq showed that AD diseased skin exhibited down regulation of CB1 and CB2 in comparison to healthy regions of skin on the same body. They also found upregulation of other "non-classical" ECS receptors, TRPV1 and TRPV2, directly related to itch (Tóth et al., 2019; Nattkemper et al., 2018). Therefore, increasing the activity or activation of CB1/CB2 receptors is associated with mitigation of both itch and pain, which can also be accomplished through the decreased activity of TRPV1 (Avila et al., 2020).

There are very few studies linking cannabinoids to the regulation of skin health and functions. Clinical studies have shown the attenuation of Asteatosis dermatitis using synthetic cannabinoids. *N*-acetylethanolamine (AEA) and *N*-palmitoylethanolamine (PEA), an endogenous cannabinoid and its derivative, were used simultaneously as a therapeutic agent for its anti-pruritic and anti-inflammatory effects (Yuan et al., 2014). Within 28 days, patients who were using the PEA and AEA topical cream saw a decrease in itch, scaling, and dryness (Yuan et al., 2014; Eagleston et al., 2018; Ring et al., 2012). This example highlights the medicinal

potential of the entourage effect and the use of topical cannabinoids (Russo, 2011; Di Marzo et al., 2001). Presumptively, phytocannabinoids can be used topically for similar applications and may have greater results due to structural diversity. For example, cannabinoids produce immunosuppressive and anti-inflammatory effects against immune mediated diseases such as atopic dermatitis (Jeong et al., 2019). Topically applied THC was shown to reduce inflammation in CB1/CB2 deficient mice through the depletion of chemokine ligand 2 (CCL2) and interferon gamma (INFγ) production in keratinocytes (Wollenberg et al., 2014; Eagleston et al., 2018). Thus, phytocannabinoids may be utilized as topical therapeutics in a variety of skin related diseases. Preliminary clinical studies have also shown that the administration of CBD ointment was useful in the mitigation of inflammation and improved patient quality of life (Palmieri et al., 2019). Although these data are promising, gaps in this study highlight the need for further clinical investigations of phytocannabinoids for the skin.

Scientific premise of this thesis

As the *Cannabis* industry continues to grow and gain popularity, we can expect more *Cannabis*-based skin care and therapeutic products. The market for these products is vast, as the *Cannabis* skin care industry is projected to generate between $135 and $155 billion (USD) globally in 2021 (Jhawar et al., 2019). It is important to understand how phytocannabinoids can be used in both the medicinal and cosmetic applications for consumer knowledge and safety. Currently there are many *Cannabis* containing topical products being advertised as muscle relaxants or treatments for severe skin disorders with no scientific evidence to back these claims. However, there

is evidence that suggests that phytocannabinoids applied topically may produce beneficial health effects (Río et al., 2018). Although this research is promising, this work is still in its infancy and needs to be further investigated to fully understand pharmacokinetics, medicinal benefits, and potential side effects of topically applied cannabinoids. Furthermore, these few studies do not take into consideration the complexity of skin permeation or how formulation environments effect the physiology of the skin and stability of active ingredient. Herein, I aim to describe the skin permeability and cosmeceutical applications of the phytocannabinoids CBD, CBG, CBN, Δ-8 THC, and CBDV.

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CHAPTER 2. MANUSCRIPT I

In vitro evaluation of Cannabidiol's Skin Permeability by Artificial Membrane Assays

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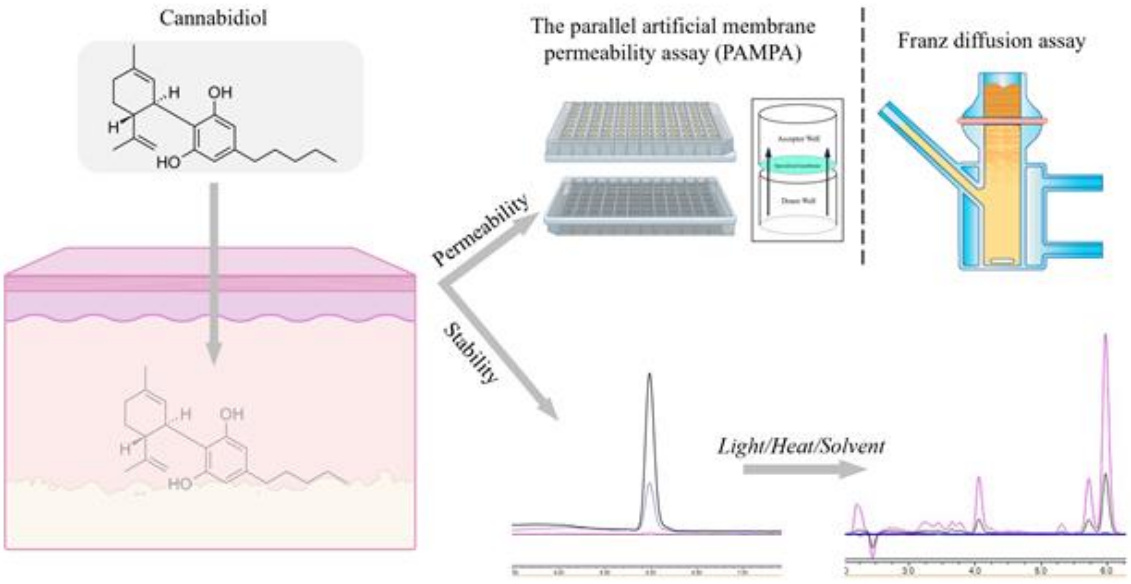
Graphical abstract



Abstract

Introduction: Cannabinoids including cannabidiol (CBD) have attracted enormous interest as a bioactive ingredient for potential dermatological and/or cosmeceutical applications . However, there is a lack of fundamental knowledge on the skin permeability of CBD and its topical formulations. Herein, we aimed to evaluate the skin permeability of CBD and CBD in a cream formulation using artificial membrane assays.

Materials and Methods: CBD and its formulations' skin permeability was assessed using artificial membrane based models including the parallel artificial membrane permeability assay (PAMPA) and using the Franz cell diffusion (*in vitro* release testing) assay.

Results: CBD isolate showed favorable skin permeability in the PAMPA assay (with a -LogPe value of 5.019 and 5.021, at pH of 6.5 and 7.4, respectively), which was supported by data from the Franz cell diffusion assay (51.8 μg CBD released over 8 hours). In addition, CBD had feasible solubility (378 μg), stability (81% stability at pH 5), in the presence of surfactant Tween-20. CBD topical cream formulations also showed promising skin permeability in the Franz cell diffusion assay (25.2 μg CBD released over 8 hours).

Conclusion: Findings from the current study suggest that CBD is a skin permeable cannabinoid and the permeability of its formulations may be influenced by several factors including surfactant, pH environment, and stability. Further biological evaluations using in vivo models are warranted to confirm these activities.

Keywords: Cannabidiol, skin permeability, PAMPA, Franz cell, cosmetics.

Abbreviations used:

CBD – Cannabidiol

PIB – poly isobutene

SLS- sodium lauryl sulfate

(w/w) – weight per weight

T20 – tween 20

T80 – tween 80

Introduction

The skin is the largest organ in the human body (Groeber, 2011). It is composed of various layers of cells that together act as a barrier to keep unwanted biological and chemical constituents from permeating and entering the body (Tadicherla 2006). The avascular outermost layer of the skin, called the epidermis, is comprised of keratinocytes, Merkel cells, melanocytes, and Langerhans cells, which participate in the protection against inflammation and immunological responses (Abels, 2019, Tadicherla 2006, Biro, 2019). The most superficial layer of the epidermis is the stratum corneum, which is the most difficult layer to permeate and is the rate-limiting step in the penetration process. Below the epidermis is the vascular dermis layer, which contains additional cell types such as fibroblasts but also free nerve endings which play a significant role in pain sensation (Abels, 2019, Tadicherla 2006). Proper formulations for skin cosmeceutical products can result in active ingredient delivery to a specific location such as muscles or blood vessels, and transdermal formulations can result in absorption and systemic circulation of the active component to achieve prolonged delivery (Ruela, 2016).

Due to the recent legalization and decriminalization status of the medicinal plant *Cannabis sativa,* as well as a general shift towards more natural ingredients in skincare, there has been a large increase in the use of *Cannabis* metabolites in skincare products (Thyagarasaiyar, 2020). *Cannabis sativa* is a member of the Cannabaceae plant family. This annual herbaceous plant has many phytochemicals unique to members of this genus, which are referred to as phytocannabinoids. The two most abundant phytocannabinoids are cannabidiol (CBD) and Δ9-tetrahydrocannabinol

(Δ9-THC) (Scherma, 2018) (Figure 1). THC is responsible for the psychoactive effects of the *Cannabis* plant, whereas CBD is the main non-psychoactive cannabinoid (Giacoppo, 2015). The human body also contains endogenous cannabinoid molecules, termed endocannabinoids, which are an integral part of the endocannabinoid system such as *N*-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG) (di Marzo, 2012 and 2015). The main molecular targets of the endocannabinoids are the cannabinoid receptors 1 and 2 (CB1 and CB2) (Stasiulewicz 2020). Much of the focus of THC has focused on the effects in the central nervous system where CB1 receptors are localized, and CBD research has largely focused on the anti-inflammatory effects involving CB2 receptors located in the peripheral nervous system and immune cells (Turcotte, 2016). CBD is a weak modulator to both cannabinoid receptors but it can modulate the release of endocannabinoids, as well as act on additional receptors such as the transient receptor potential vanilloid type 1 (TRPV1) and various G-protein coupled receptors (Giacoppo, 2015). The location of the cannabinoid receptors within the body ultimately determines the physiological reaction. The ECS in the skin is involved in various biological processes such as the proliferation, differentiation, and immune tolerance within the various types of skin cells (Bíró, 2009, Pucci, 2013). Every cell type produced by the skin relies on components of the endocannabinoid system (Justiniano, 2016), this includes the expression of cannabinoid receptors 1 and 2 on keratinocytes, hair follicles, and sebaceous glands (Scheau, 2020). Disruption and dysregulation in the ECS within the skin cells can lead to skin diseases (Chiurchiù, 2016). Other important receptors such

as the TRPV1 receptor is also highly expressed in epidermal skin cells, which facilitates the activity of CBD (Inoue, 2002).

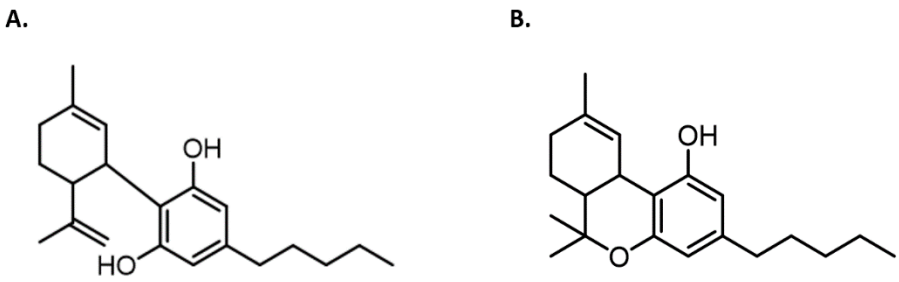


Figure 1: The chemical structure of Cannabidiol (CBD) (A) and Delta-9 tetrahydrocannabinol (Δ-9 THC) (B) isolated from the plant *Cannabis sativa*.

The medicinal benefits of CBD can be enhanced by combining CBD with THC or CBD given alone can stimulate the release of the endocannabinoid anandamide by inhibiting the FAAH (fatty acid amide hydrolase) enzyme (Scheau, 2020). Both the FAAH enzyme, which is responsible for degrading and recycling anandamide, and the MAGL (monoacylglycerol lipase) enzyme, which is responsible for removing 2-AG are also highly expressed in various constituents of the skin (Toth, 2019). Activation of CB1 receptors on the skin surface by phytocannabinoids can have therapeutic implications for diseases such as psoriasis and eczema due to the role that the endocannabinoid system plays in inflammation and immunity (Burstein, 2015). Furthermore, cannabidiol has antioxidant properties that protect the skin from oxidative stress on the epidermis caused by UVA/UVB radiation from the sun. Exposure to UVA and UVB rays shifts redox imbalance towards oxidation and ultimately leads to an increase in reactive oxygen species (ROS), which leads to cellular damage (Jastrząb, 2019).

Plant extracts and metabolites have been used for centuries as the active ingredient in creams and salves. In the 1880's Dr. Carl Koller found the local anesthetic properties of cocaine creams useful for cataract extractions and today various plant extracts are incorporated into cosmeceutical formations (Redman, 2011).

Recently, CBD has become a popular ingredient in skincare products, asserting anti-inflammatory, analgesic, hydration, and other benefits from the incorporation of this molecule. With celebrity endorsements, this is projected to become an industry worth between $135 and $155 billion (USD) globally by 2021 (Jhawar, 2019). One concern with the incorporation of cannabinoids in skincare products is the susceptibility of these metabolites to aerobic oxidation, causing a color change from a clear solution to a yellowish-brown solution resulting in the formation of hydroxyquinone (Caprioglio 2020). There remains a gap in understanding quantitatively the stability of CBD in solution, as well as the permeability of CBD in different skincare formulations. Understanding how to formulate skincare products in a way that increases the storage and stability of phytocannabinoids as well as increasing the permeation through the skin is critical. Furthermore, it is important to determine the rate at which cannabinoids passively diffuse across the skin to establish the time that would be required between dosing regimens and identify potential targets for skin therapeutics. A comprehensive understanding of the physiology of the skin as well as a physicochemical understanding of the active compound is necessary for proper formulation of products to reach the target tissue. The stability and permeability of CBD can be investigated using various *in vitro* assays, and this

information will further our understanding of this popular cosmetic ingredient for future formulation studies and ensure safety and efficacy for product use.

Materials and Methods

Reagents

Cannabidiol (CBD; purity 99.46%) was obtained from DB Labs (Las Vegas, NV, USA). The cellulose and Strat-M® membranes for Franz cell assay and ethanol were purchased from Millipore Sigma (Burlington, MA, USA). Supor PES membranes, pore size 0.1 and 0.45 μM were purchased from Pall Corporation (Port Washington, NY, USA). Phosphate buffer saline was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All materials for PAMPA were purchased from Pion, Inc. (Billerica, MA, USA). Silky Cream Base (BAS-SLKC-01) natural gel base (BAS-NATGE-01), polyisobutene, mineral oil, phytosqualane and propylene glycol were purchased from Making Cosmetics (Redmond, WA, USA). Polysorbate 20 NF was purchased from LetCo Medical (Decatur, Al, USA). Polysorbate 80 was purchased from Fisher Scientific (Waltham, MA,USA). Sodium Lauryl Sulfate was purchased from Gallpot (St. Paul, Mn, USA). Regent free water was purchased from Corning (Corning, NY, USA). Simulgel NS was purchased from Seppic inc. (Fairfeild, NJ, USA). Oleyl alcohol ethoxylate was purchased from Croda (Snaith, UK).

High-Performance Liquid Chromatography (HPLC) Analysis

Samples containing lipophilic surfactant were prepped for HPLC analysis using a C-18 100 mg (part no: 12102001) solid phase extraction (SPE) column (Agilent Technologies, Salta Clara, CA, USA) to remove residual surfactant. Analytical HPLC was carried out using a Dionex UltiMate 3000 HPLC system equipped with a micro vacuum degasser, an autosampler, and a diode-array detector.

Cannabidiol was quantified using high performance liquid chromatography. HPLC analysis was performed with an Thermo C18 column (250 × 4.6 mm, 5 μm) and a solvent system consisting of 0.05% formic acid in water (A)/Acetonitrile (B) was used as the mobile phase using a 9 minute isocratic method at 80% B and a flow rate of 0.6 mL/min.

LC-MS method

Various PAMPA samples were analyzed using both UV and LC-MS analysis (Thermo Fisher ISQ system paired with a Dionex HPLC system). LC-MS analysis was performed with an Thermo C18 column (250 × 4.6 mm, 5 μm) and a solvent system consists of 0.05% formic acid in water (A)/Acetonitrile (B) was used as the mobile phase using a 15 minute isocratic method at 80% B and a flow rate of 0.6 mL/min.

Cannabidiol Surfactant Solubility

A panel of 4 surfactants, polyisobutene (PIB), polysorbate 20 (tween 20), polysorbate 80 (tween 80) and sodium lauryl sulfate (SLS) were evaluated for their potential to solubilize cannabidiol in an aqueous solution. Each solution was prepared at surfactant concentrations of 1, 2, 5 or 10% (w/w); the final surfactant concentration used in the experiment was 10% (w/w). In a Falcon tube, 5 mg of cannabidiol and

surfactant was added to 12 mL of Phosphate Buffer Saline (PBS) or Phosphate buffer (PB). Solutions were shaken and left at room temperature for 24 hours. Solutions that did not have visible precipitation of cannabidiol were prepared for HPLC analysis using SPE and concentrations were measured using standard linear regression.

Cannabidiol Stability in Solution

The stability of CBD in a pH adjusted aqueous solution was determined through a stability test. Cannabidiol in 10% tween 20 (w/w) was added to PBS buffer that was adjusted to pH 4, 5, 6 and 7. Each solution was prepared to a final concentration of 200 µg/mL of CBD. Solutions were left at room temperature and constant light for 30 days. Samples of solution were taken at different time points. The concentration of CBD in solution was quantified using HPLC analysis and a linear regression of areas under the curve.

Parallel Artificial Membrane Permeability A**ssay (PAMPA)**

Pion's suggested procedures were followed as stated in the instruction manual, version 4. Briefly, test compounds and standards were prepared at 10 mM concentrations in DMSO. In the deep well plate provided by Pion, 1 mL of each pH adjusted buffer was added to separate wells. For each pH, 5 µL of the 10 mM sample was added to 1 mL of pH adjusted buffer then mixed thoroughly with a pipette, diluting the sample to a final concentration of 50 µM. 200 µL of the diluted sample

was added to the donor (bottom) plate from the Pion sandwich assay. Next, 200 µL of acceptor sink buffer was added in each well of the acceptor plate. The sandwich was assembled, and the plate was incubated at room temperature, with stirring for 5 hours undisturbed. After incubation, the UV profiles of the donor and acceptor plates were read on the SpectraMax plate reader connected to the PAMPA software. PAMPA software calculated the –log Pe values for each compound and standard using the UV profiles of the donor and acceptor plates. Standards were all within 0.25 of the expected value provided by PAMPA and each sample was averaged with 6 replicates for each pH tested.

Formulations of CBD creams

Two creams were used to evaluate CBD permeability in solution. The first was a silky cream base that is commercially available cream from makingcosmetics.com (BAS-SLKC-01). CBD isolate powder (1% w/w) was added directly to this base and emulsified on a shearing instrument for 2.5 minutes. The second cream was formulated using ingredients in the compounding lab at the University of Rhode Island. The cream contained equal parts propylene glycol, Phyto squalene and mineral oil to comprise 50% (w/w) of the total formulation. CBD isolate powder (1% w/w) was added directly to the propylene glycol, mixed thoroughly and added to the other oils. Next the surfactant, simulgel ns (7%) was added to the lipophilic phase mixture, followed by reagent free water (42%). The cream was homogenized on a shearing instrument for 2.5 minutes. Each formulation was physically evaluated between the

thumb and index finger to ensure a smooth texture and no signs of phase separation. Formulations were then centrifuged 9 times at 5000 rpm in 5-minute intervals to confirm stability. Both formulations were stable and did not fully separate into two phases after centrifugation.

Franz Cell Diffusion Assay

One day prior to the assay, the receptor buffer was prepared in 50 mL Falcon tubes and pre-warmed in a water bath at 32 °C overnight. 3% (w/w) surfactant was used in 1x PBS. Surfactant concentration was determined after solubility analysis. Tween-20 showed the best solubility for CBD (Figure S5). Depending on membrane brand and type, certain membranes were hydrated one day prior to running the assay by submerging the membrane in 1x PBS in a beaker with parafilm covering the top. On the day of the assay, the Franz cell water bath was also pre-warmed to 32 °C 30 minutes prior to beginning. After the water had been warmed, the pre-soaked membranes were applied to the apparatus using forceps. Next, 12 mL of acceptor buffer was added via pipette into each Franz cell, and 1 mL of pure CBD in DMSO or 200 mg of formulation was added using a syringe. The tops of the cells were clamped and covered in parafilm to prevent evaporation. At each given timepoint, 600 µL of sample was removed from the spout of the Franz apparatus and replaced with 600 µL of new acceptor buffer.

The *In Vitro* Release Testing (IVRT) Data Analysis

Each 600 μL acceptor well example for IVRT analysis was first run through a C18 SPE column to remove any surfactant from the sample. Samples were then analyzed via HPLC, and the area under the curve (AUC) for the CBD peak was recorded. Elution time was compared with that of the CBD in the standard curve. A linear regression of CBD standards was calculated (R^2= 0.9759) and concentrations of CBD were interpolated. For the initial timepoint collected in the Franz cell assay, the formula used to calculate IVRT was (drug concentration/1000 *12). For each timepoint following, the calculation was adjusted to account for the addition of new acceptor well buffer, for these timepoints the equation (drug concentration*12+0.6*(sum of sample concentrations)/1000) was used.

Results

CBD shows favorable skin permeability in the PAMPA assay

The skin permeability coefficient , Log Kp (cm/s) was used to evaluate the theoretical permeability of CBD through the skin (Table 1). This value was determined using an online computational model, SwissADME. CBD is considered to have moderate skin permeability with a Log Kp value of -3.59 cm/s. The positive PAMPA skin controls, warfarin, piroxicam, progesterone and verapamil all exhibited low skin permeability in the SwissADME predictor (Table S1). After this predictive measure, compounds were experimentally evaluated for passive permeability using the PAMPA skin model.

Table 1: *in silico* SwissADME predictive permeability of cannabidiol in the skin.

sample	-Log Kp (cm/s)	Log Po/w	-Log S (ESOL)	-Log S (Ali)
Cannabidiol (CBD)	-3.59	3.90	-5.69	-7.17

Compounds were evaluated for their permeability through a theoretical skin membrane using PAMPA at pH 6.5 and 7.4 (Figure 2). Warfarin was used as our low permeability standard, verapamil and piroxicam were used as our medium permeability standards, and progesterone was used as our high permeability standard. A pH control standard was also run. CBD showed similar permeability at both pH 6.5 and 7.4 with -LogPe values of 5.0194 ± 0.083 and 5.021 ± 0.045, respectively, suggesting it has moderate skin permeability.

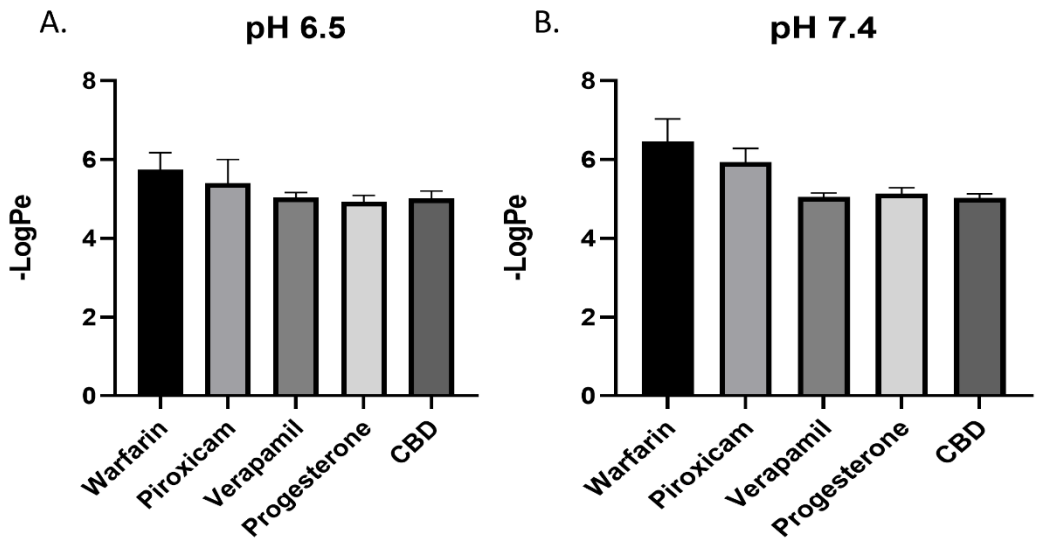


Figure 2: CBD shows passive permeability in the Skin PAMPA model. The passive permeability of CBD was evaluated at two relevant pH levels 6.5 (A) and 7.4 (B). The controls warfarin and piroxicam exhibit low permeability, while progesterone and verapamil exhibit medium permeability. -Log Pe values for CBD at pH 6.5 and 7.4 were determined to be -5.0194 ± 0.083 and –5.021 ± 0.045 respectively. (n=6).

CBD is soluble in surfactant Tween-20

CBD solubility in an aqueous solution was evaluated using four surfactants at a final concentration of 10% w/w (Figure 3) . The concentration of CBD in solution that did not out of solution or crystallize was determined using HPLC analysis. Although no apparent precipitation was observed, polyisobutene (PIB) demonstrated minimal CBD solubility in both PB and PBS buffer having 33.7 and 47.3 µg of CBD in solution, respectively. Concentrations of CBD in sodium lauryl sulfate and Tween-80 solutions were also comparable to that of Tween-20 (Appendix B). CBD solutions containing 10% Tween-20 and PBS buffers were considered the optimal surfactant-

solvent system, due to relatively high solubility and reproducibility. This solution contained about 378.4 µg of CBD per mL.

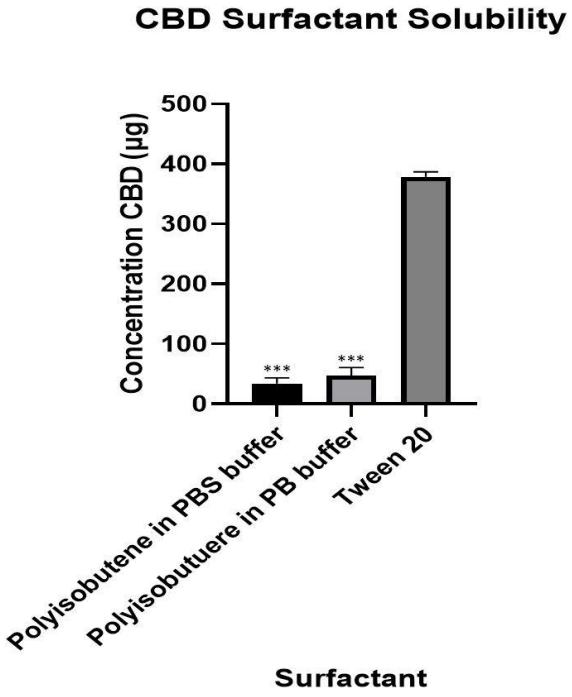


Figure 3: Tween 20 is the ideal surfactant to solubilize CBD in an aqueous solution Poly isobutene showed the least solubility potential for CBD in both PBS and PB buffer. Concentrations are expressed as mean ± standard error (n=3), statistical significance was determined by analysis of variance (ANOVA). Significance as compared with Tween 20 , $p \leq 0.01$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***) and $p \leq 0.00001$ (****).

CBD in Tween-20 is skin permeable

PAMPA was used to determine the theoretical skin permeability of pH adjusted CBD surfactant solutions (Figure 4). Concentrations of CBD in the acceptor wells were determined using LCMS analysis after 5 hours. CBD showed the least permeability at pH 5 with a maximum concentration of about 4.9 µg. In contrast, CBD in the ideal surfactant-solvent solutions (10% Tween 20 w/w) showed the most permeability at pH 7 with about 7.4 µg in solution.

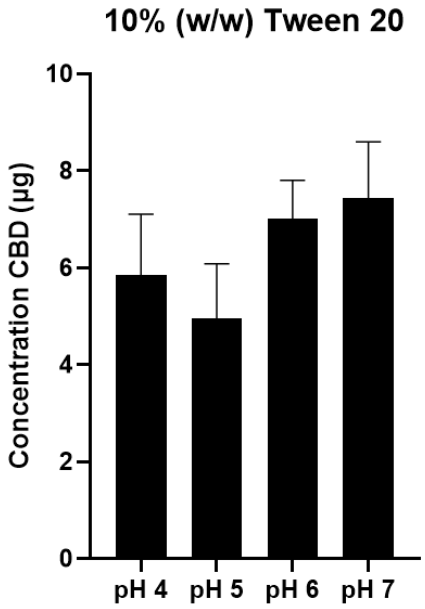


Figure 4: CBD-Surfactant solution shows passive permeability at various pH's. Assessment of skin passive permeability of CBD-Tween 20 solution determined via PAMPA. CBD exhibited variable permeability with pH change. Data is expressed as mean ± standard error (n=3), statistical significance was determined by analysis of variance (ANOVA). Statistical significance between each pH (P=0.0039).

CBD in Tween-20 is stable at pH of 5

The stability of CBD in an aqueous solution was evaluated to determine the percent degradation of CBD at different pH (Figure 5). The stability suggests the optimal pH range of formulations containing CBD. Concentration of CBD was determined through HPLC analysis and quantified through a standard linear regression. CBD was added to a pH adjusted solution of PBS with 10% Tween-20 (w/w). Overall, CBD in solution showed the least amount of degradation at pH 5 with a cumulative degradation of 19% over 30 days. In contrast, CBD in pH 7 solution showed the most degradation with 48.5% degrading over the 30-day period. CBD in pH solutions 4 and 6 showed similar trends with a degradation of about 30% and 29% over the 30 days, respectively.

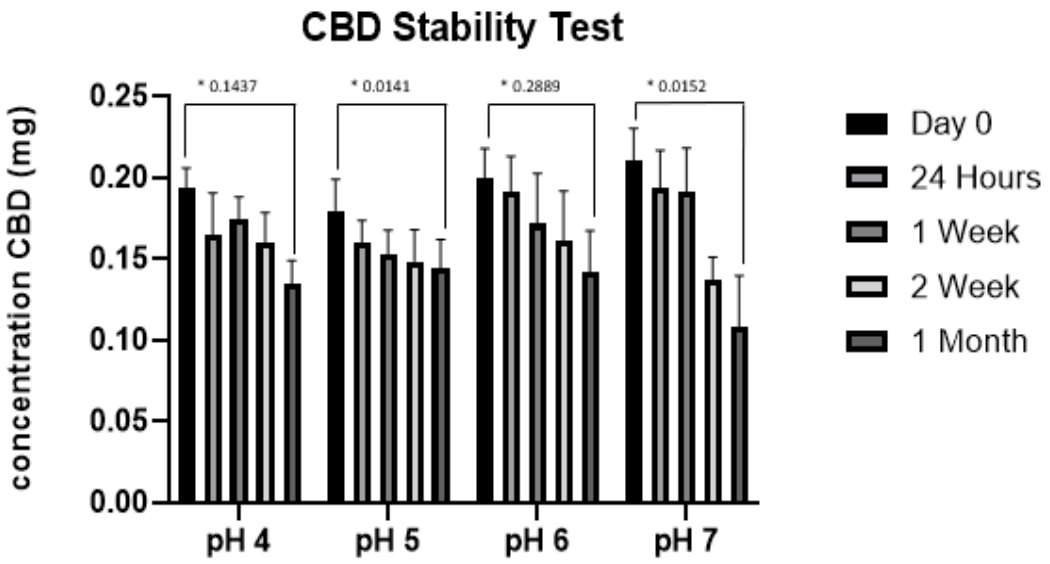


Figure 5: CBD is most stable at pH 5. The stability of a CBD-surfactant solution was evaluated at pH of 4, 5, 6 and 7 . Degradation was confirmed through the accumulation of breakdown products and is expressed as percent cumulative degradation over a 30-day timespan. Concentrations are expressed as mean ± standard error (n=3), statistical significance was determined by analysis of variance (ANOVA). Significance as compared with Day 0.

In Vitro Release Test

The permeability of CBD through a theoretical skin membrane was evaluated using the Franz cell diffusion model (Figure 6). Concentrations of permeated CBD were determined through HPLC analysis of the acceptor well. CBD showed passive diffusion through the theoretical skin membrane and reached its maximum diffusion concentration of 51.9 μg at 6 hours.

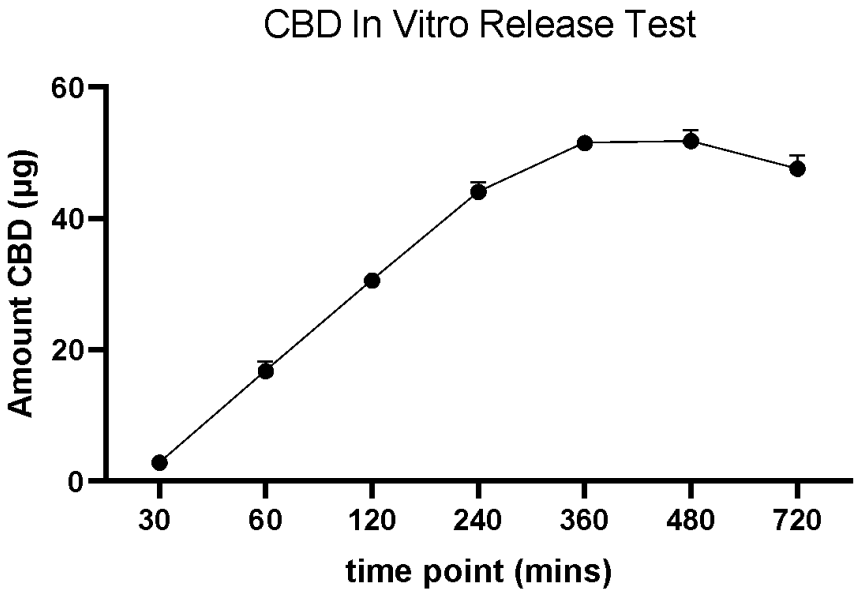


Figure 6: In vitro drug release profile of Cannabidiol (CBD) obtained using Franz diffusion cells (n = 9).

CBD Formulation In Vitro Release Test

The permeability of CBD in two different cream formulations was tested using the Franz cell diffusion assay (Figure 7) . The final CBD concentration of formulations was approximately 1% (w/w). 200 mg of cream was loaded onto each membrane, HPLC analysis was taken of the acceptor well following each timepoint. CBD showed passive diffusion through a theoretical skin membrane in a cream formulation. Further analysis is necessary to ensure CBD permeability through human skin.

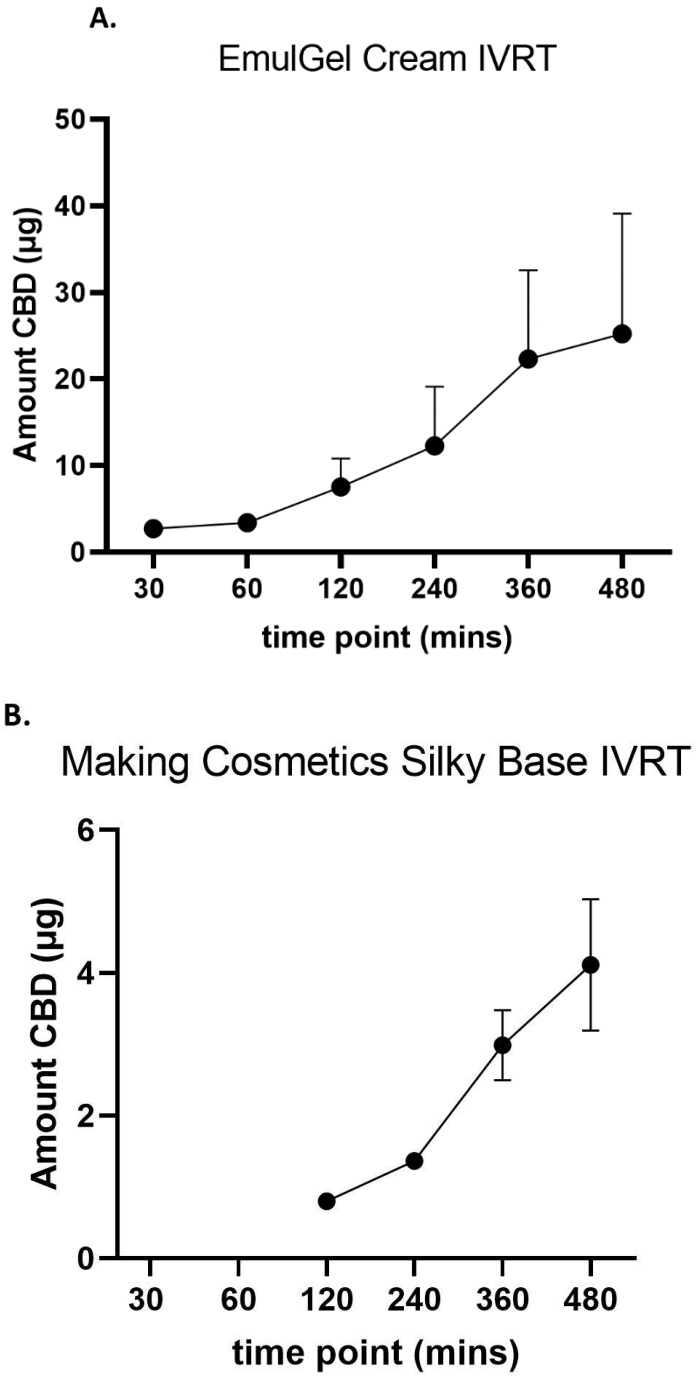


Figure 7: In vitro drug release profiles of CBD formulations obtained using Franz diffusion cells (n=3). The in vitro drug release profiles of CBD EmulGel Cream (A) and CBD making cosmetics cream (B).

Discussion

Cannabidiol's extensive use in cosmetics necessitates further evaluation into the stability and permeability of this metabolite through the skin. Cannabinoids are susceptible to UV degradation, chemical rearrangements, and oxidation (Grafstrom et al.). Lindholst determined that both temperature and light exposure influence the stability of cannabinoids when organic solvents, such as ethanol and chloroform, are used, (Lindholst 2010). However, most of the studies that have been completed investigate the resin forms of *Cannabis* or the alcohol extracts. In skincare, alcohol extracts with solvent present and pure resin are rarely used. Rather, the lipophilic cannabinoids would be incorporated into aqueous based products using surfactants, or simply added to creams without surfactants. It is well established that light and temperature play the most significant role in degradation (Lindholst 2010, Trofin 2012). We found that in a PBS and 10% w/w Tween-20 solution, which is more relevant in skincare formulations than alcoholic preparations, CBD shows the most degradation at pH 6 and pH 7 compared to pH 4 and pH 5. Many skincare products are already formulated to be slightly acidic, often using ingredients such as citric acid to maintain barrier function of the acid mantle on the skin (Schmid-Wendtner 2006).

CBD was evaluated with multiple *in vitro* assays to evaluate the passive permeability of CBD through biomimetic skin membranes. The first assay employed was the parallel artificial membrane permeability assay (PAMPA). Using this method, CBD permeability was compared to the control compounds provided with the assay with well-established pharmacokinetics. CBD showed a similar permeability profile with the endogenous steroid progesterone. A -Log Pe of 5.0194 was measured for the

pH of 6.5, and a -LogPe of 5.021 at pH 7.4 suggesting CBD is moderately permeable through the skin. This aligns well with the results from the *in silico* prediction software SwissADME which predicted the permeability to be -3.59 (cm/s).

Conceptually similar to PAMPA, the Franz cell diffusion assay was employed to determine the rate at which CBD penetrated synthetic skin membranes using in vitro release testing (IVRT). After completion of a membrane compatibility test (Figure S3), SUPOR PES membranes were the most compatible with CBD, and the membranes did not interfere with the release of the compound. Commercially available gel and cream bases were purchased from makingcosmetics.com for initial evaluation for permeability studies. Due to the popularity of CBD creams, and the higher penetration, only creams were evaluated further in this study (Figure S2). When comparing formulations of CBD in a commercially available formulation and an in-house formulation, emul-gel cream, the in vitro release testing indicated the release of CBD in our formulation was much higher compared to the making cosmetics cream (Figure 7). Discrepancies in permeability may be due to non-uniformity of CBD in formulation. Quantification of CBD initial concentrations in the commercially available product indicated that CBD isolate powder was not fully dissolved into the formulation. Excipient concentration for the making cosmetics cream is proprietary. A larger aqueous phase may account for discrepancies in uniformity and could allow for CBD to crash out of solution. This issue may be avoided in the future with the use of a carrier oil that is compatible with both the drug and formulation. Conversely, CBD isolate powder was added directly to the lipophilic phase of the emul-gel cream and concentrations of CBD in formulation were within expected range. The way in which

this emul-gel cream was formulated likely affected both stability and permeability of CBD in formulation. Permeability may have also been influenced by the presence of penetration enhancing oils in the lipophilic phase of the emul-gel cream. Propylene glycol, an excipient used in formulating, has been shown to enhance transdermal drug permeability (Babu et al., 2005). PAMPA analysis of the compounds at pH 6.5 and 7.4 showed similar permeability patterns (Figure 6). When different surfactants were used in the PAMPA assay, a difference in pH permeability dependence was observed. Tween-20 and Tween-80 showed better solubility at pH 6 and pH 7 compared to those at the lower pH values tested. Sodium lauryl sulfate showed significantly better permeation at pH 7 compared to the other pH values (Figure S4).

Conclusion

Cannabidiol's popularity in cosmeceuticals warrants an investigation into the stability and permeability through artificial skin membranes. In this study, CBD showed moderate skin permeability in the PAMPA assay using both the pure compound and cream formulations. The stability of CBD can be increased by keeping the product cool, out of light, and at lower pH. At pH 5, CBD is the most stable in a solution of PBS with Tween-20 as a surfactant. Highly lipophilic cream formulations should be considered for deeper penetration of CBD. Further Franz cell evaluation is necessary to assess CBD permeability in human skin.

Supplementary Information:

Table S1: *in silico* SwissADME predictive permeability and -Log Pe values of PAMPA standards.

PAMPA Standard	Expected -Log Pe	-Log Kp (cm/s)	Log Po/w	-Log S (ESOL)	-Log S (Ali)
Warfarin	-6.00	-6.26	2.41	-3.70	-3.77
Piroxicam	-5.67	-6.15	1.67	-4.01	-4.99
Verapamil	-5.65	-6.38	4.50	-4.46	-4.83
Progesterone	-4.92	-5.47	3.09	-4.16	-4.28

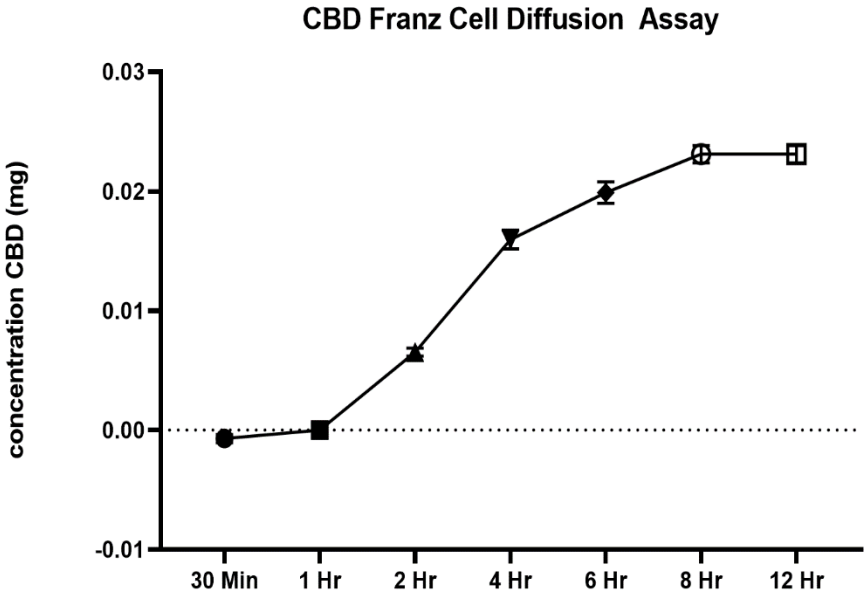


Figure S1: The permeability profile of CBD in 10% tween-20 surfactant solution through a cellulose membrane using a Franz diffusion assay. N=3.

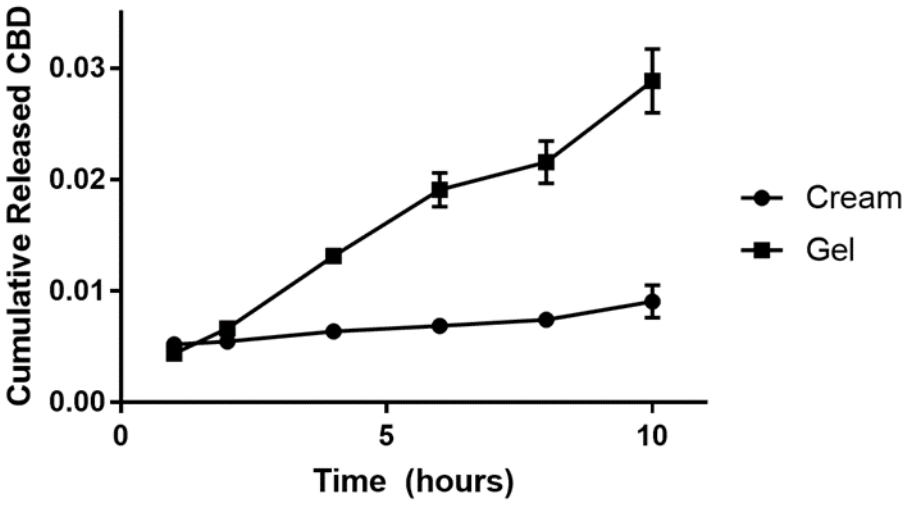


Figure S2: *In vitro* release testing (IVRT) of CBD in commercially available cream silky cream (BAS-SLKC-01) and gel (BAS-NATGE-01) formulations (Making Cosmetics) in the Franz cell assay, (n=3). CBD formulation permeability was achieved with the use of a 10% (w/w/) tween-20 carrier vehicle.

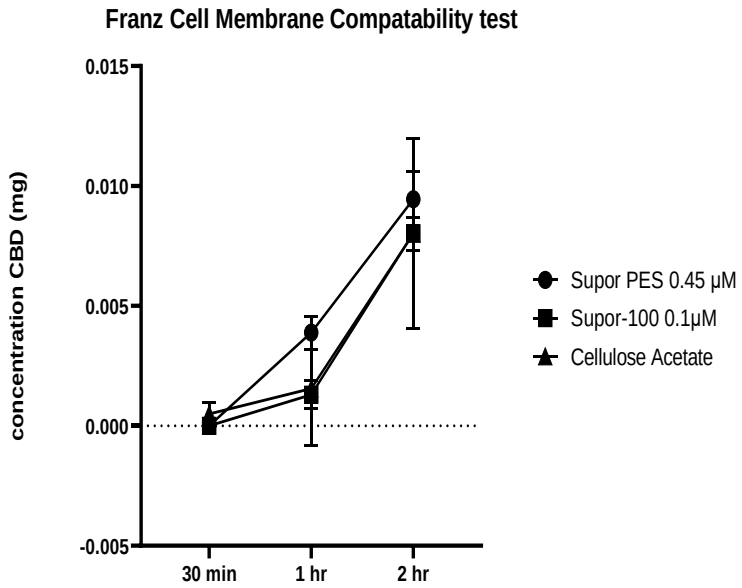


Figure S3: CBD compatibility test using the Franz cell assay (n=3). Franz cell diffusion assay optimization of the synthetic skin membranes. To ensure the passive diffusion of CBD through a synthetic skin membrane, 3 different membrane types were used to determine CBD compatibility. Supor PES 0.45 µM was determined to be the most compatible membrane.

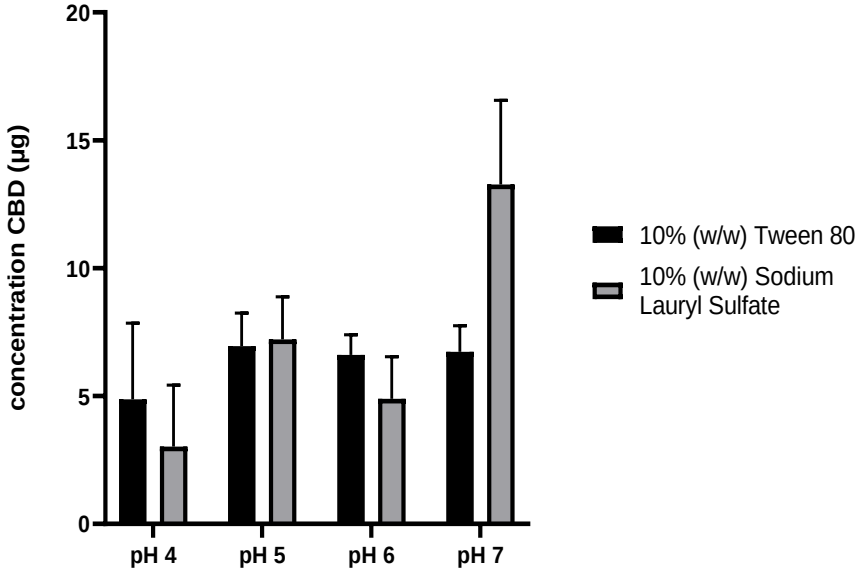


Figure S4: CBD-Surfactant solution shows passive permeability in various pH. Assessment of skin passive permeability of CBD-Tween 80 & and CBD-Sodium Lauryl Sulfate solution determined by PAMPA. CBD exhibited variable permeability with pH change. Data is expressed as mean ± standard error (n=3).

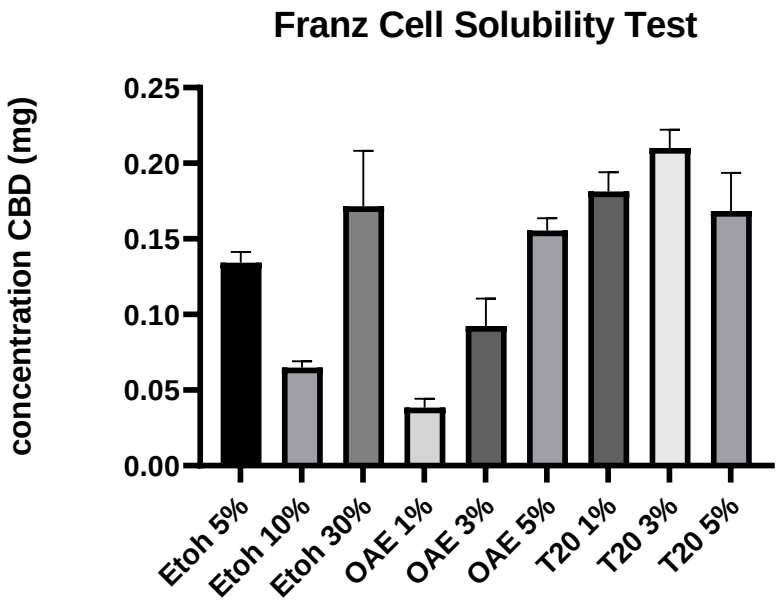


Figure S5: CBD solubility in potential Franz cell acceptor buffers. A solution of PBS buffer containing 3% (w/w) Tween-20 (T20) was determined to be the optimal solvent system that allows for the passive diffusion of CBD through a synthetic membrane. Solvent systems containing ethanol (EtOH) and oleyl alcohol ethoxylate (OAE) at various concentrations did not reach full solubility capacity.

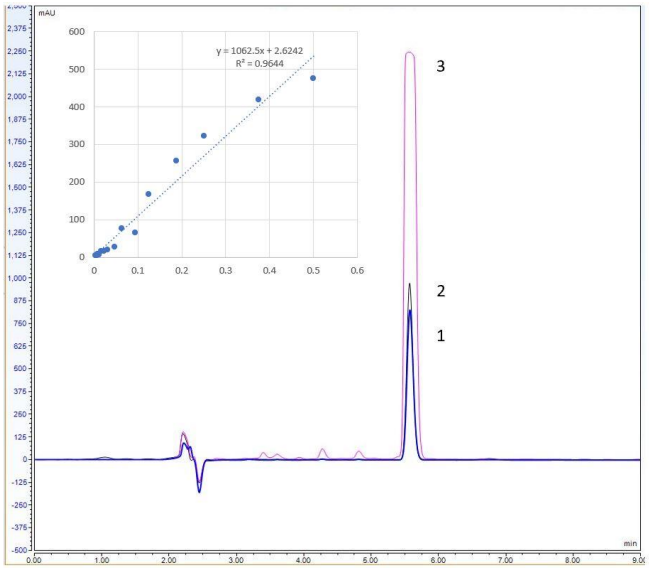


Figure S6. CBD solubility test HPLC chromatograms. CBD in a 10% (w/w) poly isobutene – PBS buffer solution (1) , 10% (w/w) poly isobutene – PB buffer solution (2) and a 10% (w/w)Tween 20-PBS solution (3) were evaluated to determine the ideal surfactant solvent system to solubilize the API. The amount of CBD in solution was determined through HPLC analysis and quantified using standard linear regression.

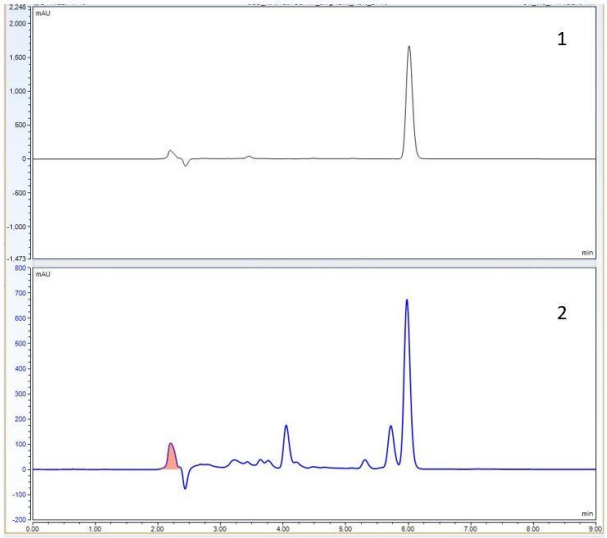


Figure S7. CBD stability test HPLC chromatogram. The stability of CBD in surfactant-buffer solution. The degradation of CBD in a surfactant-buffer solution was confirmed through HPLC analysis, using a standard elution time ~6 minutes and UV detection of 210 nm. CBD degradation was monitored from day 0 (1) to day 30 (2). Degradation of API was confirmed through reduction of CBD peak area and the accumulation of degradative product peaks eluting before CBD. CBD was quantified using standard linear regression

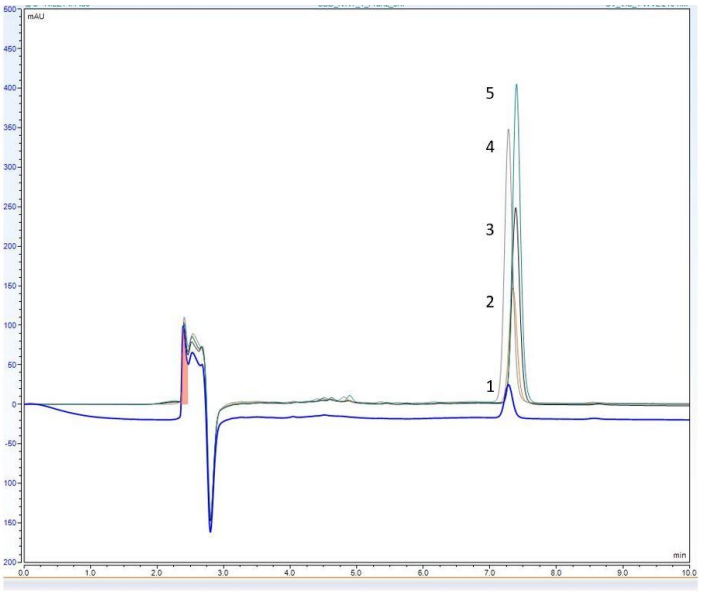


Figure S8.CBD IVRT HPLC chromatogram. CBD Showed passive permeability in the Franz Cell diffusion assay. The permeability profile of CBD through a synthetic skin membrane was determined using the Franz cell diffusion assay. The presence of CBD was confirmed through HPLC analysis, using the average elution time of ~7.2 minutes and UV detection of 210 nm. At each time point 30 minutes (1) , 1 hour (2) , 2 hours (3), 4 hours (4) and 6 hours (6) , CBD was quantified using standard linear regression.

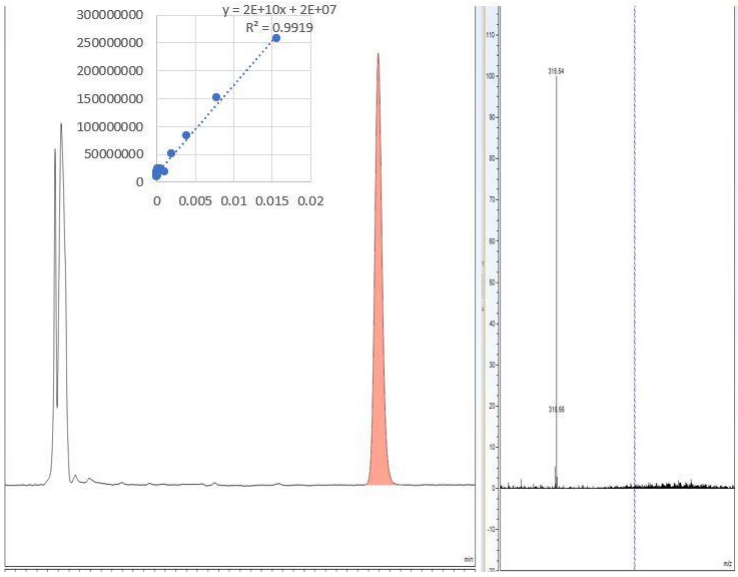


Figure S9. CBD PAMPA LCMS Chromatogram. The passive permeability of CBD through a theoretical skin membrane was determined using the PAMPA assay. The presence of CBD in the acceptor buffer was determined through LCMS analysis and confirmed with an m/z of 315. CBD was quantified using standard linear regression.

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CHAPTER 3.MANUSCRIPT II

Permeability and Cosmeceutical Evaluation of Minor Phytocannabinoids from *Cannabis sativa*

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Abstract

The majority of research for phytocannabinoids from the plant *Cannabis sativa* has focused on the plant's major constituents, Δ-9-tetrahydrocannabinol (Δ9-THC) and Cannabidiol (CBD). However, the popularly utilized plant produces over 100 phytocannabinoids that have yet to be investigated as extensively. Previously, our lab had evaluated the skin permeability of the non-psychoactive metabolite CBD, which showed promising potential as an active ingredient for cosmeceutical purposes. Herein, we aim to do the same with 4 minor phytocannabinoids, CBN, CBDV, CBG and Δ8-THC by evaluating skin permeability through an artificial membrane as well as potential skin lightening effects.

Introduction

In recent years, the plant *Cannabis sativa* has been extensively studied for its various pharmacological applications. This plant produces a class of hybrid polyketide-terpenes known as phytocannabinoids that are responsible for the psychoactive, anti-inflammatory and analgesic properties associated with its use (Hanuš et al., 2016) (Figure 1). The abundant phytocannabinoids produced, Δ-9-Tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD) have been the major focus in this field of research (Franco et al., 2020) . However, *Cannabis sativa* has been shown to produce over 100 phytocannabinoids (Hanuš et al., 2016; Sampson, 2021). Structurally these phytocannabinoids differ by changes in the isoprenyl residue, resorcinyl core, or side-chain (Hanus et al., 2016). Changes in these moieties can greatly affect bioavailability and medicinal function of each phytocannabinoid. These changes in structure may take place naturally or from auto oxidation resulting in a variety of degradative products

(Hanus et al., 2016).This is exemplified in one of the major neutral cannabinoids, cannabinol (CBN) which is the degradative product of Δ9-THC (Franco et al., 2020).These phytocannabinoids act as ligands to the Endocannabinoid system (ECS). Interference or imbalance of this ECS is directly related to the disruption of physiological homeostasis and inflammation (McPartland et al., 2014; Barry, 1991) . However, the ECS can be utilized in the treatment of various conditions such as cancer, pain, neurodegeneration, immune function, appetite, and anxiety (Husni et al., 2014). The endocannabinoid system is expressed in many parts of the body and can potentially be a target for the treatment of many different diseases (Río et al., 2018) . The lack of research on the "minor" phytocannabinoids (mPC) limits pharmacological potential of *Cannabis* metabolites and our understanding of homeostatic conditions (Franco et al., 2020). Additionally, the study of these mPCs can lead to a better understanding of drug interactions as they engage in what is known as the entourage effect. This polypharmacology phenomenon states that multiple phytocannabinoids are actually advantageous to treatment over a singularly extracted molecule, meaning they have a synergistic effect (Di Marzo et al,. 2001; Russo, 2011). This effect is reported by both consumers and experimentally (Pamplona et al., 2018; De Petrocellis et al., 2011)

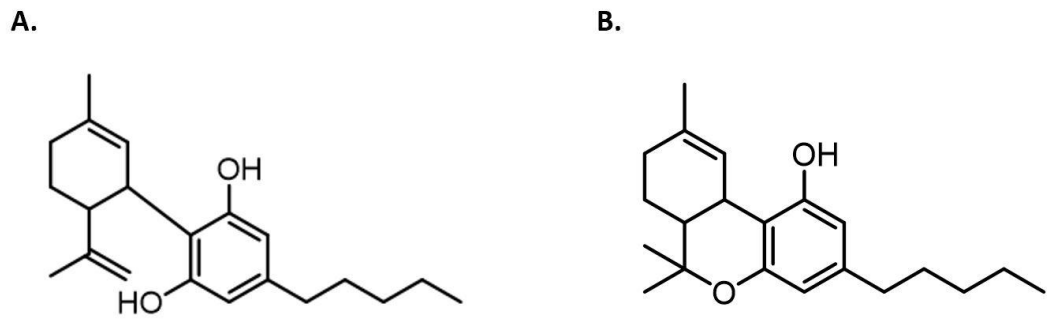

Figure 1: The chemical structure of Cannabidiol (CBD) (A) and Delta-9 tetrahydrocannabinol (Δ-9 THC) (B) isolated from the plant *Cannabis sativa*.

Although these mPCs are used to target the ECS, many of them are non-psychoactive and can therefore be considered an alternative to the federally regulated Δ9-THC. Δ9-THC is an agonist of the cannabinoid receptor 1, or CB1, which is responsible for *Cannabis* psychoactivity and the potential side effects associated with it (Franco et al., 2020; Bernadette, 2005). In situations where the CB1 receptor is the drug target, mPCs with lower binding affinities to this receptor such as D8-THC and CBN can be efficacious and reduce the chances of side effects (Pertwee, 2008).Many of the non-psychoactive phytocannabinoids such as CBD act on the cannabinoid receptor 2 or CB2. The CB2 receptors interact with the immune system and are therefore integral in the treatment of inflammatory diseases (Cabral and Griffin-Thomas, 2009). Due to their vast medicinal potential as ECS ligands, many of the mPC's are currently being entered in early stages of clinical trials and research. Recent studies have shown that CBN, cannabigerol (CBG), and cannabichromene (CBC) may be antimicrobial and have anti-inflammatory properties related to the treatment of Irritable bowel syndrome (Appendino et al., 2008; Couch et al., 2018). The application for these mPCs

are expanding as new cannabinoids are being discovered. Newer mPC's such as Tetrahydrocannabiphorol (Δ9 – THCP) and Cannabidiphorol (CBDP) are potentially more effective alternatives to the major phytocannabinoids Δ9-THC and CBD as they hold the same properties but can be used at lower doses (Sampson, 2021).

As the largest organ of the body, the skin plays a vital role in the integumentary system. Studies have found that the ECS is expressed in human skin cells, and therefore phytocannabinoids can be used to target cannabinoid receptors in the skin (Bíró et al., 2009). The majority of the research connecting the phytocannabinoids and the skin has been primarily focused on Δ9-THC and CBD. A recent study from Olah et al., has proposed the use of the mPCs as anti-acne treatments. This study showed that disrupting the endogenous cannabinoids found in sebocytes affected lipid formation and found that CBD was able to inhibit lipid production which in turn would be beneficial for the treatment of acne. CBC, CBDV and THCV also showed similar effects as potential anti-acne treatments while CBG and CBGV increased lipid production, which may be beneficial for the treatment of dry and aging skin. Further investigations also showed that THCV suppressed the production of LPS-induced pro-inflammatory cytokines related to acne such as IL-1a, IL-1b, IL-6, IL-8 and TNF-α (Olah et al.,2016). These mPC's illicit a physiological change, i.e., suppress inflammation, antimicrobial properties and management of sebum production, that have a direct cosmetic and aesthetic benefit such as reducing the appearance and instance of acne vulgaris. This shows that mPCs are the ideal active pharmaceutical ingredient for cosmeceutical applications.

Post Inflammatory Hyperpigmentation (PIH) is a skin disorder that generally affects people of African and Asian descent. Dark and discolored markings on the skin are often unsightly and result in overall patient dissatisfaction. Post inflammatory hyperpigmentation is caused by an increase in activity of the polyphenol oxidase enzyme, also known as the tyrosinase enzyme (TYR) (Ashooriha et al., 2020). This increase in enzymatic activity is also responsible for other hyperpigmentary skin conditions including melanoma, lentigines, melasma, and freckles (Ma et al., 2017; Smit, Vicanova et al., 2009; Roh et al., 2017). Located in the membrane of the melanocyte, tyrosinase is a type III copper-containing glycoprotein that catalyzes melanogenesis (Ashooriha et al., 2020; Sánchez-Ferrer et al., 1995). The tyrosinase enzyme is quintessential for this process and therefore is the rate limiting step in melanin production (Pillaiyar et al., 2017).

Current prescribed skin lightening agents are exclusively tyrosinase enzyme inhibitors as this specific enzymatic target is effective and reduces incidences of side effects (Pillaiyar et al., 2017). Until recently, hydroquinone (HQ) was a popular commercially available and medically prescribed hypopigmentation agent. Although HQ is shown to inhibit tyrosinase activity by 90%, it was recently banned by the FDA as it is possibly mutagenic in mammalian cells (Westerhof and Kooyers, 2005; Engasser, 1984; Parvez et al., 2006; Smit et al., 2009). Burning, irritation, leukoderma, hypochromia, contact dermatitis, transient erythema, and onchrosis are adverse effects associated with the long-term use of HQ (Parvez et al., 2006). More recently, kojic acid (KA) a safer alternative has become the more popular hypopigmentation agent. Simi-

lar to HQ, KA is also mutagenic to mammalian cells and potentially causes skin irritation (Saeedi et al., 2019) . Kojic acid also has poor efficacy and is unstable and therefore difficult to use in cosmetic formulations (Saeedi et al., 2019; Smit et al., 2009). Skin lightening is often time consuming and expensive as results are generally not visible for 4-6 weeks. However, HQ and KA should not be used for more than 6-month periods as prolonged use increases the likelihood of the aforementioned side effects (Westerhof and Kooyers, 2005). As current hypopigmentation agents are unsafe, time consuming and difficult to formulate, a natural alternative is necessary for the inhibition of the tyrosinase enzyme for disorders such as PIH. Natural alternatives such as mPC's are reasonably cost effective and considered to be free of harmful side effects in comparison to commercially available products (Ma et al., 2017; Zheng et al., 2008). Herein, this study aims to evaluate the tyrosinase inhibitory effects and skin permeability of CBN, CBDV, CBG and Δ8-THC (Figure 2).

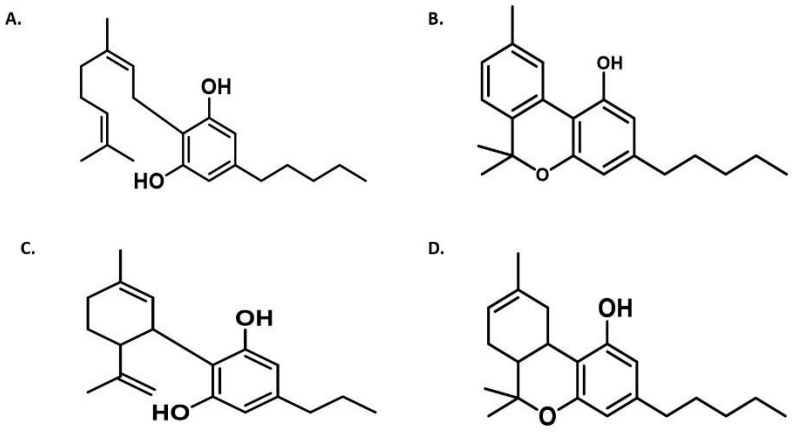

Figure 2: The chemical structure of Cannabigerol (CBG) (A), Cannabinol (CBN) (B), Cannabidivarin(CBDV) (C), Δ8-Tetrahydrocannabinol (Δ8-THC) and isolated from the plant *Cannabis sativa*.

Materials and Methods

in silico ADME Predictors

The *in silico* computer program SwissADME (http://www.swissadme.ch/) was used to predict skin permeability. Predicted permeability was assessed for each individual phytocannabinoid and control by obtaining Simplified molecular input line entry specification (SMILES) files of each compound using ChemDraw (PerkinElmer Inc.; Waltham, MA, USA). Skin permeability, Log Kp (cm/s), values were determined along with other solubility constants, Log Po/w, Log S(ESOL) and Log S(Ali).

Parallel Artificial Membrane Permeability Assay (PAMPA):

Pion's suggested procedures were followed as stated in the instruction manual, version 4. Briefly, test compounds and standards were prepared at 10 mM concentrations in DMSO. In the deep well plate provided by Pion, 1 mL of each pH adjusted buffer was added to separate wells.

About 10µL of sample for pH 7.4 was added to the 1 mL of buffer than mixed thoroughly with a pipette, diluting the sample to a final concentration of 100 µM. The final concentration of sample in pH 6.5 was prepared to 200 µM. 200 µl of the diluted sample was added to the donor (bottom) plate from the Pion sandwich assay. Next, 200 µl of acceptor sink buffer was added in each well of the acceptor plate. The sandwich was assembled, and the plate was incubated at room temperature, with stirring for 4 hours. After incubation, the UV profiles of the donor and acceptor plates were

read on the SpectraMax plate reader connected to the PAMPA software. PAMPA software calculated the –Log P_e values for each compound and standard using the UV profiles of the donor and acceptor plates. Standards were all within 0.25 of the expected value provided by PAMPA and each sample was averaged with 12 replicates for each pH tested.

Tyrosinase

Phytocannabinoids were evaluated for the inhibitory effects of mushroom tyrosinase using an L-tyrosinase substrate. The assay was conducted using a previous method with minor modifications (Ma). In a 96 well plate, 40 µl of phytocannabinoid sample was added to 120 µl mushroom tyrosinase solution (100 U/mL) and incubated at room temperature for 15 minutes. Subsequently, 40 µl of L-tyrosine solution (2.5 mM) was added and the 96 well plate was incubated at 37˚C for 30 minutes, the absorbance was then measured on a plate reader at 490 nm. Kojic acid, a common skin-whitening natural product served as a positive control. Samples were tested in triplicate and results were compared to DMSO controls. The percentage of tyrosinase inhibition was calculated as follows: $[(\Delta A control - \Delta A sample)/\Delta A control] \times 100\%$

Results

In Silico Predictions of phytocannabinoid skin permeability

In silico computational modeling, SwissADME, was used to predict skin permeability of minor phytocannabinoids (Table 1). Using physicochemical properties such as molecular weight, Log Po/w, molecular size, and lipophilicity the SwissADME determines the skin permeability coefficient (Log Kp; cm/s) of minor phytocannabinoids (Daina et al., 2017). More negative coefficient values correspond to less skin permeability of said compound. All four of the phytocannabinoids were found to have medium skin permeability. The PAMPA skin controls, verapamil, progesterone, warfarin and piroxicam, all exhibited similar skin permeability in the SwissADME predictor. Compounds were then experimentally evaluated for passive permeability in the skin after using this predictive tool.

Table 1: *in silico* SwissADME predictive permeability of minor phytocannabinoids in the skin.

Minor phytocannabinoid	-Log Kp (cm/s)	Log Po/w (iLOGP)	- Log S (ESOL)	-Log S (Ali)
Cannabigerol (CBG)	-2.96	4.31	-6.08	-8.10
Cannabinol (CBN)	-3.86	3.94	-5.74	-6.51
Cannabidivarin (CBDV)	-4.18	3.54	-4.99	-6.05
Δ8-Tetrahydrocannabinol (Δ8-THC)	-2.96	2.98	-6.39	-5.93

PAMPA: Minor phytocannabinoids are Moderately permeable through the skin

The passive skin permeability of the minor phytocannabinoids was evaluated through the parallel artificial membrane permeability assay (PAMPA) (Figure 3). Log P_e values determined through PAMPA are used to theorize the permeability potential of a sample. More negative Log P_e values correspond to lower permeability. Permeability constants of the provided control compounds were within the accepted range and were used to validate said assay. The analyzed samples showed similar permeability constants, although SwissADME predictions showed CBG having the lowest Log K_p. Therefore, theoretically CBG should show the most permeability of the mPCs studied , while Δ8-THC should show the least. The samples showed almost identical permeability to CBD, which was reported in our previous study. The phytocannabinoids also showed similar permeability in both pHs to control compound progesterone, just as CBD did. This suggests that phytocannabinoids are moderately permeable through the skin.

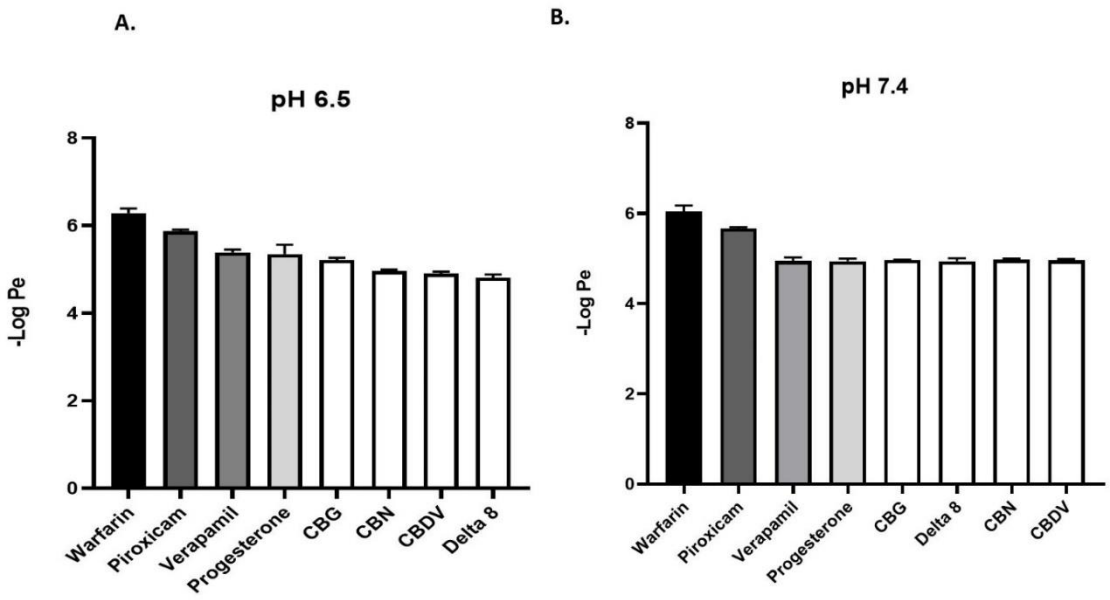

Figure 3: mPC's show passive permeability in the Skin PAMPA model. The passive permeability of CBG, CBN, CBDV and Δ8-THC was evaluated at two relevant pH levels 6.5 (A) and 7.4 (B). Control's warfarin and piroxicam exhibit low permeability, while progesterone and verapamil exhibit medium permeability.

Minor Phytocannabinoids do not inhibit Melanin Production in Mushroom model

The potential skin lightening effects of phytocannabinoids were studied using a mushroom tyrosinase model (Figure 4). The phytocannabinoids, CBDV, CBN, CBG and Δ8-THC were tested against a positive control, kojic acid. The samples showed increased enzymatic activity, rather than inhibition, at a final concentration of 500 mM. CBN showed the highest activity amongst the mPCs ,followed by CBG and Δ8-THC. CBDV showed the least activity.

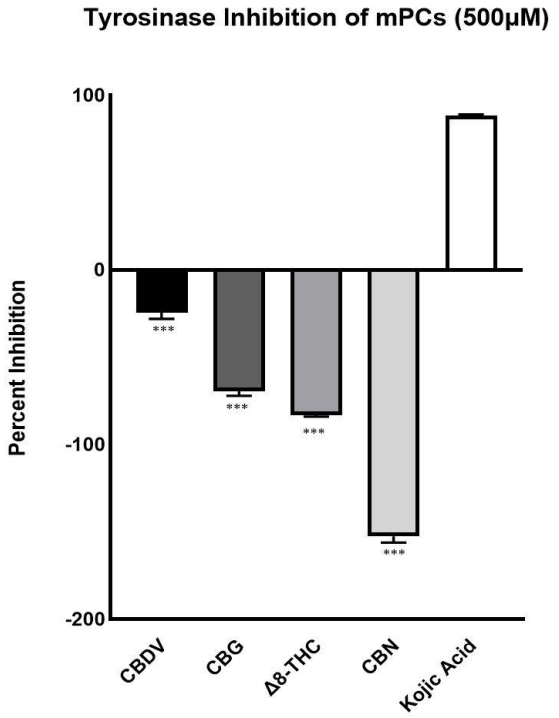

Figure 4: Modulatory effects of mPCs on tyrosinase enzyme. Minor phytocannabinoids were evaluated for tyrosinase modulatory effects (500 µM). Concentrations are expressed as mean ± standard error (n=6), statistical significance was determined by analysis of variance (ANOVA). Significance as compared with Kojic acid standard , $p \leq 0.01$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***) and $p \leq 0.0001$ (****).

Discussion

As seen with cannabidiol, the evaluation of minor phytocannabinoids from the plant *Cannabis sativa* for skin related disorders are becoming increasingly important. As the demand for *Cannabis*-based products continues to grow it is important to evaluate the skin permeability and cosmeceutical applications of the minor phytocannabinoids that are likely present in these extracts. The minor phytocannabinoids present a viable alternative for Δ9-THC as they are non-psychoactive and hold similar anti-inflammatory and antioxidant properties (Massi et al., 2006). Early studies of these minor phytocannabinoids show promising potential for pharmaceutical use, such as CBDV which is being studied for treatment against Duchenne muscular dystrophy (DMD) (Sampson, 2021). Although there is limited literature on the skin protective effects of the minor cannabinoids, it can be inferred that they hold similar medicinal benefits to CBD. Recent clinical trials have shown that CBD applied topically aided in the treatment of inflammatory skin disorders such as atopic dermatitis (Palmieri et al., 2019). Further investigation is necessary as the potential for phytocannabinoids to engage in an entourage effect has been reported multiple times (Russo, 2011).

Minor phytocannabinoids were evaluated for their passive permeability through the skin using in silico (SwissADME) and *in vitro* (PAMPA) models. Both the SwissADME and the PAMPA assay showed that mPCs have moderate skin permeability. At pH 6.4 the measured values for -Log P_e of CBN, CBDV, CBG and Δ8-THC were 4.91, 4.96, 5.21 and 4.81 respectively. Similarly, the measured -Log P_e values for CBN, CBDV, CBG and Δ8-THC were 4.87, 4.96, 4.97 and 4.94 at pH 7.4.

Likewise, the -Log P_e values of CBD were similar at 5.02 at pHs 6.5 and 7.4 (Akanji et al., 2021). This suggests that the phytocannabinoids have similar permeability profiles, which may be beneficial when considering formulating. This may also encourage the entourage effect when considering using Cannabis extracts, which is the preferred method for consumers over individual metabolites (Sampson, 2021). Skin PAMPA results coincided with the in silico predicted results, except for CBG, which was predicted to have the most permeability. Due to the high lipophilicity of their structures, it is assumed that these phytocannabinoids can also penetrate through the trans follicular route (Lodzki et al., 2003). This may account for discrepancies between the in silico and in vitro prediction methods, as the PAMPA assay only measures passive permeability. Although the PAMPA assay is a good predictive tool for skin permeability, further investigation using other skin permeability models is still necessary.

Skin lightening is becoming increasingly popular in many parts of the world including Africa and Asia. Many products on the market are not regulated and can contain high concentrations of potentially carcinogenic ingredients such as hydroquinone (Westerhof and Kooyers, 2005). The efficacy and safety of many other skin lightening agents have yet to be examined and therefore there is a need for a safe naturally derived alternative. Inhibition of the mushroom tyrosinase enzyme serves as a good screening method for potential skin lightening agents; as said enzyme is the rate limiting step in the production of melanin, melanogenesis. In this study, we proposed mPCs as potential tyrosinase inhibitors and therefore skin lightening agents. The mPCs showed no inhibition activity in the tyrosinase inhibition assay. Firstly, the

mushroom tyrosinase enzyme differs greatly from the tyrosinase enzyme found in human skin. Many skin lightening agents that inhibit the tyrosinase enzyme in vivo show no activity in the mushroom model and vice versa (Pillaiyar et al., 2017). This phytocannabinoid increase in tyrosinase activity agrees with a study done by Pucci et al that showed endocannabinoids stimulated human melanogenesis through CB1 receptors (Pucci et al., 2012). This notion was later confirmed for the phytocannabinoid CBD, which was found to upregulate melanogenesis through activation of p38 MAPK and p42/44 MAPK of the CB1 receptor (Hwang et al., 2017). Although the phytocannabinoids show activation properties of melanogenesis they may still be potentially beneficial in hyperpigmentation disorders such as PIH. PIH can potentially be prevented through alternative routes such as the mitigation of inflammation and reactive oxidative species (ROS) (Briganti et al., 2003; Smit et al., 2009). Specifically, with hyperpigmentation disorders the reduction of inflammatory mediators such as Il1α and TNF-α can limit melanogenesis (Silveira et al., 2008). Phytocannabinoids should be considered for the treatment of hypopigmentation disorders instead.

Conclusion

In summary, four minor phytocannabinoids CBN, CBDV, Δ8-THC and CBG were evaluated for their potential anti-melanogenesis effects and skin permeability. Overall, the phytocannabinoids showed favorable skin permeability in the PAMPA assay. The mPC's showed increased activity against the mushroom tyrosinase enzyme. However, lower concentrations of phytocannabinoids should be tested for any inhibitory activity. Therefore, further investigation is needed to evaluate phytocannabinoids as potential skin darkening agents. In vivo investigations are necessary to further evaluate the skin permeability and efficacy of phytocannabinoids for skin care formulations.

Supplementary Information

Table S1: in silico SwissADME predictive permeability and -Log Pe values of PAMPA standards.

PAMPA Standard	Expected -Log Pe (PAMPA)	-Log Kp (cm/s)	Log Po/w	-Log S (ESOL)	-Log S (Ali)
Warfarin	-6.00	-6.26	2.41	-3.70	-3.77
Piroxicam	-5.67	-6.15	1.67	-4.01	-4.99
Verapamil	-5.65	-6.38	4.50	-4.46	-4.83
Progesterone	-4.92	-5.47	3.09	-4.16	-4.28

Table S2: -Log Pe (PAMPA) values of minor phytocannabinoids compared to CBD.

phytocanna-binoid	-Log Pe pH 6.5	-Log Pe pH 7.4
CBD	5.02	5.02
CBG	5.21	4.97
CBN	4.91	4.97
CBDV	4.96	4.96
Δ-8	4.81	4.94

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CHAPTER 4: CONCLUSIONS

Drugs administered through the transdermal route can be used to target the ECS as potential treatments for those suffering with chronic skin conditions. Therefore, the ECS of the skin has recently become a prevalent drug target for the maintenance of skin homeostatic functions. Phytocannabinoids from the plant Cannabis sativa, have been shown to be potent modulators of ECS receptors in both the central nervous system and skin. However, lack of knowledge on proper formulations of phytocannabinoids for transdermal drug delivery proposed a significant limit for potential clinical trials. This issue is also highlighted by the fact that phytocannabinoid based skin care products are becoming increasingly popular. Due to the lack of clinical studies, many of these products include claims that have not been scientifically proven, therefore leaving consumers uninformed and self-dosing. In this study, the permeability, formulation environments and stability of phytocannabinoids are evaluated for their potential use as treatments for chronic skin conditions associated with the ECS.

In this study, CBD, CBG, CBDV, CBN and Δ8-THC were evaluated for permeability through a synthetic skin membrane. Using the skin PAMPA assay these phytocannabinoids showed moderate skin permeability capabilities, similar to that of progesterone. CBD showed further skin permeability potential by means of the passive diffusion through a biomimetic skin membrane in the Franz cell assay. Further studies using CBD formulations confirmed this notion, also showing that lipophilic formulations may be beneficial to enhance penetration capabilities. Ideal formulation

environments for CBD were determined through a series of stability and solubility experiments. It was determined that CBD is most stable in formulations at pH 5 and that the type of surfactant had a significant impact on CBD's solubility. The stability of CBD at pH 5 indicates that it is ideal for topicals as they are formulated to be slightly acidic to maintain the acid mantle of the skin. It was also demonstrated that the type of surfactant used had a significant impact on the solubility and penetration of CBD through synthetic skin-like membranes. Cosmeceutical applications for the phytocannabinoids were also investigated through the use of the tyrosinase inhibitory assay. The results indicate that minor phytocannabinoids may be used in the activation of the tyrosinase enzyme, which is responsible for the production of melanin in the skin.

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