Are all THC-Dominant Cannabis Varieties the Same? Comparing the Phytochemistry and Bioactivity of Different THC-Dominant Cannabis Samples

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Abstract

Cannabis sativa has a complex history of classification and traditional use. *Cannabis sativa ssp. sativa* and *spp. indica* are the two major lineages of cannabis and, through artificial selection, many strains or cultivars are found within each group are bred together to yield hybrid plants. New methods of classification based on Δ^9-Tetrahydrocannbinol (THC) and Cannabidiol (CBD) content as well as minor cannabinoids and terpenes have emerged as a more effective classification of cannabis. However, the fidelity of cannabis varieties relative to their respective strain names and lineages (*indica, sativa* and hybrid) based on chemistry has been brought into question. THC and more recently CBD are collectively responsible for the psychoactive and therapeutic effects of cannabis and minor cannabinoids and terpenes are emerging as having their own unique bioactivity or synergistic effects *in vitro*. Considering the variation in cannabis chemical profile and infidelity to strain names or lineages, we investigated the cannabinoid, terpene and metabolomic profiles of 33 THC-dominant strains (113 samples) to evaluate existing and alternative chemistry-based classification systems using multivariate analyses. Here, we conclude that Indica-Sativa-Hybrid designations are insufficient in describing variation in cannabinoid, terpene and metabolomic data, and that a terpene-based profile classification revealed robust groupings in cannabinoid-terpene data. However, terpene profiles were not discernable in metabolomic data. To investigate how chemical complexity and variability impacts bioactivity, we compared the activity of cannabis extracts to that of pure THC (and CBD) to determine if THC alone is driving activity. THC, CBD, and plant extracts were tested *in vitro* for cytotoxicity in BEAS-2Bs cells and for cannabinoid receptor signalling activity using a human CB_1-HEK293 cell model. THC did not completely dictate 24-hour toxicity in BEAS-2B cells suggesting that other extract components (beyond THC and CBD) are contributing to cytotoxicity. While CBD alone was 2x more toxic than THC alone, THC:CBD do no predict toxic concentration. THC within extracts appeared to drive efficacy at CB_1 receptors by reducing intracellular cAMP accumulation but did not dictate variation in EC_{50}. THC in extract also appeared to increase percent cAMP reduction in cells regardless of low CBD content but a 1:1 balanced THC/CBD extract revealed reduced percent cAMP reduction. Pure compounds compared to extracts of the same THC/CBD ratio performed very similarly at CB_1 receptors besides 1:1 extract preparation having reduced % reduction of cAMP compared to 1:1 pure compounds suggestive of NAM by extract components. Regression modelling of THC within extract revealed a significant positive relationship in % cAMP reduction (E_{max}) but no significance in TC_{50} and EC_{50}. This work demonstrates the importance of rigorous analysis of cannabis chemistry as well as evaluation of extracts in bioactivity assays.

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List of Abbreviations

^{1}H NMR	Proton nuclear magnetic resonance
2-AG	2-Arachadonyl Glycerol
$5HT_{1A}$	5-beta hydroxy tryptamine receptor 1
AEA	Anandamide
ATP	Adenosine triphosphate
BDP	Botanical drug preparation
BEAS-2Bs	Bronchial epithelial cells
BSA	Bovine serum albumin
CB_1	Cannabinoid Receptor Type 1
CB_2	Cannabinoid Receptor Type 2
cAMP	cyclic-Adenosine monophosphate
CBC	Cannabichromene
CBCA	Cannabichromenic acid
CBDV	Cannabidivarin
CBDVA	Cannabidivaric acid
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBEA	Cannabielsoic acid
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBGV	Cannabigerovarin
CBLA	Cannabicyclolic acid
CBN	Cannabinol
CBNA	Cannabinolic acid
CBT	Cannabicitran
CNS	Central nervous system
COX-1/2	Cyclooxygenase 1/2

CYP	Cytochrome p450 enzyme
DMAPP	Dimethylallyl diphosphate
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DSE	Depolarization-induced suppression of inhibition
EC_{50}	50% Effective concentration
E_{max}	Maximum effect
FBS	Fetal bovine serum
FPP	Farnesyl pyrophosphate
GC-FID	Gas chromatography- flame ionization detection
GPP	Geranyl pyrophosphate
GPCR	G-protein coupled receptor
GPR	G-protein receptor
GOT	olivetolate geranyl transferase enzyme
HEK293	Human embryonic kidney cell
HPLC- DAD	High pressure liquid chromatography – diode array detection
ICC	Immunocytochemistry
IPP	Inositol pyrophosphate
LOD	Level of detection
LOQ	Level of quantification
LTP	Long term potentiation
MeOH	Methanol
NAM	Negative allosteric modulator
O/N	Overnight
OA	Olivetolic acid
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + Tween20
PLS-DA	Partial least sum of squares-discriminant analysis

PSP	Post synaptic potential
STR	Short tandem repeats
TC_{50}	50% Toxic concentration
TRPV	Transient receptor potential vanilloid channel
THC	Δ^9-Tetrahydrocannabinol
THCA	Δ^9-Tetrahydrocannabinolic acid
THCV	Tetrahydrocannabivarin
THCVA	Tetrahydrocannabivarin acid
UPGMA	Unweighted pair group method with arithmetic mean
UPLC-HRMS	Ultra-high pressure liquid chromatography

Chapter 1: General Introduction

1.1 A Brief History of Cannabis Diversity

To evaluate *Cannabis sativa L.* classification, it is important to know the origins of its cultivation and interactions with humans through history. Cannabis appears to have been harvested for fibre in China as far back as 8500 years ago. Potential unlocking of psychoactive qualities of *Cannabis* were reported as far back as 1500 years ago based on translation of traditional Arabic literature (E. B. Russo, 2007; Small & Marcus, 2002). Through studies on early botanical collections and descriptions, *Cannabis* was distinguished as 1) a northern plant with limited intoxicant potential that had been selected for fiber and oil and 2) a southern plant with higher intoxicant potential, described as subspecies *sativa* (1) and *indica* (2) respectively (Small & Cronquist, 1976). These classifications also account for the different morphologies of cannabis where *C. sativa ssp. sativa* had more elongated leaves and were typically thinner and taller than *C. sativa ssp. indica* which present as bushier with broad leaves (Small & Cronquist, 1976). Today, following legalization in Canada after decades of illicit breeding activities, strain names are assigned to different varieties of recreational cannabis based on morphology, genetics and proportions of Δ^9-Tetrahydrocannabinol (THC) and cannabidiol (CBD) presented in percent weight/weight (Ontario Cannabis Store, 2021). Phytocannabinoids (including THC and CBD) are the dominant and distinguishing compounds in cannabis, found in specialized secretory epidermal glands termed "trichomes" (De Meijer, et al., 2009; Furr & Mahlberg, 1981; Small, 2015) which also differ between *C. sativa ssp. sativa* and *ssp. indica*. Resin in glands with extended membranes on female inflorescence has the highest cannabinoid content and therefore high THC *Cannabis* flower is harvested from clonal female plants (Kaufman, 1976). Cloning of both hemp and drug type cannabis is performed by micropropagation in tissue culture and the induction of shoots from the plant callus (Wang et al., 2009). This preserves the genotype of the

plant by avoiding random genetic events in sexual reproduction. Over the past two centuries, both *indica* and *sativa* lineages have been developed into both hemp and marijuana (drug-type) (**Figure 1-1**). The history of the *Cannabis* phylogeny is debated; however, these plants are wind pollinated and escaped or wild plants have no known barriers to hybridization with domesticated plants. Gene clusters have delineated clustering of *Cannabis* species into 3 groups: *C. indica*, *C. sativa* and *C. ruderalis* (Hillig, 2005). Conversely, studying Short Tandem Repeats (STRs) in the DNA of drug and fibre type plants revealed only a 6% difference between *C. indica* and *C. sativa* (Gilmore, Peakall, & Robertson, 2003). *Cannabis* prohibition in the southern United States in the 1920s not only halted legal cultivation of, and access to, cannabis for consumer and research purposes but further complicated cannabis pedigrees. Unlike registered hemp cultivars, which are documented with known pedigree and stable traits, drug-type "strains" were primarily developed illicitly through selective breeding and cross breeding with no documentation of specific scientific characterization. Since current regulations in North America do not define or limit the use of strains or their names, the rapidly expanding (licit) cannabis industry maintains

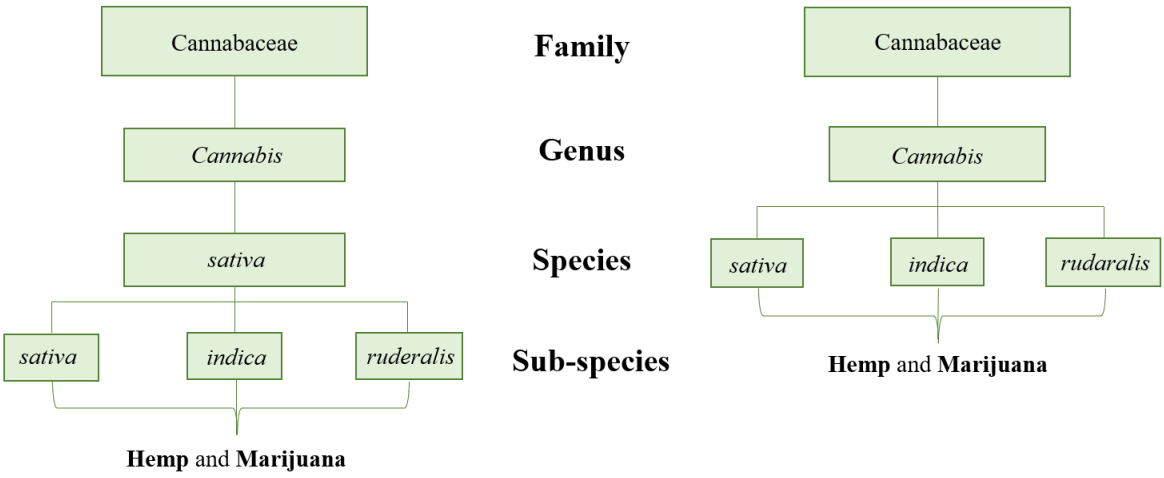


Figure 1-1: Proposed classification of *Cannabis* based on Linnaeus binomial system by different authors (Pollio, 2016)

similar practices of developing and marketing different varieties of medicinal and recreational cannabis resulting in the tremendous diversity of highly psychoactive (THC-dominant) plants and products we see today (E. B. Russo, 2007).

1.2 Ethnobotany

Cannabis sativa or 'cultivated cannabis' was first described in 1542, predating the Linnean *Genus species* binomial designation (Linnaeus, 1753). The *sativa* and *indica* gene pools have been correlated to geography by use of genetic analysis (Hillig, 2005). The *sativa* plants hailed from eastern Europe and included ruderal and hemp/fibre landraces while *indica* genes were correlated to Far Eastern fibre landraces, narrow-leaflet drug strains from Southeastern Asia, Africa and South America and wide leaflet drug strains from Afghanistan and Pakistan (**Figure 1-1**). Some authors claim that there are no truly wild cannabis strains that exist that are not a result of human manipulation (E. B. Russo, 2007). The exact origin of the species may be debated but the diversity we see today has radiated out of Central Asia (Modern day Tajikistan, Punjab, Kashmir, Uzbekistan and the Western Tian Shan mountain ranges) (Vavilov, 1951).

Traditional use of *Cannabis* appears in Uttaranchal (modern India, Western Himalayas). Folk taxonomy describes 2 classes, *Ghar-Banghau* known as the cultivated one and 'Kath-Bhanghau', the wild one, which mostly grows near cattle sheds and habitations (Shah, 2008). Cultivated plant seeds and oils are used in a myriad of traditional dishes in this region and plant fibres are utilized for their strength in cattle rope named *Bhangu-Pakhuli*. *Ganja* is the collection of dried inflorescences from female plants which are absent of leaves. The wild plant inflorescences (Kath-Bhanghau) are harvested and rubbed between the palms to yield a substance called 'Attar', a resinous exudation which is smoked by groups of habitual users called *Attarchi*. *Ganja* use was also reported in Southeast Asian cultures in food preparations and was

recognized as an analgesic used to treat cholera, malaria, dysentery, anorexia, asthma, indigestion, coughing, dizziness, convulsions and elimination of intestinal parasites (Martin, 2011). Oral use of *Cannabis* has been reported as Jamaican tea preparations, however, very low THC solubility in H_2O has led to incorporation of lipid carriers such as ghee and clarified butter in traditional oral preparations (Balant et al., 2021)

Ritual cannabis smoking was identified by findings of charred cannabis plants in wooden braziers in a tomb in the eastern Pamirs region (Ren et al., 2019). It is postulated that cannabis was used in mortuary ceremony dating back to 500 BCE. Ancient preserved *Cannabis* plants were also discovered in tombs of north western China (2800-2400 BCE) indicative of ritual use in death ceremonies (Hongen Jiang et al., 2016). The *Atharva Veda*, a text of Hindu hymns contains some of the oldest reference to the use of cannabis deeming it one of the five sacred plants (Ferrara, 2021). The second book of the *Atharva Veda* goes on to describe cannabis preparations as 'sacred grass' and for its power to vanquish sickness, despair and calamity. Aside from ritual use, *Cannabis* seeds contain 30% oil and 25% protein with a preferable ratio of omega-6 and omega-3 fatty acids. Seeds or fruits have alimentary use by roasting, pickling, grinding and pressing for oil (Balant et al., 2021).

Marijuana became popular in Mexico in the early 19th century which was a preparation of cannabis leaves and flowering tops. This narcotic preparation percolated up through the southern United States quickly through the 1930s travelling up the Mississippi River. During this time there were a recorded some 28 different over the counter pharmaceuticals which contained in part cannabis, often described as *indica* (Sasman, 1938). In 1937 U.S Congress passed the Marihuana Tax Act, the final move to prohibit the use of cannabis as a therapeutic medicine (Mikuriya, 1969). Canada added 'marijuana' to the Confidential Restricted List in 1923

under the *Narcotics Drug Act Amendment Bill* during a time in which the House of Commons was cracking down on opium preparations post WWI (House of Commons, 1923). Cannabis remains federally illegal in the U.S today. However, on October 17th, 2018 Canada was the first G7 country to permit the sale and consumption of cannabis products for the adult recreational user. These products include: Cannabis oil, fresh cannabis, dried Cannabis, Cannabis plant seeds and Cannabis plants (Codification, 2019). A year later, edible cannabis products have become available commercially as well. Statistics Canada reported that Cannabis use among all age groups was higher in the third quarter of 2019 as compared to 2018 with seniors aged 65 and over having the largest increase (1-7%) (Statistics Canada, 2019). The Canadian government made 107.1 million CAD in revenue in the first quarter of 2019 and has reported 598000 'new' cannabis users in the second and third quarter of 2019 (Statistics Canada, 2018). Statistics Canada also reported an increase in Cannabis use in all age groups during the COVID 19 pandemic (Statistics Canada, 2021). The government of Canada would benefit from stronger quality control/ quality assurance bolstered by diligent research in *Cannabis* bioactivity.

Recreational *Cannabis* strains are commercially available at high Δ^9-Tetrahdrocannabinol (THC) (15.0-30.0 % w/w) with usually low but sometimes intermediate levels of CBD (0.0- 20.0 % w/w). Industry today defines recreational cannabis as Indica and Sativa or some mix of the two (hybrids). Retailers and the Ontario government will describe varieties as being 'Sativa-dominant' or 'Indica-dominant' as to avoid using the debatable and potentially unprovable classification of 'Indica' and 'Sativa' (Cannabis Act, 2018). The development of illicit marijuana was a process of artificially selecting *C. indica* plants that yielded the highest levels of Δ^9-tetrahydrocannabinol (THC) and, thus, the most potent "high". Most collections of commercial strains consist of predominantly THC-dominant varieties with a small proportion

being cannabidiol (CBD) balanced or CBD dominant (Choi et al., 2004; Ciolino, Ranieri, & Taylor, 2018; Jikomes, Christina, Vergara, & Keegan, 2021). Interestingly, cannabis fields in Morocco and Afghanistan of wild breeding plants yield equal parts CBD to THC (E. B. Russo, 2007). As CBD has emerged as a therapeutic in recent years, the development of higher CBD strains via crossing with *sativa* dominant plants has emerged quickly, leading to further diversification of products and product chemistries (Aliferis & Bernard-Perron, 2020). Selection of strains with more balanced THC:CBD may better correlate to varieties that were being selected for by traditional peoples.

1.3 Cannabis Phytochemistry

Δ^9-tetrahydrocannabindiolic acid (THCA) and cannabidiolic acid (CBDA) represent the highest proportion of cannabinoids in trichomes but there are other minor cannabinoids such as cannabigerolic acid (CBGA), cannabinolic acid (CBNA), cannabichromene (CBCA), Δ^9-Tetrahydrocannabivarinic acid (THCVA) and cannabidivarinic acid (CBDVA) **(Figure 1-2)**. Cannabis has been classified into three predominant chemotypes based on THC/CBD ratios: Type I (drug type, High THC), II (intermediate with similar balanced THC/CBD) and III (fibre type, CBD is the dominant cannabinoid) (Hillig & Mahlberg, 2004; Piluzza, Delogu, Cabras, Marceddu, & Bullitta, 2013).

The biosynthetic pathway for cannabinoids has been well described (Andre, Hausman, & Guerriero, 2016; Gagne et al., 2012; E. B. Russo, 2011) and is transcriptionally governed by locus *O* in the *Cannabis* genome (De Meijer et al., 2009). The B_T and B_D alleles are codominant and determine 3 different possible chemotypes. Hemp chemotype I plants possess B_T/B_T alleles with high THCA (>>0.1 THCA:CBDA), chemotype II is heterozygote with B_T/B_D resulting in a

moderate level of THCA (0.5-2.0 THCA:CBDA) and chemotype III, B_D/B_D, with low levels (<<0.1 THCA:CBDA) (De Meijer, 2014; Small & Beckstead, 1973).

Geranyl pyrophosphate (GPP) is synthesized from dimethylallyl pyrophosphate (DMAPP) and isopentenyl phosphate (IPP) in plastids by the geranylpyrophosphate: olivetolate geranyl transferase enzyme (GOT) (Fellermeier, et al., 2001). GPP may be coupled with olivetolic acid (OA) to produce the pentyl side chain cannabinoids: cannabigerolic acid (CBGA), delta-9-tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA) and cannabichromic acid (CBCA) (Fellermeier & Zenk, 1998). THCA and CBDA are synthesized by THCA and CBDA synthases, respectively, from CBGA (Gagne et al., 2012). These acidic cannabinoids are readily decarboxylated to their more active forms (THC and CBD) by temperatures greater than 100°C (Baker, et al., 1981). Incorporation of divarinic acid instead of olivetolic acid with geranyl pyrophosphate creates CBGVA instead of CBGA, the propyl analogue precursor. From this, tetrahydrocannabivarin (THCV) and cannabidivarin (CBDV) are synthesized by the same pathways (**Figure 1-2**) (Flores-Sanchez & Verpoorte, 2008). Compounds such as CBN, CBL, CBT and CBC are isomers and degradation (oxidation/ photochemical reaction) products of these cannabinoids (Berman et al., 2018) which may also accumulate in plant tissues. These compounds, however, only provide a glimpse of the phytochemical diversity of *Cannabis*, with over 150 unique cannabinoids described to date, some with known biological effects (Brenneisen, 2007; Koltai & Namdar, 2020). Along with cannabinoids there are also over 200 different terpenes that have been isolated from the plant (E. B. Russo, 2011). Terpenes are highly volatile compounds that give cannabis its pleasing aroma. Monoterpenes are synthesized from the same substrate as cannabinoids (GPP) while the sesquiterpenes and triterpenoids derive

from farnesyl diphosphate and squalene respectively (**Figure 1-3**) (Aliferis & Bernard-Perron, 2020; Jin et al., 2020).

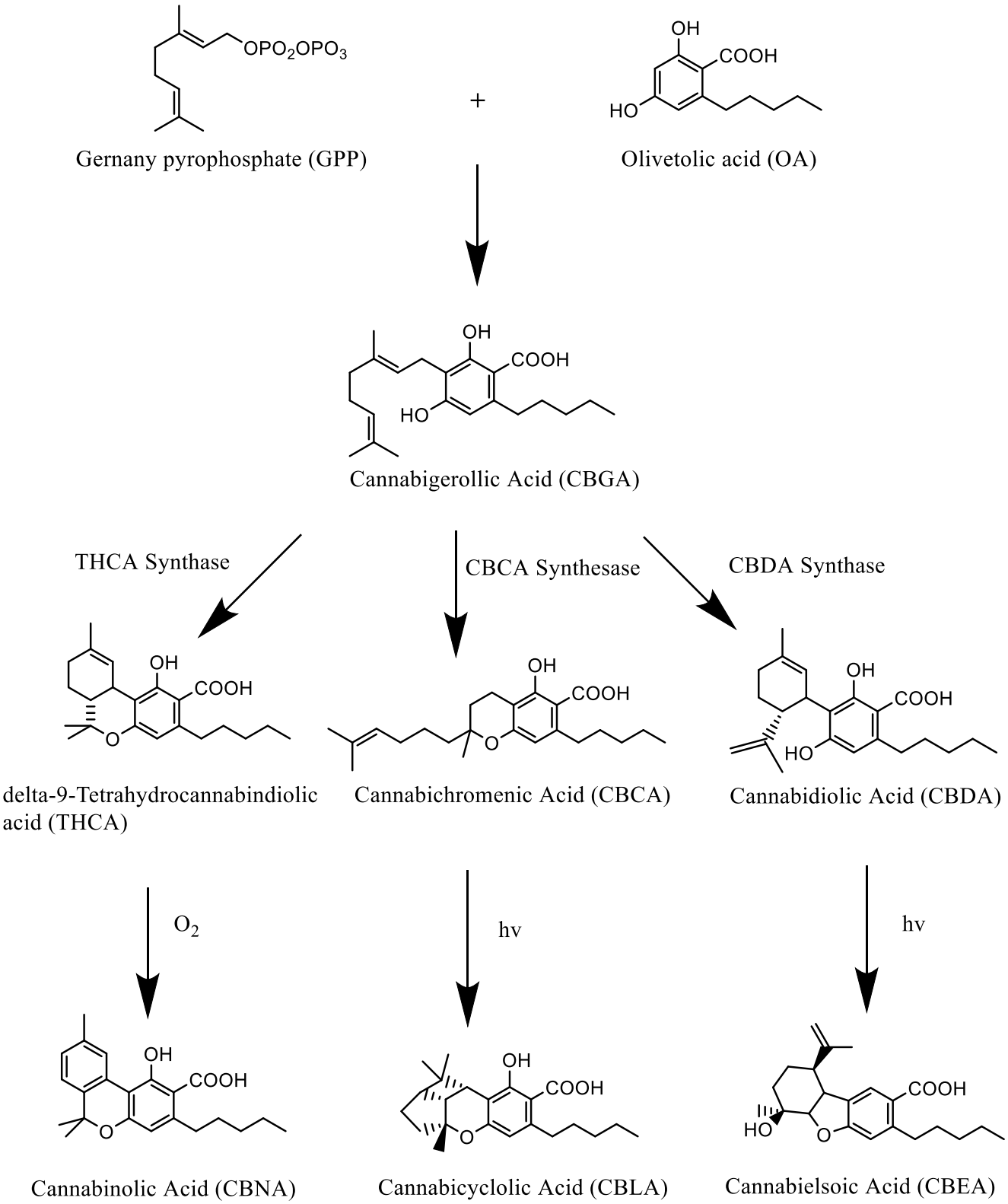


Figure 1-2: The synthesis of major cannabinoids Δ^9-THCA, CBDA, CBGA, CBCA and there resulting degradation products CBNA, CBLA and CBEA. O_2 refers to oxidation and hv revers to photochemical reactions. Not shown is the incorporation of divarinic acid instead of OA to generate the propyl side chain analogues (CBGVA, CBDVA and THCVA)

1.3.1 Extraction and Analysis

A variety of extraction methods have been employed to characterize, quantify, and isolate phytocannabinoids. Neutral cannabinoids are highly lipophilic (Compton, 1990). The acid precursor cannabinoids in plant tissue however are more polar due to the carboxylic acid group (Hanuš et al., 2016). A few polar organic solvents are typically used for cannabis inflorescence (bud) extraction followed by High-Performance Liquid Chromatography (HPLC) analysis. A 9:1 methanol/chloroform or 80% methanol solution at 10 ml per 100 mg ground plant flower were effective in cannabinoid recovery (Elizabeth M Mudge et al., 2017). Another study used grounded dried buds and 99% ethanol at 10 ml per 100 mg cannabis (Ciolino et al., 2018). Both studies performed the extraction in a sonicating water bath after which, samples were filtered at 0.22 µm. To control for moisture content liquid nitrogen added to cannabis buds, which allows for straightforward pulverization in mortar and pestle (Lodhi et al., 1994). Extraction methods have been paired with analytical methods and reviewed (see Aliferis & Bernard-Perron, 2020). HPLC coupled with diode array detection (DAD) as well as Gas Chromatography (GC) coupled with Flame Ionization Detection (FID) are compatible with EtOH extracts of cannabis plant material to characterize cannabinoids and terpenes respectively

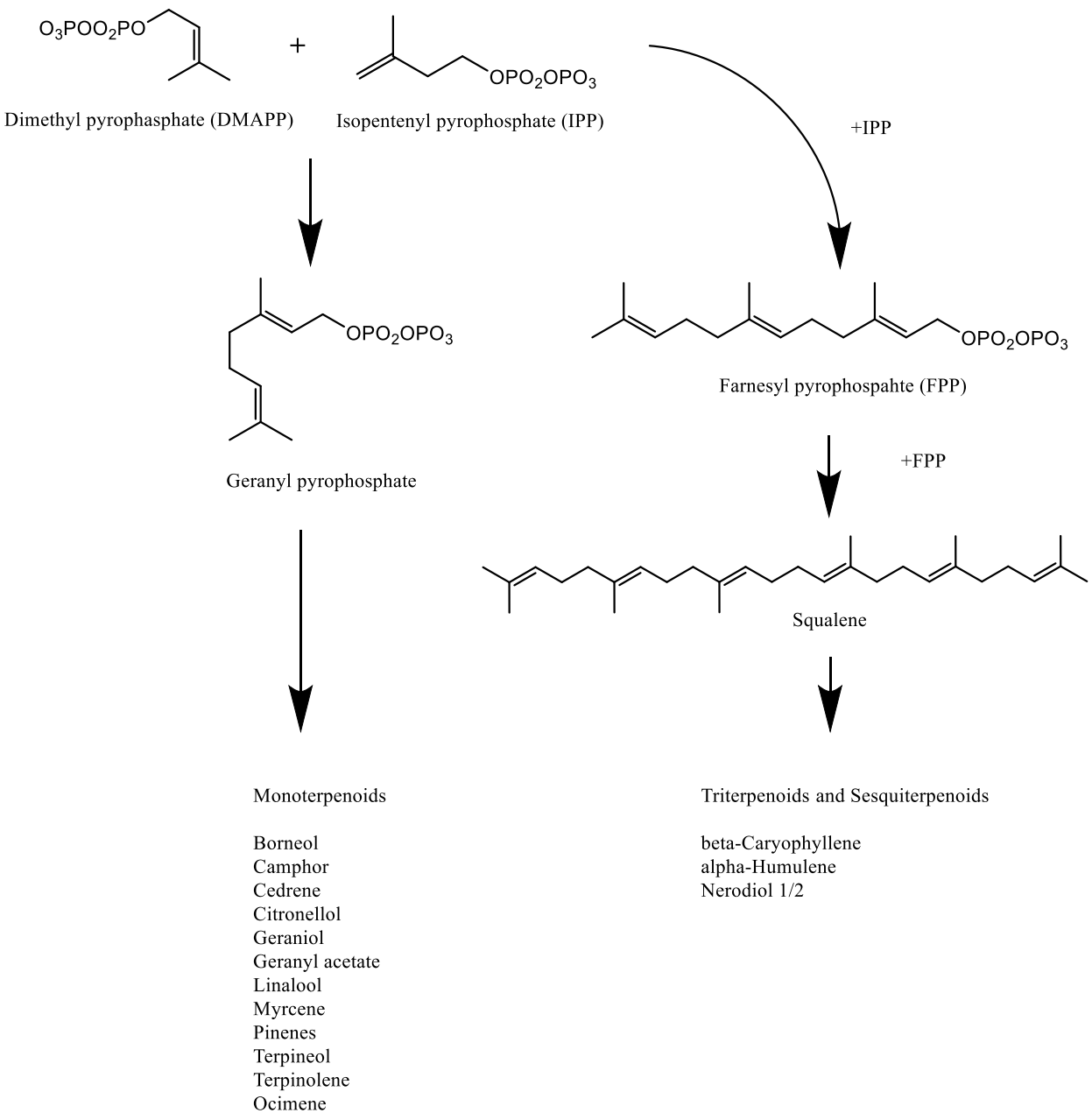


Figure 1-3: The synthesis of major terpenes found in *C. sativa* +IPP refers to the addition of another Isopentenyl to geranyl pyrophosphate. +FPP refers to the addition of farnesyl pyrophosphate to farnesyl pyrophosphate to yield squalene

Publications seem to go back and forth on if Indica and Sativa should be classified as unique subspecies (Mcpartland, 2017). A potential answer to this is the emerging use of metabolomics or metabolomic finger printing as means to completely map the complexity of cannabis chemistry (Aliferis & Bernard-Perron, 2020). Metabolomics captures all the metabolites which exist within a sample which is more powerful than just studying the plant genome. Conventional strain names such as "Bubba Kush", "Northern Lights" and "Sour Diesel", as well as plant morphology, capture only a portion of the potential variability in phytochemistry between plants. Where strains with their associated strain ID are sourced, grown, cultivated and cured can introduce differences in final product In this study targeted high pressure liquid chromatography with diode array detection (HPLC-DAD) data with cannabinoid standards and gas chromatography with flame ionization detection (GC-FID) data with terpene standards as well as untargeted ultra-high pressure liquid chromatography with high resonance mass spectroscopy UPLC-HRMS, Orbitrap MS) data was collected. Consistent analysis protocols on samples from a single licensed grower should reduce the sources of variation and provide a clearer indication of strain variability. Using metabolomics allows us to detect intra and inter strain differences in cannabinoids and terpenes but also in unknown mass features which may be driving some of the existing variation

1.4 Cannabinoid Pharmacology

Two cannabinoid receptor types have been characterized: CB_1 and CB_2, cloned in 1990 (Matsuda, Lolait, Brownstein, Young, & Bonner, 1990) and 1993 (Munro, Thomas, & Abu-Shaar, 1993), respectively. THC is responsible for the psychoactive effects of *Cannabis* (Gaoni & Mechoulam, 1964) due to it preferentially binding and activation of CB_1, which is highly

prevalent in the central nervous system (CNS) (Matsuda et al., 1990). CB_2 receptors are localized to peripheral immune cells and have therefore been implicated in the immunomodulatory effects of cannabis (Gonsiorek et al., 2000). CBD has micromolar affinity for both CB_1 and CB_2 but appears to antagonize THC at CB_1 receptors (Pertwee, 2008; Russo & Guy, 2006). Whereas CBD has been reported to have multiple pharmacological effects in the fields of inflammation, neurodegeneration, epilepsy and autoimmune disorders (Pisanti et al., 2017), which appear to be CB_1 receptor-independent.

1.4.1 Endocannabinoid system

Phytocannabinoid discovery predates the recognition of the human endocannabinoid system and why it is thusly named. The endocannabinoid system consists of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) and their coordinated release from neuron terminals. Phytocannabinoids (THC and CBD) essentially mimic these signalling molecules and bind the same receptors, to varying degrees. CB_1 is highly expressed in all areas of the CNS and PNS and modulate sleep, appetite and mood as well as pain sensation, cardiac function and energy metabolism (Zou & Kumar, 2018). Endocannabinoids regulate neuronal firing through a process called retrograde synaptic signalling where they may inhibit the further release of excitatory or inhibitory neurotransmitters (Howlett et al., 2002; Kreitzer, 2005). Endocannabinoids regulate neuronal homeostasis as they are synthesized in a response to neuronal excitation and inhibit further neuronal synapses excitatory or inhibitory signalling. Administration of THC and the endocannabinoids AEA and 2-AG inhibits the induction of long-term potentiation (LTP) in the hippocampus which regulates learning and memory (Stella, Schweitzer, & Plomelli, 1997). Furthermore, AEA and 2-Arachadonyl Glycerol (2-AG) also inhibit long term depression (LTD)

in cerebellum and nucleus accumbens (Hoffman et al., 2003; Lévénès et al., 1998). This is due to AEA being a full agonist at CB_1 at nanomolar affinities (Deutsch & Chin, 1993; P. Reggio, 2010) and 2-AG having nanomolar affinities for both CB_1 and CB_2 but much higher agonist activity at CB_2 (Gonsiorek et al., 2000).

1.4.2 Phytocannabinoid Activity

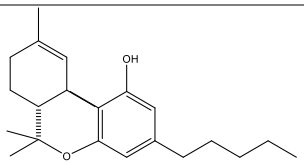
THC and CBD essentially mimic the actions of the endocannabinoids due to their similar structure and polarity (**Table 1-1**). THC alone has nanomolar affinity for CB_1 and CB_2 receptors as a partial agonist (Friedman & Devinsky, 2015; Iwamura et al., 2001). CB_1 and CB_2 are GPCRs coupled to a $G_{\alpha i/o}$ subunits which upon receptor activation will inhibit adenylyl cyclase (AC) and reduce intracellular cAMP (Childers & Deadwyler, 1996; Slipetz et al., 1995). Ligand binding at CB receptors stimulates the β-Arrestin recruitment cascade that deactivates and internalizes the receptor (Ibsen et al., 2019; Nogueras-Ortiz & Yudowski, 2016; Tham et al., 2019). More forward, cannabidiol (CBD) has been shown to be a negative allosteric modulator (NAM) of CB_1 receptors (Laprairie et al., 2015; A Thomas et al., 2007) but will also bind CB_1 and CB_2 receptors at micromolar affinities (Devane et al., 1988). There is still no definitive mechanism of CBD due to its affinity for multiple binding sites, on multiple receptors at multiple cell types (Elsaid & Le Foll, 2019).

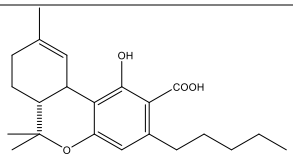
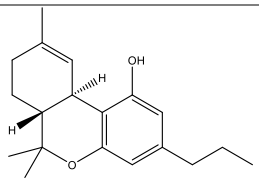
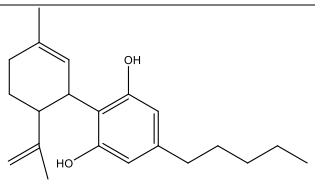
1.4.3 Phytocannabinoid Activity at Other Receptors

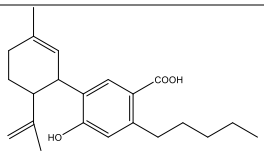
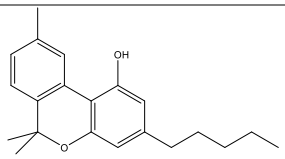
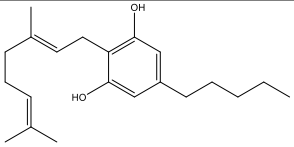
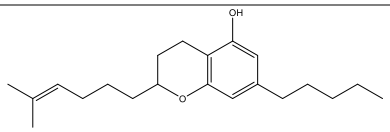
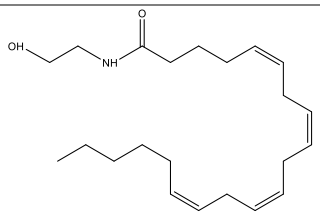
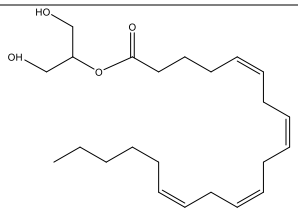
Cannabinoid receptor activity of synthetic, plant and endogenous cannabinoids has been studied and reviewed (Pertwee, 2006, 2008; P. Reggio, 2010; Soethoudt et al., 2017; Zou & Kumar, 2018). Many CB receptor ligands, including phytocannabinoids, are active at other receptors including GPR55 (Kapur et al., 2009), GPR119 (Overton et al., 2006), $5HTA_1$ (Cascio,

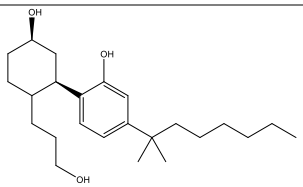
Gauson, Stevenson, Ross, & Pertwee, 2010; E. B. Russo, Burnett, Hall, & Parker, 2005), FAAH, MAGL (Di Marzo, 2006), COX-1 and COX-2 (Ruhaak et al., 2011) and p450 CYP liver enzymes (Jaeger et al., 1996; R. Jiang et al., 2013), which are not called 'cannabinoid' receptors but contribute to endocannabinoid function and cannabinoid pharmacology. Cannabinoid activity has been reported at receptors such as TRP channels (TRPV1, TRPV2, TRPM8, TRPA1) resulting in an increase of $[Ca^{2+}]_i$ (Bisogno et al., 2001; De Petrocellis et al., 2011) and reduction neuronal excitability (Lévénès et al., 1998; Whalley et al., 2004). The combination of GPCR dependent adenylyl cyclase inhibition and inhibition of voltage gated Ca^{2+} is the mechanism in which endocannabinoids suppress signalling in synaptic terminals (Busquets-Garcia et al., 2018; Zou & Kumar, 2018) and release of neurotransmitters across the synaptic cleft. CB_1 receptors are localized to neuron terminals and mediate the release of acetylcholine, noradrenaline, dopamine, 5-hydroxytryptamine (5-HT), GABA, glutamate, D-aspartate and cholecystokinin (Freeman et al., 2019). The result is regulation of postsynaptic neuron excitability (Lévénès et al., 1998) and what is termed 'depolarization induced suppression of inhibition (DSI) and depolarization induced suppression of excitation (DSE) (Diana & Marty, 2004).

Table 1-1: Summary of Cannabinoids and efficacies at CB_1 and CB_2 receptors

Name	Structure	Receptor	Activity	Publication
Δ^9-THC		CB1	Partial Agonism K_i CP-55,950 human CB1 CHO cells: 5.05 nM	(Friedman & Devinsky, 2015) (Iwamura et al., 2001)
			Receptor agonist, 15 µM % cAMP reduction EC_{50}	(Yang et al., 2020)
		CB2	Partial Agonism K_i CP-55,940 human CB2 CHO cells: 3.13 nm	(Friedman & Devinsky, 2015) (Iwamura et al., 2001)

			Receptor agonist, >10 000 nM % cAMP reduction EC_{50}	(Yang et al., 2020)
Δ^9-THCA		CB1	Receptor agonist, 1800 nM % cAMP reduction EC_{50}	(Yang et al., 2020)
		CB2	Receptor agonist, 30 000 nM % cAMP reduction EC_{50}	(Yang et al., 2020)
Δ^9-THCV		CB1	Competitive receptor antagonist K_i = 75.4 nM	(Adele Thomas et al., 2005)
		CB2	Competitive receptor antagonist K_i = 62.8 nM	(Adele Thomas et al., 2005)
CBD		CB1	Inverse Agonist by ability to antagonize (R-(þ)-WIN55212) and (CP55940). Negative allosteric modulator K_i = 4900 nM K_i = 1690 nM (CP-55940) K_i = >30000 nM (WIN-55,212-2) Receptor agonist, >10 000 nM % cAMP reduction EC_{50}	(A Thomas et al., 2007) (R. B. Laprairie et al., 2015) (Navarro et al., 2018) (Yang et al., 2020)
		CB2	K_i= 4.2 uM on hCB_2-CHO cells (displacement of CP55490) K_i = 1714 nM (CP-55940) K_i = >4019 nM (WIN-55,212-2) Receptor agonist, >10 000 nM % cAMP reduction EC_{50}	(A Thomas et al., 2007) (Navarro et al., 2018) (Yang et al., 2020)

CBDA		CB1	Receptor agonist, 17 000 nM % cAMP reduction EC_{50}	(Yang et al., 2020)
		CB2	Receptor agonist, 17 000 nM % cAMP reduction EC_{50}	(Yang et al., 2020)
CBN		CB1	Partial Agonism K_i = 211.2 nM in COS CB1 cells. % cAMP reduction EC_{50} of 120.0 nM	(Rhee et al., 1997)
		CB2	Partial Agonism K_i = 126.4 nM in COS CB2 cells. % cAMP reduction EC_{50} = 261.2 nM	(Rhee et al., 1997)
CBG		CB1	K_i = 1045 nM (CP-55940) K_i = >30000 nM (WIN-55,212-2)	(Navarro et al., 2018)
		CB2	K_i = 1225 nM (CP-55940) K_i = 2656 nM (WIN-55,212-2)	(Navarro et al., 2018)
CBC		CB1	No Activity	(Udoh, Santiago, Devenish, McGregor, & Connor, 2019)
		CB2	Selective agonist (30 µM)	(Udoh et al., 2019)
Anandamide		CB1	Agonist in cAMP reduction at forskolin stimulated N18TG2 cells, EC_{50}= 540 nM	(Allyn C. Howlett & Mukhopadhyay, 2000)
		CB2	Agonist, affinity K_i= 795 nM (HU-210)	(Gonsiorek et al., 2000)
2-AG		CB1	Agonist effects, 170 times more present than AEA (binds just as well to CB2)	(P. Reggio, 2010) (Mechoulam et al., 1995)
		CB2	Agonism, High affinity K_i= 949 nM (HU-210)	(P. Reggio, 2010)

			Agonist, % cAMP reduction EC_{50} = 1300 nm	(Gonsiorek et al., 2000)
CP 55,940		CB1	Receptor agonist, 2.5 nM % cAMP reduction EC_{50} K_d = 2.4 nM	(citti, 2019; A Thomas et al., 2007; Yang et al., 2020)
		CB2	Receptor agonist, 2.4 nM % cAMP reduction EC_{50}	(A Thomas et al., 2007; Yang et al., 2020)

1.5 Cannabis extracts vs pure cannabinoids: is the sum equal to the parts?

Synergism from *Cannabis* extract components could be a result of binding on common or off-target (non-cannabinoid) receptors of different cell types (Laprairie & Kelly, 2013) which modulate intracellular calcium, cAMP, CB receptor translocation and other downstream signalling proteins (Laprairie et al., 2014). THC, CBD, minor cannabinoids and endocannabinoids and their activity at CB_1 and CB_2 receptors have been summarized **Table 1-1**. Synergism between THC and CBD has been demonstrated by (D. Baker et al., 2000) in which 5 mg/kg cannabis extract was more efficacious than 1 mg/kg pure THC to reduce muscle spasticity in mice over time (D. Baker et al., 2000; Williamson, 2001). *Cannabis* extracts have also been effective in the treatment of intractable neurogenic symptoms (Wade et al., 2003) and seizures (Friedman & Devinsky, 2015; Kaplan et al., 2017; Press et al., 2015). A comprehensive study by De Petrocellis et al., outlining 11 cannabinoids from botanical extracts demonstrates that components which exist in smaller concentrations such as CBG, CBGV, CBC and THCV have potent effects at TRP channels (De Petrocellis et al., 2011). Extracts or Botanical Drug Preparations (BDP) have been investigated in an antitumour study in which BDPs were more potent in producing antitumor responses *in vitro* than pure THC (Blasco-Benito et al., 2018). The superior anticancer activity of extracts was further evaluated in a murine model where a

THC dominant BDP was more effective in reducing cancer cell viability than THC alone (Scott, Dalgleish, & Liu, 2014). A recent study published in 2020 evaluated the percent reduction of intracellular cAMP of extracted Cannabis at CB_1 and CB_2 receptors with varying ratios of THC/CBD. It was discovered that there was no significant relationship between phytocannabinoid content and CB_1 activity using a partial least sum of squares regression analysis but correlation was found at CB_2 with an increasing THC:CBD ratio in extract (Yang et al., 2020). Another *in vitro* study found a 1:1 mix of pure CBD and THC was more effective than CBD or THC alone but less effective than a 1:1 CBD/THC extract in increasing intracellular calcium concentrations in neurone and glial culture (Ryan, Drysdale, Pertwee, & Platt, 2006).

Results such as this led to the clinical development of Sativex® (Scott et al., 2014) from GW Pharmaceuticals which is a 1:1 concentration of THC:CBD (27mg/ml THC and 25 mg/ml CBD) while Epidiolex® is a highly purified CBD tincture formulation. These products effectively reduce seizure frequency and severity in people with Dravet and Lennox- Gastaut syndrome. Reviews of CBD and THC interaction suggest that CBD can modulate the effects of THC by decreasing the harmful effects and increasing clinical efficacy, which is likely related to CBDs interaction at the allosteric site of CB_1 lowering the affinity of THC and endocannabinoids at the receptor (Bonn-Miller, ElSohly, Loflin, Chandra, & Vandrey, 2018; Freeman et al., 2019). CBD was also found to antagonize FAAH increasing available AEA (Watanabe et al., 1998).Conversely, THC alone depresses evoked depolarizing post synaptic potentials (PSPs) in rat olfactory cortex neurones while the THC-plant extract, as well as a THC-free extract showed potentiated evoked PSPs (Whalley et al., 2004) suggesting that compounds beyond THC in extracts are elliciting effects.

Allosteric pharmacology at CB_1 and CB_2 has been established (Pertwee, 2006; Tham et al., 2019). Allosteric ligands that change the affinity (strength of ligand docking in the orthosteric site), but not the efficacy (strength or resulting signalling), are interesting because of their ability to increase or decrease the activity of endogenous ligands (Price et al., 2005). Strong evidence of negative allosteric modulation at CB_1 is demonstrated by radioligand displacement assay (Price et al., 2005) with synthetic cannabinoids as well as with CBD (R. B. Laprairie et al., 2015). Reduction of agonist E_{max} or maximal effect fit the model of allosteric modulation, whereas dextral shifts in concentration-response curves with a CB_1 inhibitor present would be indicative of competitive inhibition (Atwood et al., 2012). Fitting concentration-response data to a log-logistic model allows for both maximum agonist effect (E_{max}) and EC_{50} values to be compared.

Most studies in the field of cannabis pharmacology are done on pure or synthetic cannabinoids at specific cellular targets (De Petrocellis & Di Marzo, 2010; A. C. Howlett et al., 2002; Lucas, Galettis, & Schneider, 2018a; Pertwee, 2006, 2008). Although research on complex crude or formulated cannabis products is becoming more common, the majority of pre-clinical data are derived from individual compounds, mixtures of two compounds, or a single (or small number) of products, extracts, often poorly characterized beyond THC/CBD content. In the real world, however, cannabis industry sells – and users consume – cannabis flower, extracts and concentrates, and edibles that, whether smoked, vaporized, or ingested, deliver a complex (and variable) mixture of compounds, not individual cannabinoids. Once further, the terpene profile that gives cannabis its diversity of aromas also has potential bioactivity and interaction with cannabinoids. Recently (-)-β-Caryophyllene, a known cannabis terpene, has been shown to be a CB_2 selective receptor agonist (Alberti et al., 2017). β-Caryophyllene was identified as

being neuroprotective and BDPs with β-Caryophyllene content along with THC were more effective than THC alone in the reduction of breast cancer tumors (Blasco-Benito et al., 2018). Terpenes studied alone *in vitro* and *in vivo* showed cannabimimetic activity namely α-humulene, geraniol, linalool and β-pinene. These effects were additive with WIN55,212 at CB_1 receptors *in vitro* suggestive of a terpene 'entourage' (LaVigne et al., 2021). This and other bioactive terpenoids coupled with cannabinoids have been previously reviewed (E. B. Russo, 2011).

1.6 Research Question and Study Objectives

Given the tremendous diversity of drug-type cannabis products available to consumers, the variability of cannabis phytochemistry, the lack of research on complex crude extracts, and the potential for poly-chemical interactions, my overarching research question is, *are all varieties of THC-dominant cannabis the same?* Investigating how THC-dominant cannabis varieties are related (or differ) at the level of the phytochemistry and bioactivity, my thesis encompasses five primary research objectives:

Objective #1: Characterize and compare the phytochemistry of different THC-dominant cannabis samples using HPLC-DAD and GC-FID for cannabinoid and terpene profiling as well as UPLC-HRMS for untargeted metabolomics.

Objective #2: Employ multi- and univariate analysis techniques on HPLC-DAD quantification data as well as untargeted HRMS data to analyse *Indica-Sativa-Hybrid* and terpene profile classification strategies

Hypothesis 1: THC-dominant strains cannot be distinguished by classical *Sativa*, *Indica* and *Hybrid* naming convention

Prediction 1: Indica, Sativa and *Hybrid* strains will not cluster together respectively in principal component analysis of cannabinoid, terpene, cannabinoid + terpene and metabolomic data.

Hypothesis 2: Strains will follow an established "terpene profile" clustering scheme in multivariate analysis (1) High β-Myrcene/pinene, (2) High terpinolene and (3) High β-Caryophyllene /Limonene

Prediction 1: Multi and univariate analysis of quantified cannabinoid and terpene data will reiterate already established dominant terpene profile classifications

Prediction 2: The same groupings based on terpene profile will be discernible in metabolomic analysis

Hypothesis 3: Strains will exhibit low fidelity to strain name based on metabolomic data

Prediction 1: Strains will group poorly within clades using hierarchical cluster analysis of scaled metabolomic data

Prediction 2: Strains will not cluster exclusively with samples of the same strain name in multivariate analysis of metabolomic data

Objective #3: To investigate if THC content predicts the cytotoxicity of THC-dominant cannabis extracts, I evaluated the *in vitro* cytotoxicity of pure THC and 6 extracts of THC-dominant strains in human bronchial epithelial cells (BEAS-2B).

Hypothesis1: Cytotoxicity of plant extracts will be greater than that of pure THC (on a weight-by-weight basis)

Prediction: TC_{50} values generated by extract concentration-response curves will be lower than those predicted by the concentration-response of pure THC

Hypothesis 2: THC will be more toxic than CBD

Prediction: THC concentration-response curves will generate TC_{50} values below that of CBD

Objective #4: To investigate if THC content predicts the CB_1 agonist activity of cannabis extracts, I evaluated *in vitro* activation of CB_1 by pure THC and six extracts of THC-dominant strains in human recombinant CB_1-HEK293 cells.

Hypothesis 1: Pure THC will be more potent than crude extracts

Prediction 1: THC concentration-response curves will yield lower EC_{50} values than that of heated extracts in cAMP assay

Prediction 2: THC concentration-response curves will be more effective than crude extracts in reducing intracellular cAMP (increased E_{max})

Prediction 3: Hemp (low-THC) extracts and pure CBD will not be effective in reducing intracellular cAMP

Objective #5: Evaluate intracellular cAMP accumulation (receptor efficacy) of a heated extract spiked with CBD to obtain 100:1, 10:1 and 1:1 THC/CBD ratio vs pure compounds of the same ratio

Hypothesis: Pure compounds will be more efficacious than extract preparations

Prediction: Extract constituents will have shifted concentration-response curves to the right (increased EC_{50}) and potential interaction of the constituents at the allosteric binding site will reduce E_{max}.

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Chapter 2: Multivariate Analysis of High THC strains of *Cannabis*

2.1 Introduction

The terms *indica* and *sativa* are the two main designations commonly used by consumers and industry to describe the lineage, pedigree and/or appearance of high THC cannabis strains (Sawler et al., 2015). However, as there is only a weak correlation between cannabis genes and reported lineage groupings (Gilmore et al., 2003; Sawler et al., 2015), these terms often do not accurately reflect the originally described botanical subspecies *indica* and *sativa*. Recent work by Jikomes et al., 2021 analyzed the chemical profiles of ~90 000 commercial *Cannabis* samples of different varieties, binning them into 3 chemotypes with 96.5% falling into a THC-dominant group, 1.4% into CBD-dominant and 2.2% THC:CBD balanced. Supported by multivariate analyses, the authors proceed to describe a classification method that further separates THC-dominant varieties into 3 categories based on dominant terpene content: high caryophyllene-limonene, high myrcene-pinene and high terpinolene-myrcene. Studies on terpenoid chemoprofiles have found that THC-dominant cannabis samples that are <0.5 CBDA:THCA ratio can also be organized into 3 major groups based on terpene content alone, these being a β-myrcene dominant, γ-terpinene/ terpinolene dominant and a β-caryophyllene/limonene dominant (Orser et a., 2018; Reimann-Philipp et al., 2020). Despite the apparent emergence of different chemotypes across large phytochemical datasets of different cannabis samples, neither varying ratios of THC:CBD nor terpene profiles alone have proven effective in distinguishing clear

lineage groups or strains of commercial cannabisas there is high inter- and intra-strain variation due to genetics, epigenetics, growth conditions and curing process (Bonn-Miller et al., 2018; Citti et al., 2020; Fischedick J, 2015; Jikomes et al., 2021; Orser et al., 2018).

Such chemotypic variability among strains and products marketed under the same lineage or strain name may result in variability in bioactivity. Product consistency is important to ensure predictable effects for consumers. Ratios of THC and CBD at the level of cannabinoid receptors ($CB_{1/2}$) have been studied and the biopharmacodynamics may lead to different downstream effects within the cell (Laprairie et al., 2015; Laprairie et al., 2014; Nogueras-Ortiz & Yudowski, 2016; H. Reggio, Elphick, & Egertova, 2013; Tham et al., 2019; Van Der Lee et al., 2009; Yang et al., 2020).

Consumers choose strains based on personal and anecdotal experience in combination with recognition of familiar commercial names and aroma. There is a huge diversity of THC-dominant strains however little fidelity of THC/CBD concentrations to strain name (Reimann-Philipp et al., 2020). This becomes an issue for industry and regulation as naming convention is not a robust predictor of THC or CBD content and therefore intoxicating effects. The current solution in Canada is to mandate labelling of THC and CBD content of all cannabis products, but if the vast majority are THC dominant (e.g >10:1 THC:CBD), these details still leave consumers to rely on marketed names and related information with limited insight into chemical similarity to other products.

Research on phytochemical relationships within and among drug-type cannabis varieties has focused on cannabinoid content and ratios, primarily THC and CBD but also minor cannabinoids, as well as terpene profiles. Prior to legalization, such studies were limited to a small number of compounds profiled and compared in a small number of varieties/ samples

which typically could only be confiscated material (ElSohly & Slade, 2005). With hundreds of cannabinoids and terpenes previously reported in cannabis (Brenneisen, 2007; Russo, 2011), improved methods and technologies for analysis (Aliferis & Bernard-Perron, 2020), and more access to different varieties thanks to regulations, researchers can now investigate the phytochemical diversity of cannabis at larger scales, leading to large data sets and more sophisticated statistics, notably multivariate analysis.

Some approaches in multivariate analysis involve grouping samples in hierarchical cluster analysis using unweighted pair group method with arithmetic mean (UPGMA) and Principal component analysis (PCA) which is an unsupervised clustering method which reduces dimensionality of multivariate data while preserving most of the variance based on phytochemicals. Unsupervised multivariate analysis of cannabinoid and terpene are not able to group THC-dominant strains into their respective strain names (Gilmore et al., 2003). To potentially capture more explained variance, extracted plant material processed using UPLC-HRMS yields a large cloud of mass features to where PCA may allow for parsing out data structure from the noise. ^{1}H NMR data has been used to discriminate *C. sativa* cultivars based on cannabinoid content as well as proteins, lipids and fats in different plant tissues (Choi et al., 2004). Metabolomic analysis of *Cannabis indica* terpene content also showed clustering based on unique terpene fingerprint with 41.6% of variance explained (Fischedick, Hazekamp, Erkelens, Choi, & Verpoorte, 2010). Once further, total cannabinoid content of cannabis samples was shown to be positively correlated with total terpene content and specific cannabinoids and terpenes can be correlated as evidenced by heat mapping analyses (Jikomes et al., 2021). Hierarchical clustering analysis of a sample of high THC varieties of *Cannabis* revealed that these strains cluster into a 'Δ^{9}-THCA' group and a 'CBDA' group demonstrating

differences between THCA dominant and 'balanced' CBDA groupings in targeted analysis. This is partially due to CBN and CBT type cannabinoids accumulating into the THC/A group and CBND and CBE into the CBDA group (the CBDA groups has a high CBDA:THCA ratio but is still high THC/A) (Berman et al., 2018). The power of metabolomic analytics has been demonstrated in separating out high THC strains into more advanced groupings based on underlying chemical diversity and is an important tool in building a chemotaxonomy of *Cannabis* in the future. Analyzing more mass features may better validate the classification schemes used in the past or better define a new classification system,

Considering the established variation and inconsistency in the phytochemistry of high-THC recreational cannabis strains the objective for this chapter was to characterize and compare the cannabinoid, terpene, and untargeted metabolomic profile of different THC-dominant cannabis samples to investigate phytochemical relationships within and between lineages (*indica, sativa, hybrid*), chemotypes (terpene dominance), and strains.

Unlike recent similar studies based on large heterogeneous datasets generated from samples grown and analysed in numerous different production facilities and laboratories, this study is unique in the fact that all plant samples were acquired from a single licenced grower where all plants and inflorescences were, respectively, grown under the same conditions and harvested then processed using standardized methods. All extraction and analyses were similarly carried out in the same laboratory using the same equipment and analytical method. Each of 113 high-THC plant samples representing 33 different strains as well as *sativa, indica,* and *hybrid* types was harvested from a mature plant grown from seed (for example "AlienOG" seeds were grown then five separate (female) "AlienOG" plants and were sampled by a licenced grower (Flowr, BC). Ethanolic extracts of dried inflorescence were analysed for cannabinoid and

terpene content, as well as untargeted metabolomic profiling, followed by multivariate approaches to determine how phytochemical variability relates to lineage groups, chemotype, or strain name.

2.1.2 Study Objectives and Hypotheses

Objective #1: Characterize and compare the phytochemistry of different THC-dominant cannabis samples using HPLC-DAD and GC-FID for cannabinoid and terpene profiling as well as UPLC-HRMS for untargeted metabolomics.

Objective #2: Employ multi- and univariate analysis techniques on HPLC-DAD quantification data as well as untargeted HRMS data to analyse *Indica-Sativa-Hybrid* and terpene profile classification strategies

Hypothesis 1: THC-dominant strains cannot be distinguished by classical *Sativa*, *Indica* and *Hybrid* naming convention

Prediction 1: Indica, Sativa and *Hybrid* strains will not cluster together respectively in principal component analysis of cannabinoid, terpene, cannabinoid + terpene and metabolomic data.

Hypothesis 2: Strains will follow an established "terpene profile" clustering scheme in multivariate analysis (1) High β-Myrcene/pinene, (2) High terpinolene and (3) High β-Caryophyllene /Limonene

Prediction 1: Multi and univariate analysis of quantified cannabinoid and terpene data will reiterate already established dominant terpene profile classifications

Prediction 2: The same groupings based on terpene profile will be discernible in metabolomic analysis

Hypothesis 3: Strains will exhibit low fidelity to strain name based on metabolomic data

Prediction 1: Strains will group poorly within clades using hierarchical cluster analysis of scaled metabolomic data

Prediction 2: Strains will not cluster exclusively with samples of the same strain name in multivariate analysis of metabolomic data

2.2 Methods

2.2.1 Sample collection and preparation

High THC variety *Cannabis* samples were provided by a licenced cannabis producer (n=113, Flowr, British Columbia). Each of these samples were grown from seed and are therefore non-clonal individuals. The company used a dedicated grow room for experimental plants and, following proprietary growth, harvesting, and processing practices, shipped dried inflorescence samples to the university of Ottawa. The samples consisted of strains with conventional names, such as "Chem 4 OG", and lineage groups, as either an *indica, sativa* or hybrid (a cross of Indica and Sativa). Samples were given a unique name based on internal ID and strain name. In addition to strain names provided by the licensed producer, each sample was assigned a lineage group (*sativa, indica* or hybrid) based on available information collected from Leafly.com, an open access industry web resource for cannabis users and education. Independent of strain name, each sample was also designated into one of four groups based on observed terpene dominance (as described and represented in Results, **Table 2-1**)

Table 2-1: Dominant terpene profiles as determined by GC-FID data and multivariate analysis.

Cluster	Dominant Terpenes	Colour

1	β-Myrcene-α/β pinene	Black
2	Terpinolene-menthol	Green
3	β-Caryophyllene-Limonene	Red
4	Other (a different dominant terpene)	Blue

Extraction technique was adapted from (Lodhi et al., 1994; Elizabeth M Mudge et al., 2017) in which 99% ethanol at a ratio of 80 ml to 2 g dried inflorescence was used. Weighed inflorescence samples were dehydrated using liquid nitrogen, re-weighed, then powdered in clay mortar and pestle and washed out with 40 ml of 99% ethanol. These were then loaded into 50 ml polypropylene syringes with 0.22 µm filters and injected into round bottom flasks. The plant material was then extracted a second time with another 40mls of ethanol by the same process. 1 ml of this taken from the resulting 80 ml and filtered again at 0.22 µm and put in HPLC vials for HPLC-DAD cannabinoid quantification. The remainder was rotary evaporated (Yamato RE 500) down to ~10 mL in a 40°C water bath with -4°C ethanol condenser. The solution was then speed vacuumed for 24hrs and later stored in polypropylene falcon tubes and frozen at -20°C. This extraction removes any water and fibrous plant material and therefore concentrates some cannabinoids while other more volatile compounds may be lost.

2.2.2 Cannabinoid Quantification by HPLC-DAD

Prepared extracts were analyzed on an Agilent 1100 series HPLC system with a Quaternary pump (G1311A), a Solvent degasser (G1322A), a Column oven (G1316A), and a Photodiode array detector (G1315A). The separation of the target compounds was carried out on with a Kinetex C18 column (150 x 2.1 mm, 2.6 µm particle size, Phenomenex, Mississauga, Ontario, Canada). Mobile phase was H_2O (A) and methanol (B) with 0.1% trifluoroacetic acid in both. The gradient elution method was initialed with 68 % B and gradually change to 85% B in

13 min and maintained at this ratio for 7 min. At 20.1 mins, 100 % B was used to flush the column for 5 min and system was re-equilibrate for 8 mins before the next injection. The flow rate was set at 0.25 ml/min and column oven was set at 60ºC. DAD was set at wavelength 210nm to monitor CBD, THCV, CBG, CBDA; 230nm for CBDV and THC; 260nm for CBNA and CBCA; 270nm for CBDVA, canflavin A, CBGA, CBN, THCVA and CBC; 320nm for THCA. Please note that due to the dominant concentration of THCA in plant extract a less absorbing 320nm was selected to monitor this compound to avoid oversaturation of the detector.

ChemStation software (version B 3.02) was used to analyze the data. Linear calibration curves were generated by injecting each pure compound at different concentrations. Compounds were quantified based on the area under the peak that bracketed the response obtained from the calibration range of each compound.

These data were collected in a matrix and quantified in mg/g (%w/w) inflorescence. For total cannabinoid acid precursor amounts were added to neutral cannabinoid while considering molecular weight (Total THC = (0.877)THCA + THC).

Table 2-2: List of targeted cannabinoids and terpenes which were standardized in HPLC-DAD and GC-FID calibration curves

Cannabinoids	Terpenes
THCA	α-pinene
CBDA	β-pinene
CBGA	Myrcene
CBNA	Limonene
CBCA	Ocimene

THCVA	Terpinolene
CBDVA	Linalool
THC	Fenchol
CBD	Borneol
CBG	Menthol
CBN	Terpineol
CBC	Citronellol
THCV	Geraniol
CBDV	Geranyl acetate
	Cedrene
	β-caryophyllene
	α-humulene
	cis-nerolidol
	trans-nerolidol

2.2.3 Terpene Quantification by GC-FID

Terpenes were quantified by an adapted method described by Supelco in their data packet. 2.5µL cannabis extract samples were injected on a GC instrument (Agilent 6890N Network GC) with an autosampler (7683 series) and dual injectors (7683B series) fitted with an FID detector and SLB-5MS GC Column (30m x 0.25mm x 0.25µm) at a concentration of 1 mg/mL in 99% ethanol. The injector temperature was set to 230°C in splitless injection. The carrier gas was hydrogen set to a flow rate of 1.4 mL/min, and the oven was programmed for 60°C for 3.5 min, 3.5°C/min to 155°C, 30°C/min to 300°C with a post run at 340°C for 10 minutes. The needle was programmed to rinse before and after injection 3 times, at 1.5 µL volume in methanol.

Terpene standards were obtained from Sigma-Aldrich and calibration curves were prepared in 99% ethanol solution at concentrations of 250, 200, 150, 100 and 50 ng/mL (**Table 2-2**). Calibration curves were obtained each day with correlation coefficients greater than 0.99. The data were processed on ChemStation using 0.25% error for peak detection. The limit of detection (LOD) and the limit of quantification (LOQ) were evaluated as per ICH guideline

Q2(R1) (Branch, 2005) based on standard deviation of the response and the slope. A low concentration cannabinoid mix was injected seven times to calculate the standard deviation (σ). The LOD was determined to be 3.3 σ /S where S is the slope of calibration curve, and LOQ was determined to be 10 σ/S.

2.2.4 Metabolomics

The strains selected for study were represented by >2 unique strain IDs, for example if "Chem 4 OG" had only 2 strain IDs in our collection, it was excluded. Samples were prepared for UPLC-HRMS by first weighing out triplicates of dried extract at 5mgs x 3 in polypropylene Eppendorf tubes for each unique strain ID. These were then dissolved in MS grade MeOH (Fisher) followed by vortexing and sonication at 37°C for 10 minutes. A 1:10 dilution in LC grade methanol was then performed to yield 500 µg/ml dilutions for each sample. Samples were filtered by 0.22 µm PTFE syringe filter into amber HPLC vials and kept at -20°C until analysis. Standards for CBDV, THCV, CBN, CBG, CBC, CBD, Δ^9THC, CBDVA, THCVA, CBNA, CBGA, CBCA, CBDA and Δ^9THCA were prepared at 100 µg/ml. Neutral and acid cannabinoids were dissolved in 200 µL of MS grade MeOH and 200 µL acetonitrile, respectively. Methanol blanks were processed as per initial extraction procedure (Extraction Blanks) and methanol from metabolomics (Method blanks). In total 332 samples were sent for processing. A subset of samples were then chosen for this analysis. Extracts in triplicate and standards were analysed in random injection order with MeOH blanks ran every 8 samples on the UPLC-HRMS. Following data collection, the average was taken of the 3 triplicates for each returned mass feature. The UPLC column is a Phenomenex Kinetex 1.7 µm C18 100Å, 50 mm x

2.1 mm (part number 00B-4475-AN) with C18 guard cartridge (part number Aj0-8782), oven temp 30°C at a flow rate of 0.35 mL/min. HRMS is an Orbitrap XL positive mode electrospray ionization (ESI), resolution = 30000, scan range = 50 – 2000 m/z, MS runtime = 10 minutes.

Raw data files were provided by Amanda Sproule at Agriculture and Agri-Foods Canada which were then converted to a NetCDF file format using Mzmine 2.53 and reimported. Mass detection of raw data was done by local minima search. For the entirety of the data preprocessing an m/z tolerance of 0.005 was set. ADAP chromatogram builder was used to generate chromatograms. The minimum threshold was set to 1.0 E +06 and minimum peak intensity to 2.0 E +06. Chromatogram deconvolution consisted of visualizing individual spectral scans to capture unique peaks, changing the chromatographic threshold to 20%, minimum RT range to 0.05 min, minimum relative height of 15%, minimum absolute height to 3.0 E +06, and a minimum ratio of peak top edge to 1. The major cannabinoids have similar monoisotopic weights and therefore peak will be grouped very close together, lowering these parameters was integral to selecting unique peaks. Isotopic peak grouper was used with a maximum charge of 1 and monotonic shape applied to peak. Join aligner allowed for buckets to be made to create a 2 dimensional aligned chromatogram and gap-filling using the peak finder function with 5.0% Intensity tolerance and 0.05 RT tolerance. The joined and gap filled chromatogram was then exported to CSV with the peak ID, retention time and m/z value. Data was transposed so that each column represented a mass feature with the naming conventions “RT_m/z”. Data pruning consisted of removing features which do not reach the minimum threshold of 2.0 E +06 in samples or are abundantly present in methanol blanks

2.2.5 Statistical Analysis

Principal component analysis (PCA) is an unsupervised clustering method which reduces dimensionality of multivariate data while preserving most of the variance. Unweighted pair group method with arithmetic mean (UPGMA) is a hierarchical cluster analysis which was performed on metabolomic data to evaluate distance connectivity of strains. Individual points in PCA appear to be independent, but strains may be represented by 3-5 samples in plots which may explain some of the resulting clustering patterns. Studying extracted plant material using LCMS yields a large cloud of mass features and PCA allows parsing out the data structure from the noise. Upon generating PCA plots, all scree plots were observed to confirm inflection occurs after PC1 and PC2. Cannabinoid, terpene, cannabinoid + terpene and metabolomic data were analyzed using an unsupervised clustering method. Either an *indica-sativa-hybrid* or terpene classification (i.e., "high β-Myrcene/pinene", "high terpinolene/menthol", "high β-caryophyllene/limonene" and "other") was applied to each strain.

Univariate analysis on cannabinoid and terpene content is used to determine the relative difference in phytochemicals between groups and further evaluate any emerging correlations in the data set. F statistics and significance can be extracted from the univariate analysis to identify differences between groups based on targeted or untargeted phytochemicals.

2.3 Results

2.3.1 Cannabinoid and Terpene Profiles

Targeted profiling of cannabinoids and terpenes in cannabis tinctures, quantified relative to reference standards, was calculated relative to dry inflorescence mass (mg/g dry weight) (**Figure 2-1**). To control for potential differential conversion of cannabinoids from acid to neutral form during processing, the detected concentrations of acids were adjusted then added to

neutral cannabinoid concentrations to generate total values for each quantified cannabinoid. Seven pairs of acid-neutral cannabinoids were quantified (THC/A, CBD/A, CGB/A, CBC/A, THCV/A, CBDV/A and CBN/A). All samples were confirmed to be THC-dominant with total THC levels ranging from 43.9-232.2 mg/g (4.3 – 23.2%) and an overall mean (±SEM) of 155.3 ± 3.8 mg/g (15.5%) across samples (n=113). Averaged across all samples the next most abundant cannabinoids were CBG (12.2 ± 0.7 mg/g), THCV (10.6 ± 0.4 mg/g), CBC (4.6 ± 0.3 mg/g) and CBD (3.4 ± 0.2 mg/g) with lower levels of CBN (0.20 ± 0.01 mg/g) and CBDV (0.05 ± 0.004 mg/g). THC to CBD ratios ranged from 13:1 to 273:1 with a mean of 72:1.

Targeted profiling of terpenes revealed much lower amounts of terpenes in dried cannabis samples. Average total terpene content across all samples was 8.51 ± 0.37 mg/g (0.9%), with values ranging from 1.68 to 23.75 mg/g (0.2 – 2.4%). The most abundant terpene, on average, was myrcene 3.17 ± 0.29 mg/g, followed by β-caryophyllene (1.39 ± 0.07 mg/g), limonene (1.14 ± 0.1 mg/g), α-pinene (0.93 ± 0.13 mg/g), β-pinene, α-humulene (0.34 ± 0.03 mg/g), nerolidol-2 (0.31 ± 0.02 mg/g) and terpinolene (0.26 ± 0.09 mg/g)

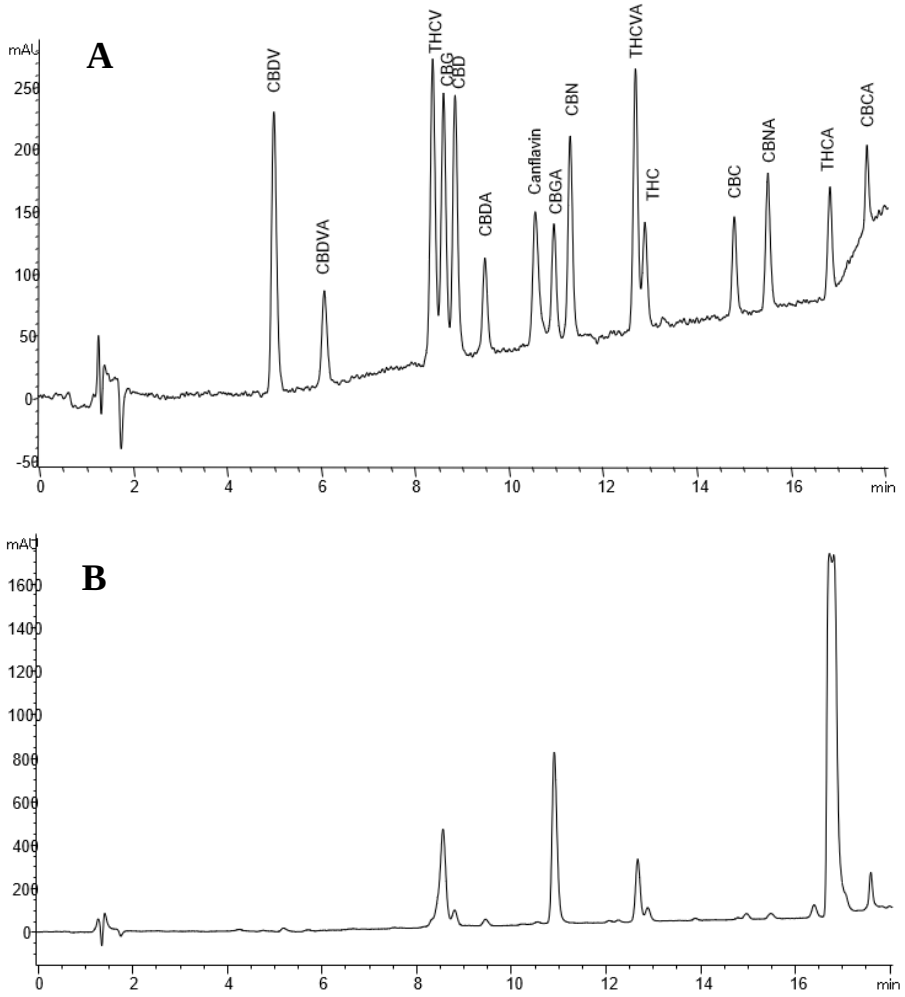


Figure 2-1: (A) Chromatograms of peak intensity (mAU) over time (min) of cannabinoid standard mix (THC/A, CBD/A, CBG/A, CBC/A, CBN/A, CBDV/ and THCVA) and **(B)** sample 125. Targeted compounds separated by HPLC and captured by DAD signal at 230 nm. Upper panel shows standard mix of 15 cannabinoids with labelled peaks. Lower panel shows a replicate from sample 125 injected at 1 mg/ml

THC dominant strains organized into "High β-myrcene - α/β-pinene" group (34.5%), the "High terpinolene – menthol" group (13.3%), the "High β-caryophyllene – Limonene" group (24%) and the "Other" group (28.2%) when appraising terpene only data. β-myrcene was very abundant in the myrcene dominant group and as a result this cluster also had the highest total terpene content when compared to the others ($p<0.001$). In this data set it appears nerolidol2

accumulates higher in the "Other" group ($p<0.05$) which is causing a negative shift in the principal component 2 of the presented terpene data set (**Figure 2-4**).

2.3.2 Multivariate Analyses of Cannabinoid and Terpene Profiles

Cannabinoid data alone in PCA was able to capture 38.1% of the variation in samples through PC1 and PC2. Terpene data alone captured 28.3 % and Terpene + Cannabinoid data only 22.8%. This is the result of the give and take relationship between data scaling and explained variation in multivariate analysis. When cannabinoid + terpene data was not scaled, 69.3% of variation was captured by PC1/ PC2 **(Appendix 1-1)**. However, since terpene content is proportionally much lower than cannabinoid content in extract, the separation between groups is mostly driven by cannabinoids CBG, THC, and CBD as evidenced by loadings plot. Here we see that the Terpinolene-Menthol group does not separate from the Myrcene-Pinene nor the Caryophyllene-Limonene groups. To potentially drive separation a partial least sum of squares supervised clustering analysis was performed (**Appendix 1-2**) where 67.1% of variation was explained in PC1 and PC2. Still poor separation of the Terpinolene-Menthol group was observed, however, and Caryophyllene-Limonene groups still appeared distinct. Non-scaled data revealed that myrcene and pinene drive variation in the data set and are correlated to CBD content just as in scaled data. To consider minor terpene content and gain stronger insight into terpene contributions of sample variability, all data were scaled moving forward, including untargeted metabolomic data.

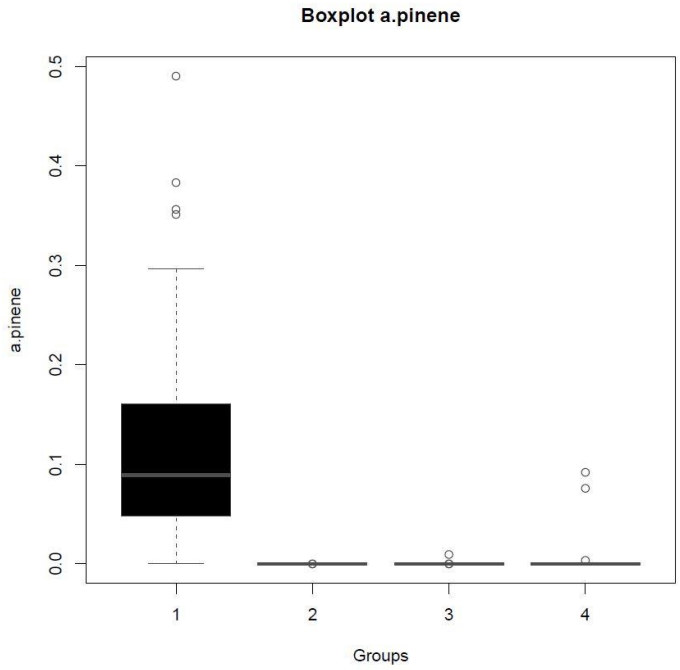
Principal Component Analysis (PCA) of cannabinoid data for 113 samples revealed an even distribution without clear clustering across PC1 and PC2, which cumulatively explained 38.1% of variation. Neither classification type (*indica, sativa, hybrid*) nor terpene-dominance appears associated with cannabinoid profile (**Figure 2-3**). Following PCA of terpene data, no

distinction (or near complete overlap) was observed between samples classified as indica, sativa or hybrid types (**Figure2-4B**) but discrete clusters were associated with terpene-dominance, particularly previously described chemotypes. The fourth, "Other", overlaps with both High β-myrcene – α/β pinene and β-caryophyllene – limonene groups but not the High terpinolene-menthol group (**Figure2-4C**).

When terpene and cannabinoid data were combined strains were again classified by either *indica, sativa* or hybrid with no obvious clustering pattern (**Figure 2-5B**). Univariate analysis revealed that, based on combined cannabinoid-terpene clustering data, *indica, sativa, hybrid* classification was insufficient in defining differences in plant terpene and cannabinoid content as no significant differences were seen across groups, for each quantified cannabinoid and terpene, no significant difference was seen between groups (data not shown). However, when dominant terpene classification was applied, still without supervision (PCA), boundaries between three groups appear more confident in the score plot (**Figure 2-5C**). Univariate analysis confirms that the dominant terpenes are in fact significantly higher in their designated groups (**Figure 2-2**). CBD was also the only cannabinoid that was significantly higher in one of the terpene groups. The high β-myrcene – pinene group had elevated CBD ($p<0.001$) which is confirmed in loadings plot as CBD and myrcene have very similar PC1 and PC2 values.

The observed ratios of THC:CBD ranged from 13:1 to 273:1 over the entire data set and THC:CBD ratio was not significantly different between *indica*, *sativa* and *hybrids*. When terpene profile classes were compared, strains in the high caryophyllene-limonene class had higher THC:CBD ratios as compared to all other groups using one way ANOVA and Tukey's HSD *post hoc* test ($p<0.05$) (**Figure 2-7)**. High β-myrcene/pinene groups have proportionately

higher CBD content which is driving this difference as there is no significant difference between THC content across these groupings (**Figure 2-6**).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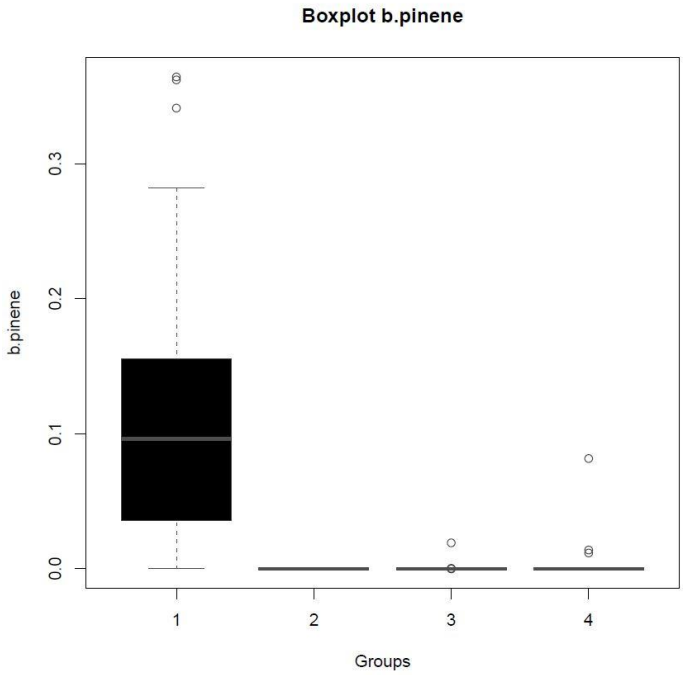
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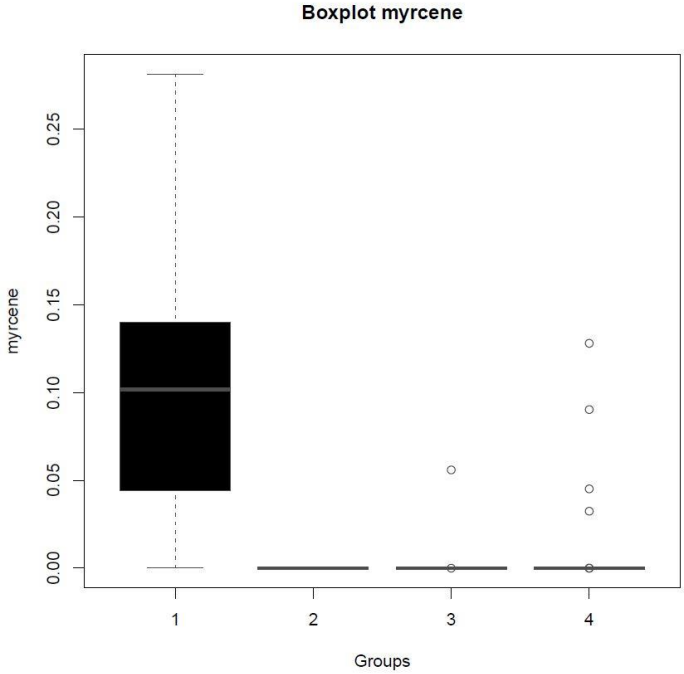
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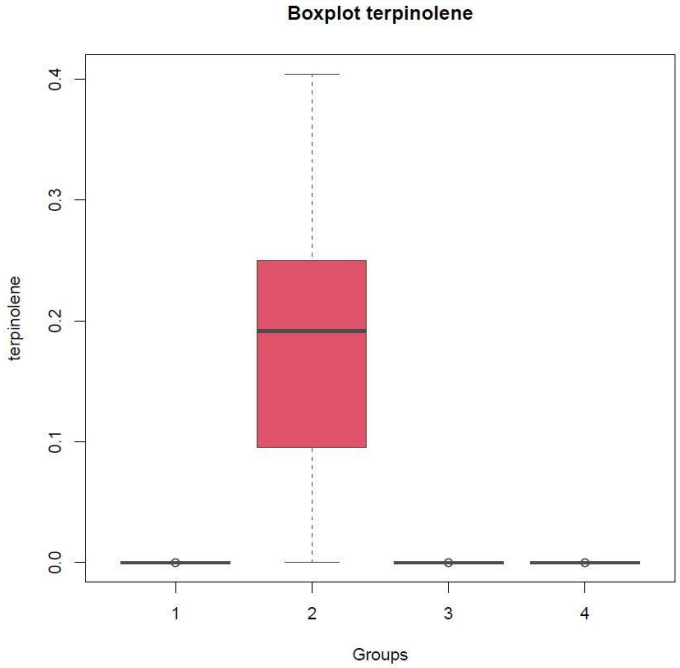
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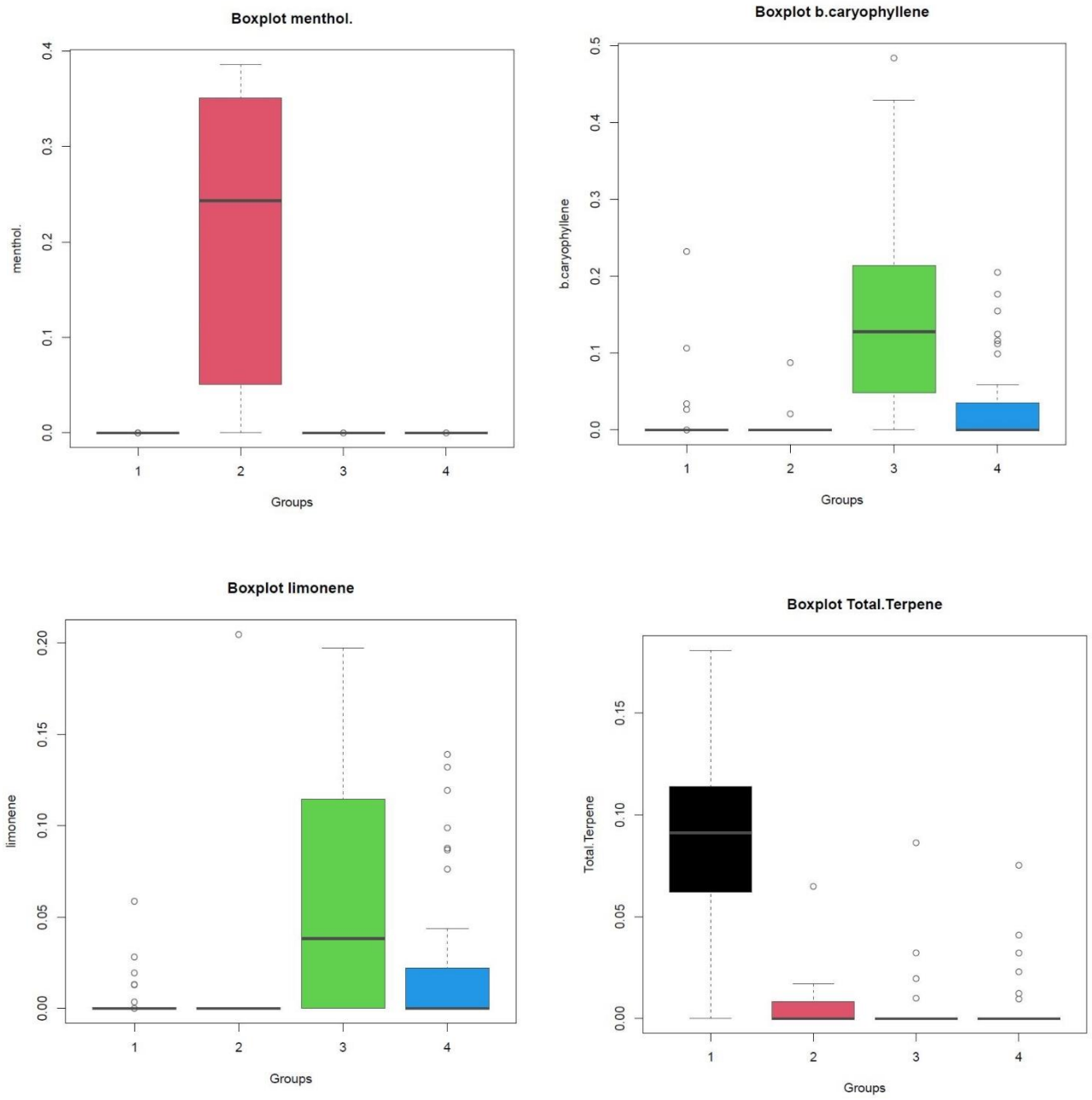


Figure 2-2: Boxplots representing scaled terpene content as defined univariate analysis of combined cannabinoid and terpene data. Colours represent groups of "High myrcene – pinene" (Black), "High Terpinoline-menthol (Red), "High β-Caryophyllene-Limonene (Green) and "Others" (Blue). For each indicated terpene, the dominant group has significantly higher quantity of terpene ($p<0.01$)

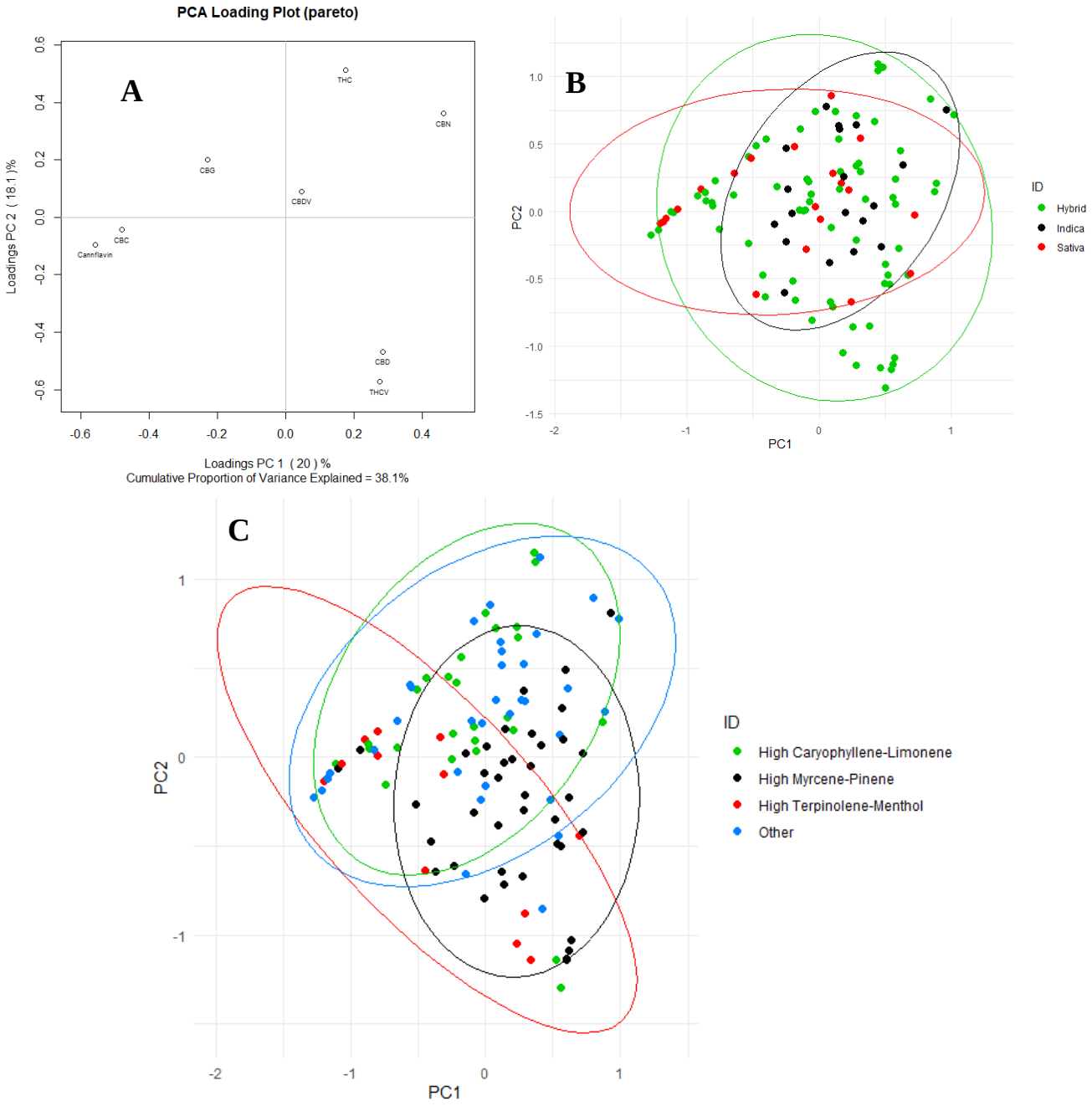


Figure 2-3: Loadings and Principal Component Analysis (PCA) plots of 113 high THC variety Cannabis extracts based on quantified cannabinoid profile. Extracts were quantified using HPLC-DAD and analytical standard calibration curves. Data was pareto scaled in R and unsupervised clustering performed. PCA plot with colours representing Sativa, **Indica**, and Hybrid (B). PCA with colours colours representing "High myrcene – pinene" (Black), "High Terpinolene-menthol (Red), "High β-Caryophyllene-Limonene (Green) and "Others" (Blue). Ellipses represent 95% confidence interval (C).

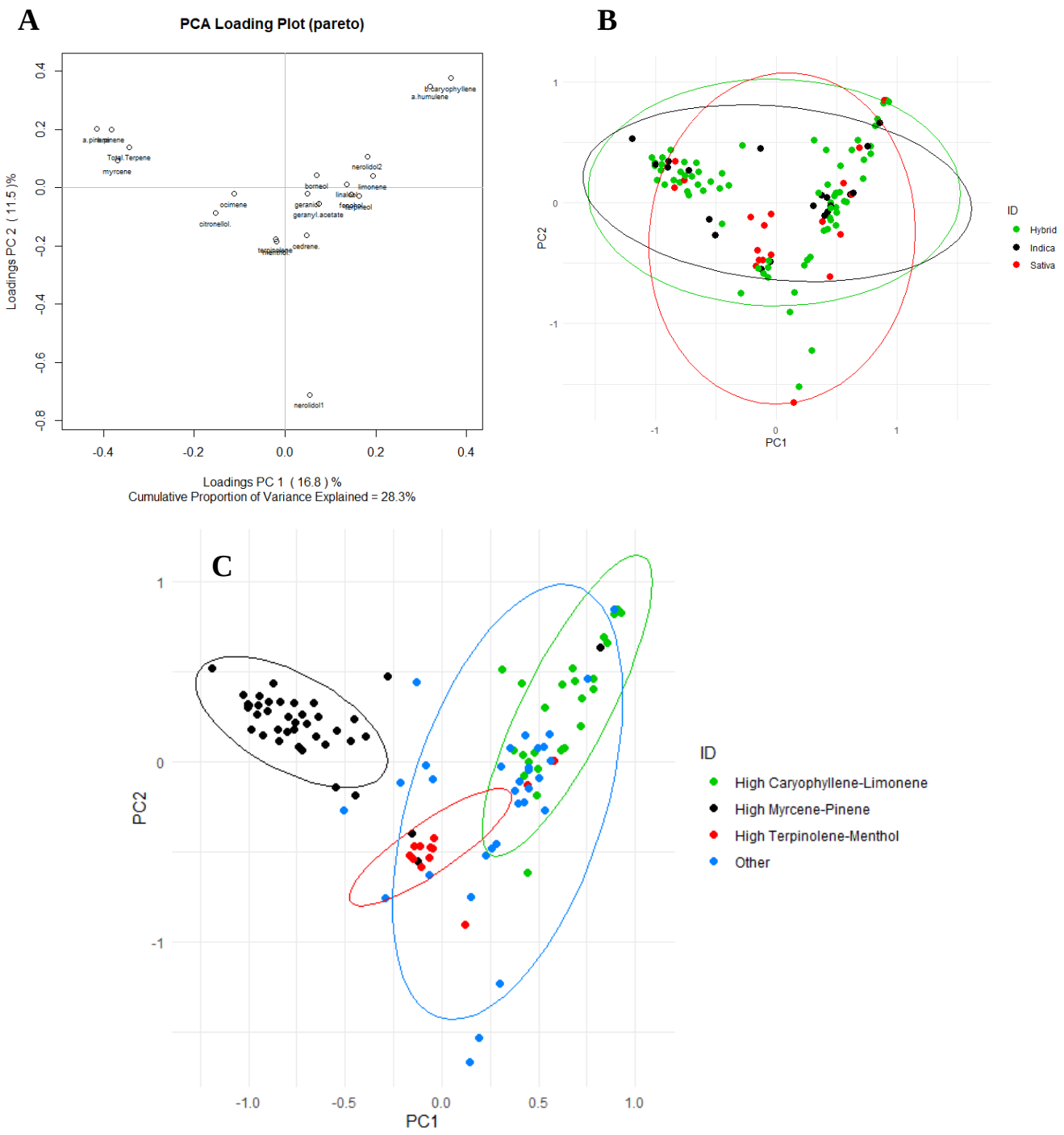


Figure 2-4: Loadings and Principal Component Analysis (PCA) plots of 113 high THC variety cannabis extracts based on quantified terpene profile. Extracts were quantified using GC-FID and analytical standard calibration curves. Data was pareto scaled in R and unsupervised clustering performed. PCA plot with colours representing Sativa, **Indica**, and Hybrid (B). PCA with colours colours representing "High myrcene – pinene" (Black), "High Terpinolene-menthol (Red), "High β-Caryophyllene-Limonene (Green) and "Others" (Blue) (C). Ellipses represent 95% confidence interval.

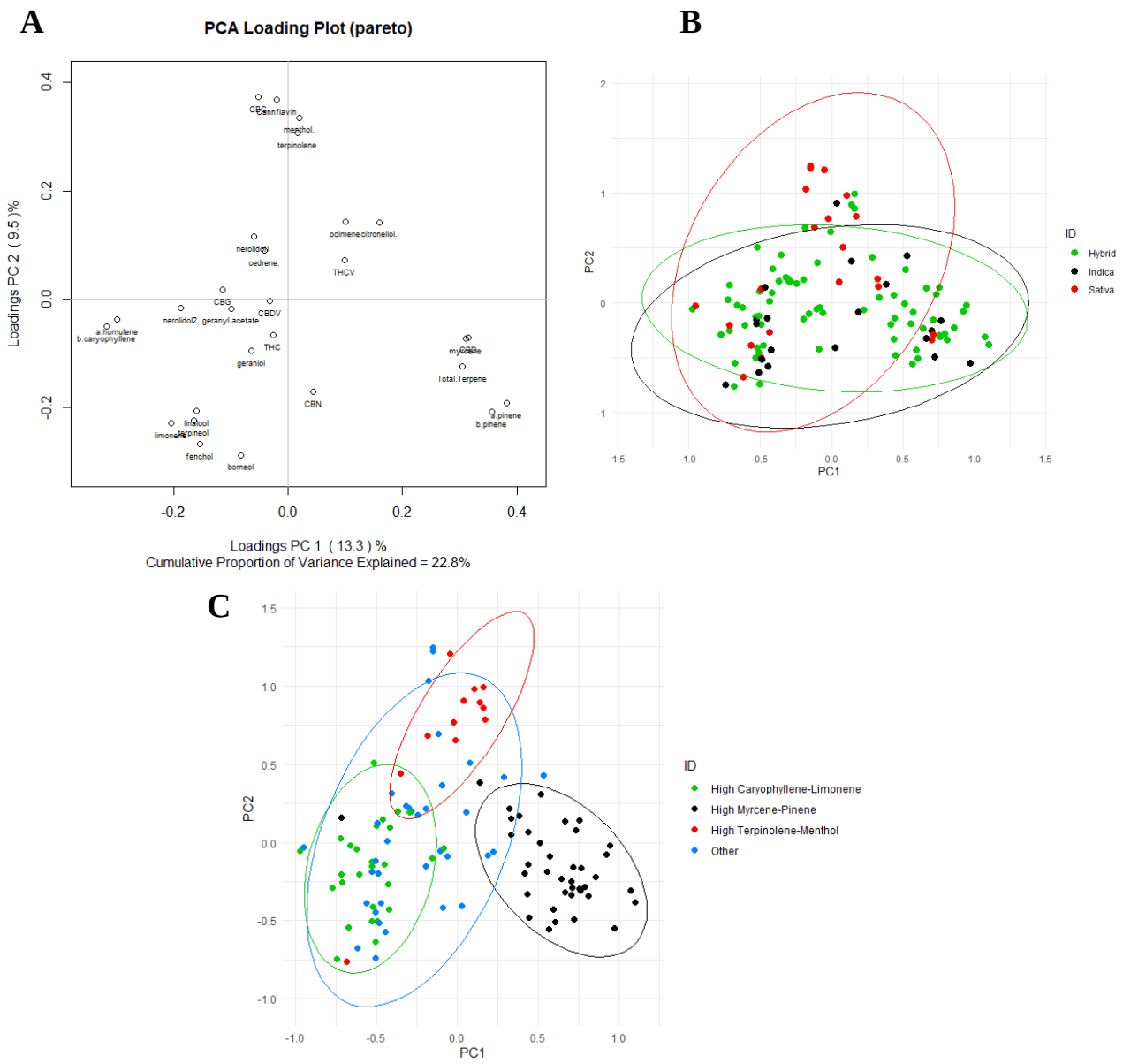


Figure 2-5: Loadings and unsupervised principal component analysis plot of 113 high THC variety cannabis extracts on quantified scaled cannabinoid-terpene profile. Extract cannabinoid content was quantified using HPLC-DAD and terpene content by GC-FID corresponding standards. Data was pareto scaled in R and unsupervised clustering was performed. PCA plot with colours representing **Sativa**, **Hybrid**, and **Indica** (B). PCA plot with colours representing "High myrcene – pinene" (**Black**), "High Terpinoline-menthol (**Red**), "High β-Caryophyllene-Limonene (**Green**) and "Others" (**Blue**) (C). Ellipses represent 95% confidence interval.

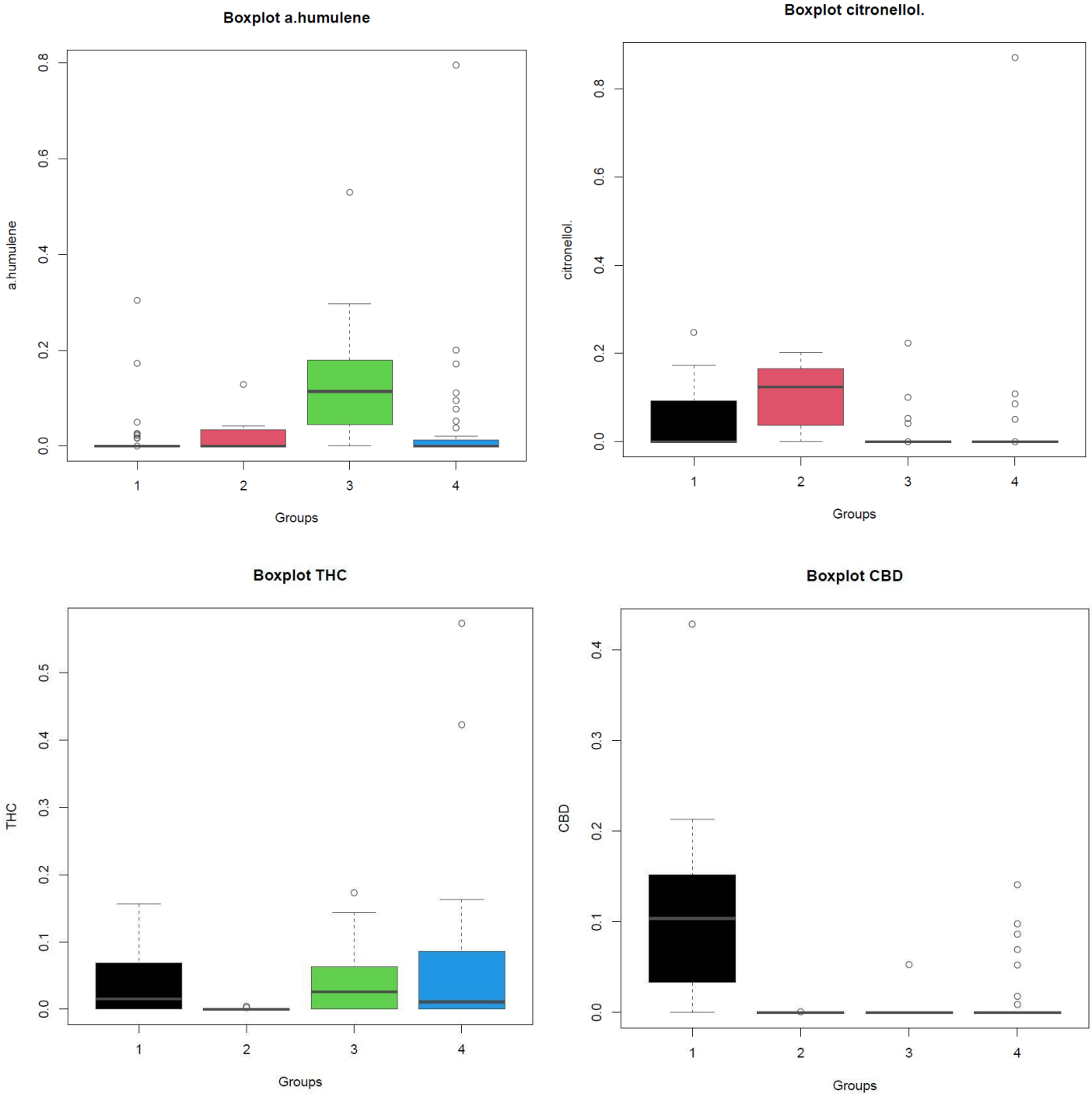


Figure 2-6: Boxplots derived from univariate analysis of scaled cannabinoid-terpene content for α-humulene, citronellol, THC and CBD. Colours representing (1) High myrcene – pinene (Black), (2) High Terpinoline-menthol (Red), (3) High β-Caryophyllene-Limonene (Green) and (4) Others (Blue). Significant lower THC content ($p<0.01$) was identified in the High-Terpinolene-menthol group between and elevated CBD in the high β-myrcene – pinene group had significantly elevated levels ($p<0.001$)

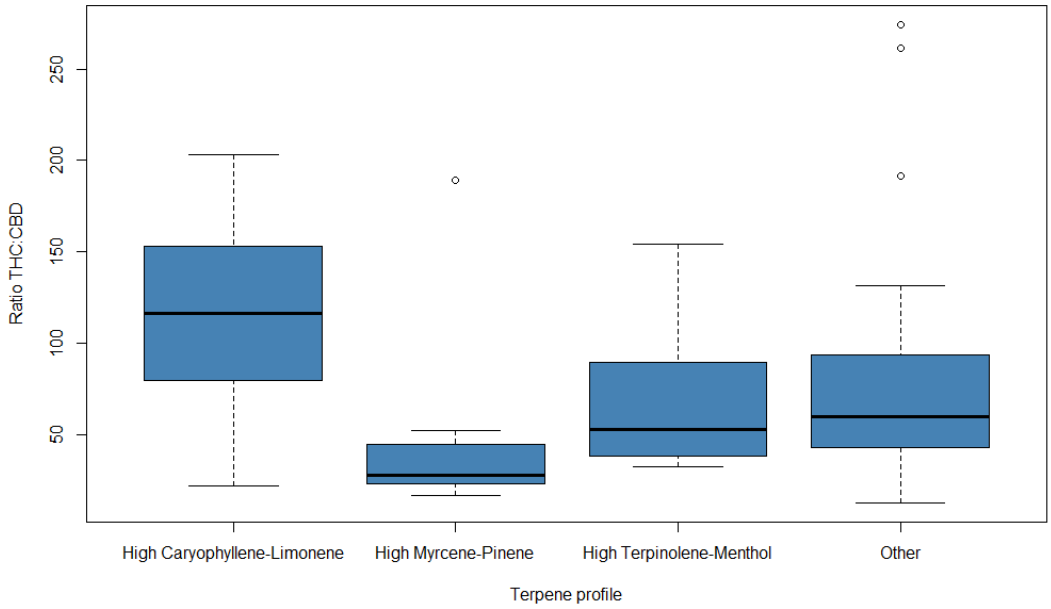


Figure 2-7: THC:CBD ratio of cannabis extracts as determined by HPLC-DAD analysis. Extracts were injected 3 separate times and averages taken. Calibration curves were prepared with standard THC and CBD to quantify THC and CBD content. The relationship between terpene profile and THC:CBD ratio is significant by ANOVA ($p<0.001$)

2.3.3 Untargeted Metabolomics

Untargeted metabolomic data was collected for reconstituted dried extracts (0.5 mg/ml) of 105 different cannabis samples. Ion chromatograms and MS data were extracted and imported into mzML for data preprocessing and for identifying major cannabinoids relative to standards. Monoisotopic masses of cannabinoid standards are summarized (**Appendix Table 1**). The major mass adduct was found to be $M+H-H_2O$ and these peaks were tagged along with their retention times. A total of 284 mass features were considered in multivariate analysis and chromatographs as other mass features were pruned out as they were either artifacts from HRMS or appeared in method/ analytical controls based on minimum threshold of 2.0 E+06 m/z .

Unsupervised principal component analysis was then performed on metabolomic data using terpene profile classes revealing no confident separation of strains based on these classes

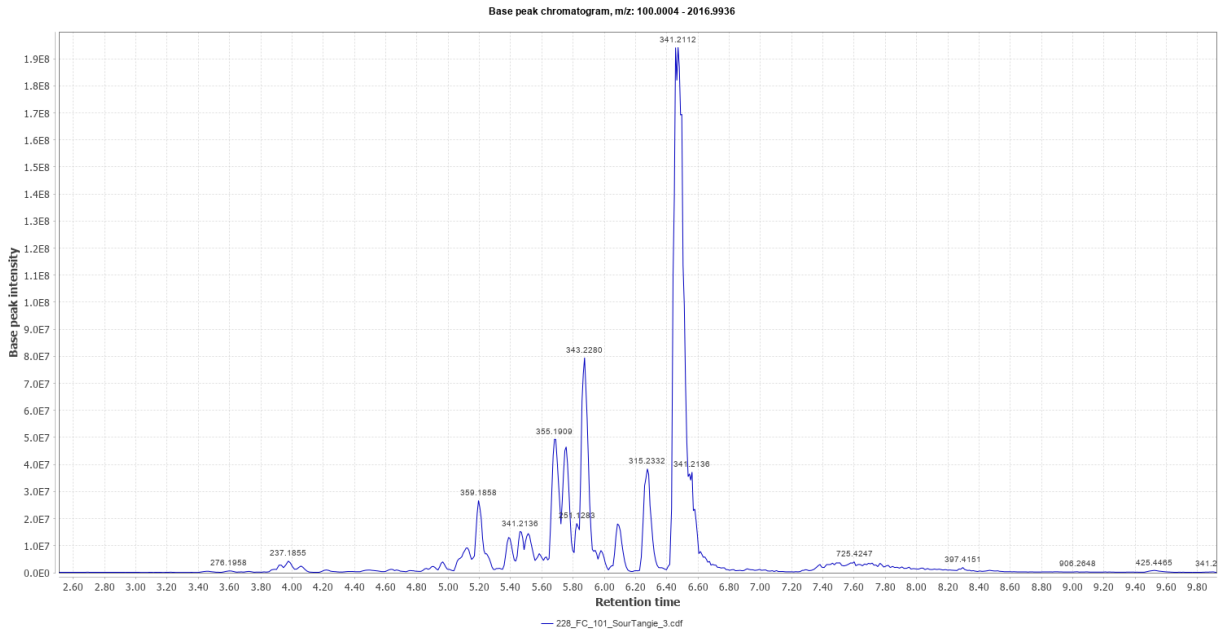


Figure 2-7: Chromatogram of base peak intensity and retention time from UPLC-HRMS analysis of 101_Sour_Tangie cannabis metabolome

(**Figure 2-9**) with 20.7% of variation explained.

Principal component analysis was also performed on strains which had 5 or more unique strain_IDs in our library (**Figure 2-10**) to observe if strains separate confidently based on strain name classification. Whereas replicates of no strain clustered independently from those of other strains, the degree of intra-strain variability (as visualized by coloured ellipses representing 95% CI) and overlap with other strains differed considerably among strain names (**Figure 2-10**). Whereas the metabolomes of TahoeOG, Sour Tangie, Violator Kush, and Kosher Kush were highly variable and overlapped with those of all represented strains, the metabolomes of White Russian, Grapefruit God and Headband samples clustered more closely together and overlapped with fewer other strains.

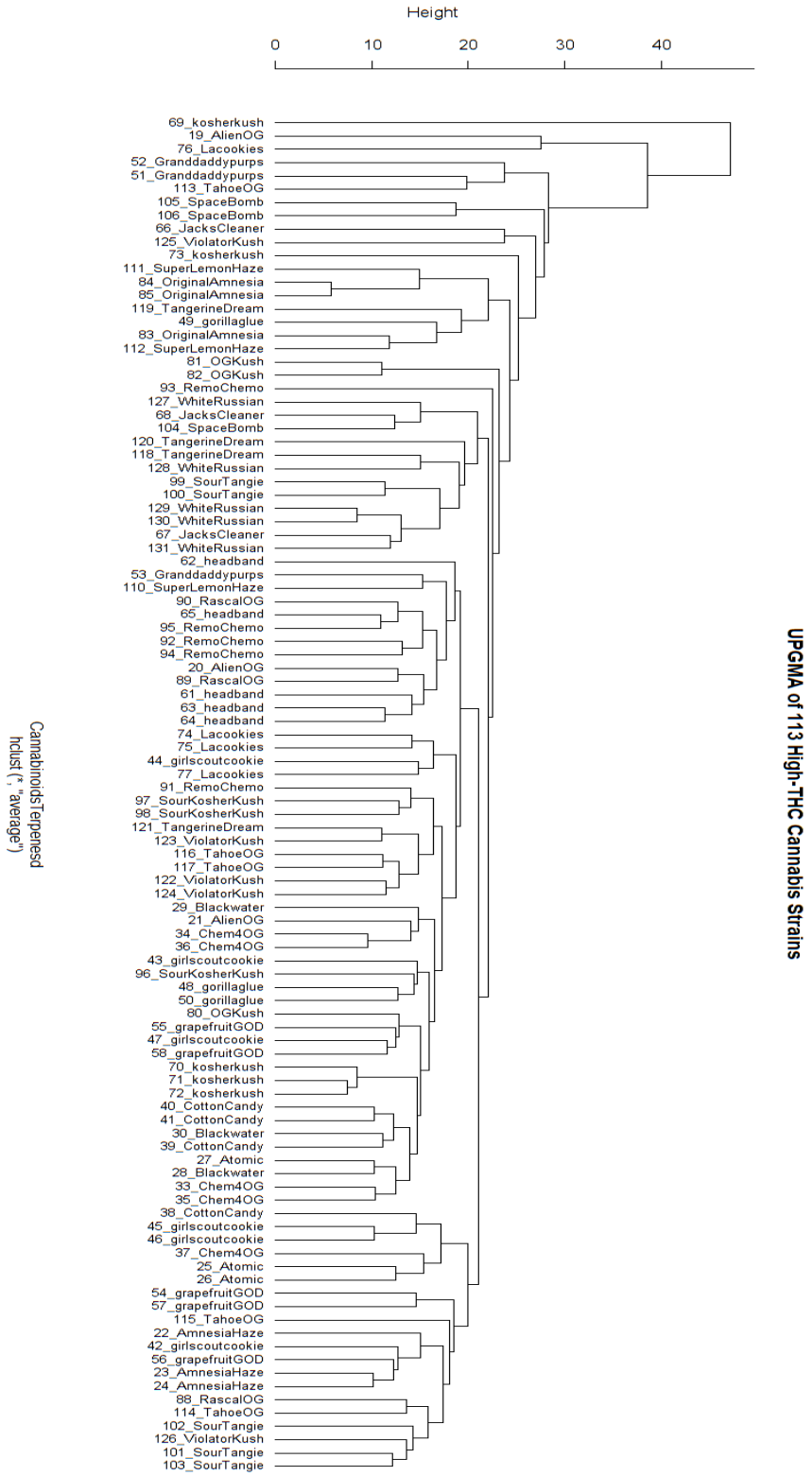
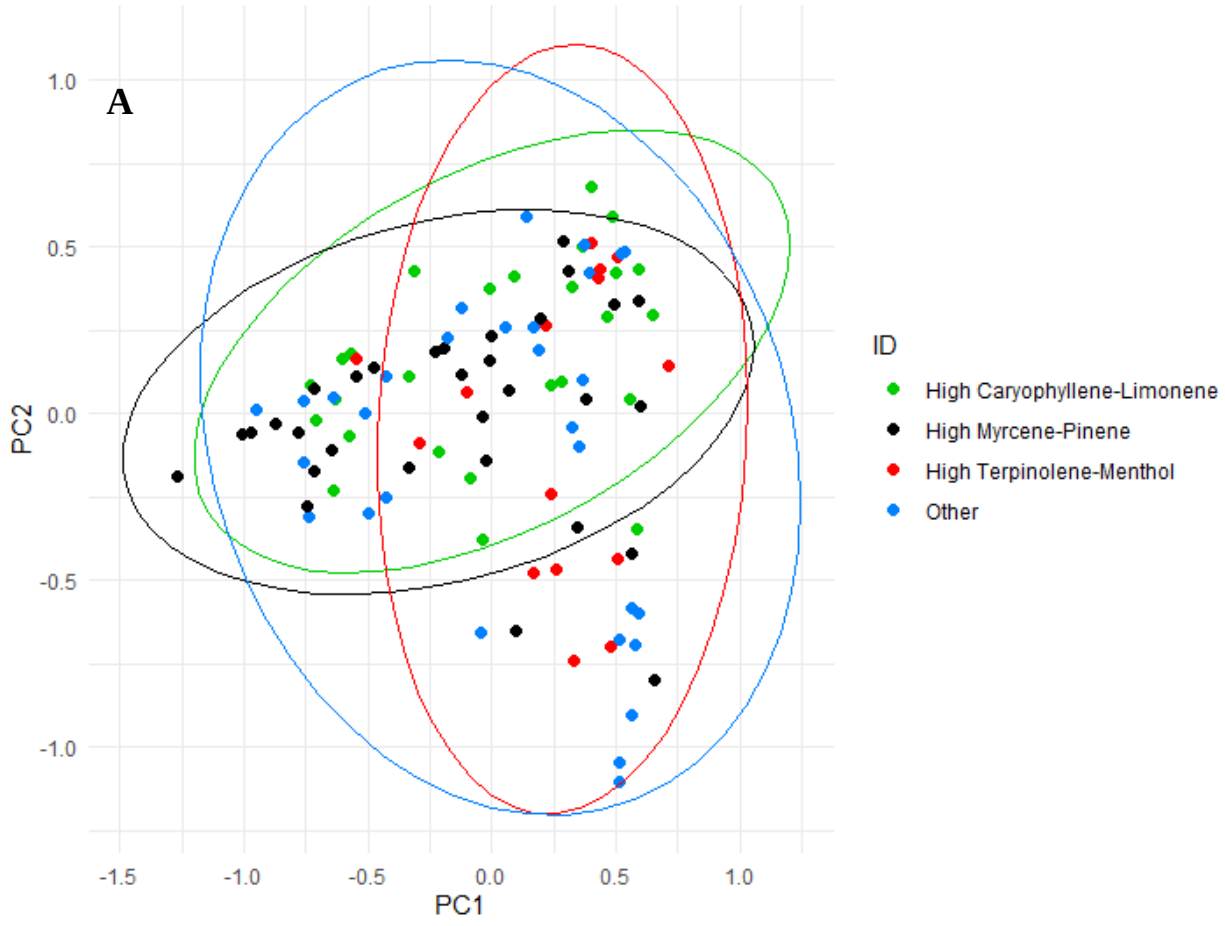


Figure 2-8: Hierarchical cluster analysis of strain metabolomes (UPGMA) or average linkage clustering. Strains represented as their ID_strainname. Each ID represents the average of 3 replicates (n=3)

To gain additional insight into the relationship between sample metabolome and strain

name, hierarchical cluster analysis (UPGMA) was performed to generate clades of samples based on phytochemical similarity (**Figure 2.8**) in which strain IDs of the same clade are more closely related. No clade was populated by samples representing one strain name, meaning all samples of a particular name were the most related to each other *and* only each other. For example, Amnesia Haze clustered into a clade of 5 samples including 1 Grapefruit GOD and 1 Girl Scout Cookie. For most IDs 2 or 3 of the strains would define a clade but the remainder clustered out a great distance from this clade. Notably 3 headband IDs formed an exclusive clade as well as Kosher Kush. Others had IDs which formed an exclusive clade of 2 IDs included: Atomic (25,26), Chem4OG (33,35) and (34,36), Gorilla Glue (48,50), Grapefruit GOD (54,57), OG Kush (81,82), Original Amnesia (84,85), Remo Chemo (95,92), Sour Kosher Kush (97,98), Space Bomb (105,106), Tahoe OG (116,117), Tangerine Dream (120,118), Violator Kush (122,124). Strains that did not cluster together whatsoever were: Alien OG, Black water, Girl Scout Cookie, Jacks Cleaner, Rascal OG and Super Lemon Haze.


A
1.0
0.5
0.0
-0.5
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PC2
-1.5
-1.0
-0.5
0.0
0.5
1.0
PC1
ID
High Caryophyllene-Limonene
High Myrcene-Pinene
High Terpinolene-Menthol
Other

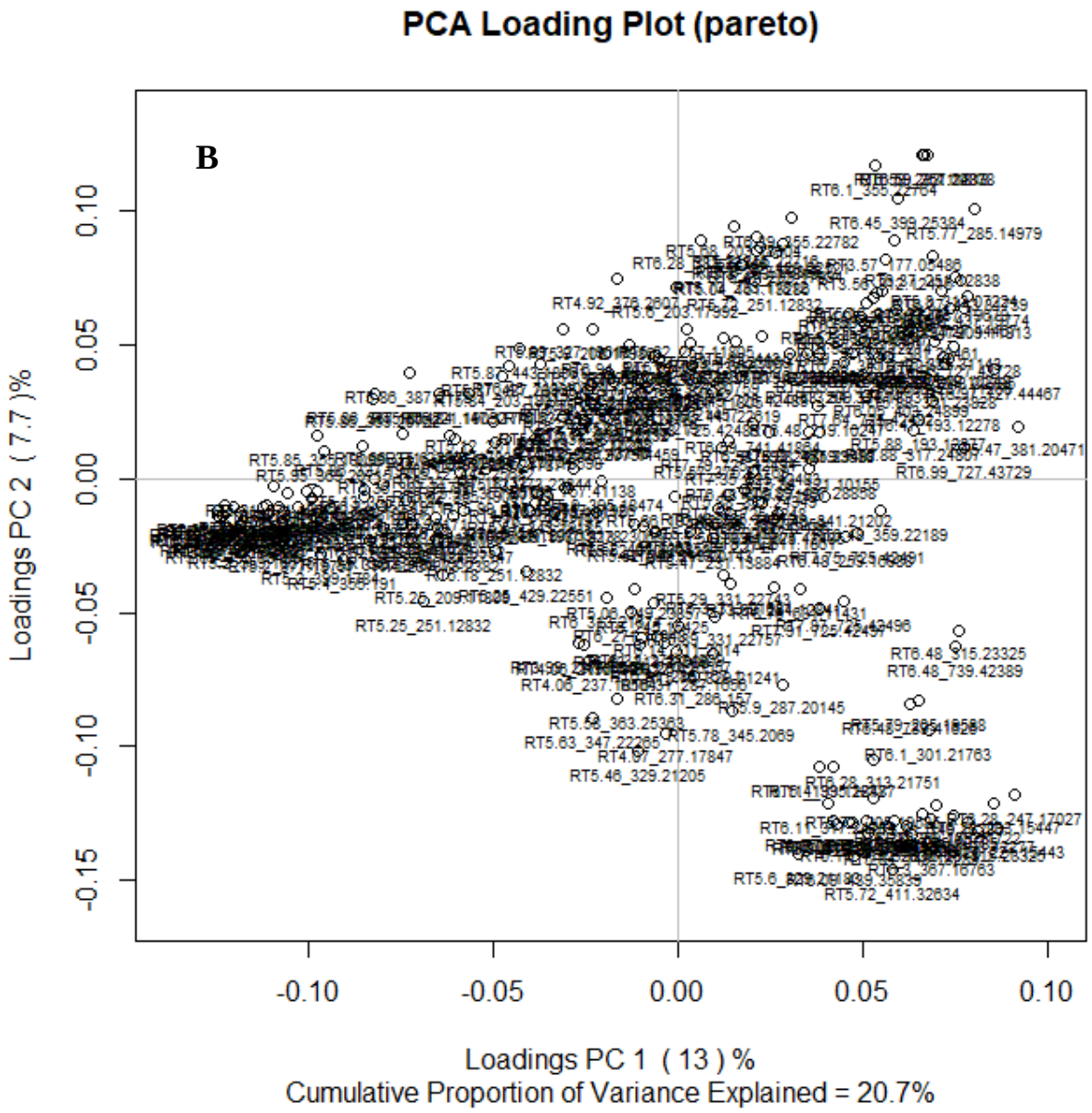

Figure 2-9: Score and Loadings plot derived from metabolomic data of 113 Cannabis strains. Colours representing (1) High myrcene – pinene (Black), (2) High Terpinoline-menthol (Red), (3) High β-Caryophyllene-Limonene (Green) and (4) Others (Blue) (A). Strains represented as their ID_strainname. Each mass feature is denoted by a retention time (RT) and mass to charge ratio (m/z). Loadings represent peak area for each mass feature in chromatograms (B).

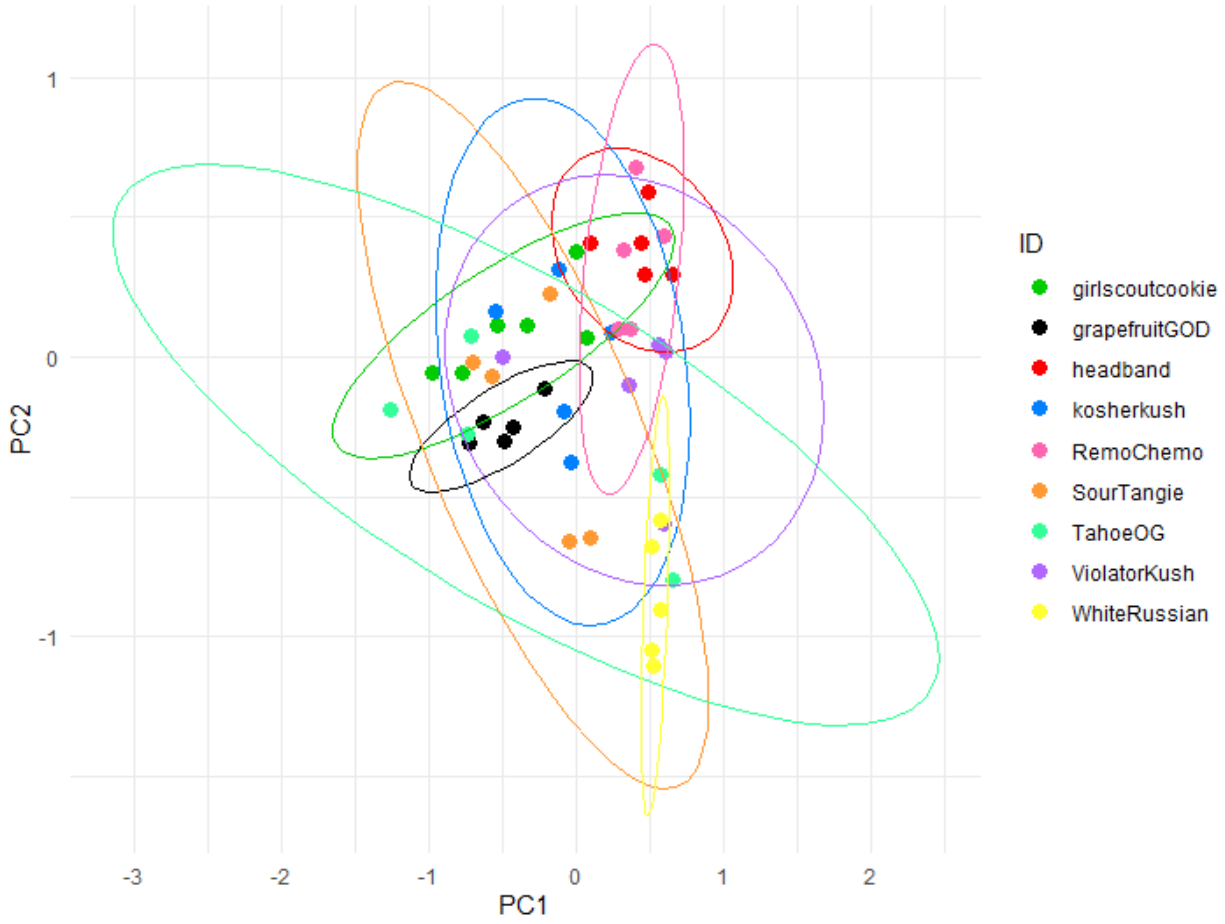

Figure 2-10: PCA of 9 selected strains with 5 or more strain replicates loaded with metabolomic data (n=9). Clustering was performed unsupervised, and data was pareto scaled before analysis. Ellipses represent 95% confidence interval.

2.4 Discussion and Conclusions

Our targeted results support previously defined chemotypes of high-THC cannabis based on terpene dominance. Terpene data alone showed an obvious 3 way branching pattern that followed established terpene profiles in literature (Aliferis & Bernard-Perron, 2020; Hazekamp & Fischedick, 2012; Jikomes et al., 2021). Based on terpene PCA and univariate analysis these

groupings appeared to describe data structure in this study as well. "High myrcene – pinene", "High Terpinolene-menthol" and "High β-Caryophyllene-Limonene" accounted for 69% of all strains with the remaining 31% representing a different dominant terpene. Pareto scaling of terpene and cannabinoid data is done for each phytochemical individually, irrespective of the abundance of other potentially more or less abundant phytochemicals. As a result, THC and β-myrcene appear to have relatively moderate levels in univariate analysis box plots (**Figure 2-6**), whereas both these compounds accumulate at higher levels in inflorescence when compared to other phytochemicals. Reimann-Phillep et al., 2020 found that by using k-means clustering approach on quantified terpene data alone, three natural clusters emerged. The clusters each contained a profile of most abundant terpenes, however, the authors go on to define them as a β-myrcene dominant (59.4%), terpinolene dominant (7.5%) and limonene dominant (33.0%) groups. It should be noted that even though individual samples may fall out of the 95% confidence interval in PCA for these groups, they were still included in that cluster for analysis. The relatively low variability could be explained by PCA 1 and 2 (28.5%) and ellipses representing 95% intervals show some overlap in plots. Jikomes et al., 2021 combined dominant terpene groups to again create 3 clusters: High caryophyllene – limonene, high myrcene - pinene, and high terpinolene – myrcene. However, of this data set containing commercial high THC cannabis samples, 3.9% of strains had a different dominant terpene. Here we also see nerolidol1 pulling variation in the 'other' dominant terpene cluster in terpene data (**Figure 2-4C**), this may be since nerolidol1 may define a more minor terpene group in future which may be further parsed out from the 'other' group. More research into terpene composition of THC dominant extracts with a wider range of terpene standards may define samples that exist in the 'other'

group, however these findings do in fact point out the inaccuracy of *indica*, *sativa* and *hybrid* designations to describe phytochemistry.

Cannabis samples classified as *sativa* were indistinguishable from *indica* and hybrid types as indicated by multivariate analysis of terpenes or cannabinoids alone, terpenes together with cannabinoids and metabolomics. Similarly, there were no significant quantitative differences in terpene and cannabinoid content across *indica*, *sativa* and *hybrid* designations. This is conflicting with findings that *spp. indica* have higher B_T allele frequency and higher THC:CBD ratios than that of *spp sativa* (Hillig & Mahlberg, 2004) but in line with findings that THC and CBD content varies among strain IDs making *spp indica* and *spp sativa* difficult to distinguish (Fischedick J, 2015; Sawler et al., 2015). It is difficult to track the history of recreational THC dominant cannabis and the resulting pedigree of strains due to the overwhelming amount of hybridization, first illicitly and now legally, by growers over the past century. The botanical description of *sativa* and *indica* Cannabis plants has also become obscured, reflecting the variable and indistinctive chemistry of *sativa* and *indica* plants we see today. It is entirely possible that traditional landraces of these plants may become extinct due to extensive human influenced hybridization and the fact that escaped artificially selected strains can easily interbreed with traditional ones (Mcpartland, 2017). It is also possible that different seed collections of the same strain name are misnamed or simply genetically heterogenous, confounding attempts to associate chemistry with pedigree. Unlike most THC-dominant strains, hemp cultivars show a relatively conserved phytochemical profile with CBD being the dominant cannabinoid and limited variation in terpene content (Hazekamp & Fischedick, 2012), which is logical as these varieties have been selected for fibre or seed oil production. With different degrees of hybridizations and no true definitive groups of recreational THC dominant strains it is

not surprising that clouds of data are apparent in multivariate analysis rather than clearly defined clusters.

Capturing and analyzing untargeted metabolomic data is a more exhaustive approach to chemotyping relative to targeted profiling as UPLC-HRMS data yields all detectable compounds found in cannabis extracts. Following PCA analysis of 284 mass features across 105 cannabis extracts, PC1 and PC2 only captured 20.7% of the variation of scaled metabolomic data. It appears that as more compounds are added to the data set, the amount of data structure that can be explained by principal components decreases. What is interesting is when a more expansive analysis consisting of 284 mass features is used in metabolomics, the terpene classification became insufficient in defining groups (*indica, sativa* and *hybrid* classifications were insufficient as well). Terpene profiles seem to define groups when considering only the most commercially relevant phytochemicals (cannabinoids and terpenes) but these terpene-dominant chemotypes differences are lost at the level of the metabolome. A potential confounding factor in this analysis is that metabolomics or UPLC-HRMS analysis was performed on dried down cannabis tinctures, which likely resulted in the loss of volatile terpenes in the drying process. This indicates that, for dried ethanol extracts, THC-dominant strains are indistinguishable chemically and that variance will be driven primarily by major cannabinoids seeing as they account for a high proportion of cannabis bud extracts.

Figure 2-8 and **Figure 2-10** shows some strains exhibit high fidelity to their respective strain names as represented by tight clustering of strain replicates. The strains “grapefruit GOD”, “headband” and “White Russian” form tighter clusters in which 95% confidence intervals do not overlap in principle components. However, most of the remaining strains do overlap and intrastrain variability appears to be high even when these strains have been subject

to the same growing, curing and extraction protocols. Why some strains are more tightly linked than others may be due to how robust the back-breeding process was to establish cultivars for a given strain name. Seeing that we do not know breeding history, tighter clusters could allude to more stable genetics in that strain's population. Unlike other data sets, in which usually many dried cannabis samples are analyzed in multiple lab settings under different protocols, our samples were collected from a single seed source a grower providing added insight on the consistency of strain chemistries. Untargeted metabolomics may be a viable tool to identify strains with more stable chemistries such as "grapefruit GOD", "headband" and "White Russian". Consumers can benefit from knowing that certain strains names will provide a reliable experience rather than trusting *indica* and *sativa* designations, this is just as useful for regulators to parse out 'good' and 'bad' strains in terms of chemical diversity and eventually start tagging these strains with a more complete terpene or metabolomic profile.

Loadings plot of cannabinoid-terpene data reveals a correlation between CBD and Myrcene. Univariate analysis on terpene profiles in cannabinoid-terpene data revealed high myrcene-pinene strains also had higher CBD when compared to the remaining groups. Another consideration is there appearing to be a correlation between α-humulene and β-Caryophyllene. This can be corroborated with findings by (Smith et al., 2021). This is potentially due to cannabis having a diverse terpene profile and many terpenes being positively or negatively correlated to one another due to shared biosynthetic pathways (Luo et al., 2019). It should also be noted that in this data set 'balanced' and CBD-dominant strains had much lower terpene diversity with most samples being myrcene dominant. Scaling of terpene and cannabinoid data may have influenced the resulting data structure where more cannabis samples do appear to be myrcene dominant in non-scaled data. Higher CBD and balanced strains appear to exhibit

myrcene dominance which could allude to plants that have been selectively bred for high THC content were also selected for more diverse terpene profiles and therefore exists the complex cannabis terpene repertoire we see today.

Moving forward it is important to note that THC, CBD, and CBC appear to polarize data in terpene + cannabinoid loadings plot. This may be due to fact that these are the 3 major cannabinoids synthesized from a common substrate of CBG (**Figure 1-2**). Development of illicit marijuana strains selectively bred for high THC content may have led to a lowering of genetic diversity among THC-dominant strains (Elizabeth Margaret Mudge, 2019). It is a shame that a bottlenecking of available cannabis cultivars occurred and that subspecies of cannabis that display more balanced or CBD dominant chemistry may have been lost, these strains may no longer exist or only exist around the geographic origin of *indica* and *sativa* in central Asia. Incorporation of these balanced and high CBD plants if accessible would be interesting to observe how they vary compared to high THC samples using metabolomic data and if similar or new terpene classes emerge.

Annotating mass features with UPLC-MSMS and corroborating these results with a data base of cannabis phytochemistry will allow for identification of compounds driving variation in metabolomic data. Fragmenting compounds with MS-MS analysis would allow for identification of structures. Perhaps metabolites other than cannabinoids and terpenes are better suited to explain true variation, or potentially contribute to the two species hypothesis of cannabis classification. More rigorous methods of cannabis identification will lead towards a more harmonious chemotyping system. It would then be pertinent to study the difference

between high caryophyllene/limonene and high myrcene/pinene groups *in vitro* to investigate differences in CB_1 activation.

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Chapter 3: Cytotoxicity and CB_1 receptor activation of High THC Cannabis Varieties

3.1 Introduction

It is well established that Δ^9-tetrahydrocannabinol (THC) mediates the psychoactive effects of Cannabis, primarily its partial-agonist activity at CB_1 receptors (Friedman & Devinsky, 2015; Iwamura et al., 2001). Research however has found it difficult to quantify 'psychoactivity' or the feeling of being high as dosing with cannabis depends on the user's personal experience, tolerance, mode of consumption (smoking, vaporizing, edibles, etc.), body mass, mood and so on (Lucas, Galettis, & Schneider, 2018b; Zhao, Feng, Tian, Taylor, & Arden, 2021). Users also have developed preferences which have been connected to either percent THC/ CBD content as well as choosing between *indica* and *sativa* (Pearce, Mitsouras, & Irizarry, 2014). *Indica* strains are usually associated with a 'body-high' which can be described as calming and grounding while *sativa* strains are associated with a 'head-high' with effects being more uplifting and energetic (A Hazekamp & Fischedick, 2012). Considering that THC content dominates cannabis phytochemistry across *indica*, *sativa* and hybrid strains, and across terpene based chemotypes with no significant differences between groups, the various different effects of cannabis may be attributed to the 'entourage effect' from other minor cannabinoids or terpenes (Ben-Shabat et al., 1998; E. B. Russo, 2011). 'Entourage' is basically the synergism between plant extract active compounds and other minor compounds which enhances the total effect of the plant extract (Wagner & Ulrich-Merzenich, 2009). This provides the foundation in which traditional medicine practitioners believe plants have better therapeutic activity than the compounds isolated from them (Koltai & Namdar, 2020).

Cannabis has a complex composition of over 150 unique cannabinoids (Brenneisen, 2007; Koltai & Namdar, 2020) and over 200 terpenes (E. B. Russo, 2011), along with plant phenolics and flavonoids (Cannflavin A and Cannflavin B) (Flores-Sanchez & Verpoorte, 2008). The interactions between phytocannabinoids at the level of CB receptor signalling have been studied, primarily with respect to THC and CBD but also the interaction of other extract components such as minor cannabinoids and major terpenes that may change the potency of a cannabis extract (Finlay, Sircombe, Nimick, Jones, & Glass, 2020; LaVigne et al., 2021). To investigate the potential "entourage" effects among different THC-dominant cannabis extracts, and to determine if activity is driven by THC, THC:CBD ratio, or other extract components, I have evaluated the *in vitro* tracheobronchial cytotoxicity and CB_1 receptor signalling activity of heat-activated cannabis extracts of known THC and CBD content.

3.1.1 Tracheobronchial Cytotoxicity

Cannabis products, although consumed in a variety of formats through different routes of administration, are most frequently consumed by smoking or vaping dried cannabis inflorescence (bud) or extracts/condensates (Rotermann, 2019). Lung endothelial cells are therefore exposed repeatedly to high doses of neutral cannabinoids. The potential cytotoxicity of cannabinoids and other cannabis constituents on lung epithelial cells are poorly studied but, as with nicotine and tobacco, cytotoxicity depends not only on the desired bioactives but on other compounds and by-products inhaled at the same time. Carriers (e-liquids) used in cannabis vape-pens as well as e-cigarettes may contain flavorings which have been identified as cytotoxic in a fibroblastic lung cell line, namely butterscotch, caramel and cinnamon flavourings (Bahl et al., 2012). Menthol and tobacco flavoured e-liquids were also identified as cytotoxic to BEAS-2B human lung epithelial cells, as evidenced by non-mitochondrial oxygen consumption increase (Stefaniak,

Lebouf, Ranpara, & Leonard, 2021). Diluents in which nicotine crystals may be dissolved have been studied, revealing a growing body of evidence for the cytotoxicity of propylene glycol and vitamin E acetate (Huanhuan Jiang et al., 2020). However, no study has compared the toxicity of pure cannabinoids and complex cannabis extracts using *in vitro* or *in vivo* models of pulmonary delivery.

In the same way that CBD modulates THC at CB_1 it is pertinent to investigate if CBD has protective effects when it comes to BEAS-2B viability. CBD as a therapeutic has been chosen due to its lack of psychoactive effects but apparent wide range of molecular targets and ability to treat seizure disorders like Lennox Gastaut syndrome and Huntington's disease (Brenneman, Petkanas, & Kinney, 2018; Press et al., 2015). CBDs dissociation constant at CB_1 was reported in the 2-30 μM range for radioactive ligand displacement assays (CP55,940 or HU-243) at CB_1 and CB_2 whereas THCs range was 3-40 nM in the same selected assays (Pertwee, 2008). THC has the most potent psychoactive effects and CBD seems to attenuate some of these effects as well as posses some anti-inflammatory effects when studied *in vitro* (Booz, 2011; Pisanti et al., 2017). Considering THCs higher affinity for CB receptors studying THC, CBD and plant extracts in cytotoxicity may reveal if THC/CBD are driving toxicity or potentially other components of extracts.

3.1.2 Activity of THC, CBD, and Cannabis Extracts at CB_1

To approximate CB1 receptor activation, intracellular cAMP accumulation has been employed to evaluate THC and heated plant extracts for CB_1 agonist/ antagonist activity (A. C. Howlett et al., 2002; Yang et al., 2020). Other studies in this field also employ a [^{35}S]GTPγS assay which monitors the amount of G-protein liberate from the GPCR receptor complex

(Devane et al., 1988; Rubino, Forlani, Viganò, Zippel, & Parolaro, 2005). Ultimately, the liberated $G\alpha_{i/o}$ protein will inhibit AC on cell membranes which will decrease the amount of cAMP generated from ATP in cells (Rhee et al., 1997). Binding of agonists at CB_1 and resulting signalling cascades modulates N- and P/Q-type Ca^{2+} channels. This is thought to underlie the mechanism in which cannabinoids inhibit the further release of neurotransmitters from pre-synaptic cells (Demuth & Molleman, 2006). Interactions of cannabinoids, as well as synthetic ligands such as CP55 490 (Price et al., 2005; Tonini et al., 2006), WIN55,212 (Sugiura et al., 1996) and HU-210 (McKallip et al., 2002), with cannabinoid receptors (CB_1 and CB_2) have been studied using numerous *in vitro* pharmacological models focusing on both direct ligand-receptor activity and downstream signalling cascades. However, whereas most research related to cannabis has assessed only pure cannabinoids administered alone or in simple combinations (e.g. THC:CBD) (Laprairie et al., 2014; Pertwee, 2008; Russo & Guy, 2006), few studies have considered the interplay between THC, CBD, minor cannabinoids and other metabolites as found in extracts and other cannabis products and would better simulate exposure to recreational cannabis.

Therefore, for this study, we compared the CB_1 signalling activity of cannabis extracts compared to that of pure THC and CBD to determine if efficacy is driven by THC alone or if there are additive, synergistic, or antagonistic effects of extracts. First extracts were chosen based on a range of THC:CBD ratios in unheated extracts. These were then heated (decarboxylated) and evaluated relative to pure THC in intracellular cAMP accumulation assays. Next, pure THC and CBD at a ratio of 100:1, 10:1 and 1:1 was compared to pure THC and heated extracts spiked with CBD to obtain the same 100:1, 10:1 and 1:1 ratios. Pure compounds compared to quantified heated extracts will allow for speculation on if negative allosteric

modulation (NAM) occurs in extracts as well. Acid cannabinoid precursors (Yang et al., 2020) and propyl side chain derivatives THCV, CBDV, CBGV (Adele Thomas et al., 2005) as well as CBG, CBN and CBC (**Table 1-1**) all have activity at CB_1 and exist in minor concentrations in extracts. Although acid and minor cannabinoids appear to posses weak or no agonist CB_1 agonist activity (Zagzoog et al., 2020)**,** they may compete with THC for binding at the active (orthosteric) site and act as an antagonist or, like CBD, may bind CB_1 at an allosteric site and negatively (or positively) modulate THC-mediated activation. The activities can be investigated by comparing concentration-response curves on an agonist with a potential antagonist or NAM. Rightward shifts in response curves are indicative of competitive inhibition at the orthosteric site of the receptor, lowering median effective concentration (EC_{50}) without impacting maximum agonist response (maximum effect, E_{max}). A reduction in E_{max} would suggest non-competitive antagonism or NAM (Wouters, Walraed, Banister, & Stove, 2019). Cannabis extracts are quantified before and after heating (decarboxylation of acid precursor cannabinoids) and tested in assay as if they are 100% THC (THC concentration ranges from 34-60%). Values therefore are presented in µg/ml throughout data collection. EC_{50} and E_{max} values can be collected after experimentation and corrected for THC content (µg THC/mg).

3.1.3 Study Objectives and Hypotheses

Objective #1: To investigate if THC content predicts the cytotoxicity of THC-dominant cannabis extracts, I evaluated the *in vitro* cytotoxicity of pure THC and 6 extracts of THC-dominant strains in human bronchial epithelial cells (BEAS-2B).

Hypothesis1: Cytotoxicity of plant extracts will be greater than that of pure THC (on a weight-by-weight basis)

Prediction: TC_{50} values generated by extract concentration-response curves will be lower than those predicted by the concentration-response of pure THC

Hypothesis 2: THC will be more toxic than CBD

Prediction: THC concentration-response curves will generate TC_{50} values below that of CBD

Objective #2: To investigate if THC content predicts the CB_1 agonist activity of cannabis extracts, I evaluated *in vitro* activation of CB_1 by pure THC and six extracts of THC-dominant strains in human recombinant CB_1-HEK293 cells.

Hypothesis 1: Pure THC will be more potent than crude extracts

Prediction 1: THC concentration-response curves will yield lower EC_{50} values than that of heated extracts in cAMP assay

Prediction 2: THC concentration-response curves will be more effective than crude extracts in reducing intracellular cAMP (increased E_{max})

Prediction 3: Hemp (low-THC) extracts and pure CBD will not be effective in reducing intracellular cAMP

Objective #3: Evaluate intracellular cAMP accumulation (receptor efficacy) of a heated extract spiked with CBD to obtain 100:1, 10:1 and 1:1 THC/CBD ratio vs pure compounds of the same ratio

Hypothesis: Pure compounds will be more efficacious than extract preparations

Prediction: Extract constituents will have shifted concentration-response curves to the right (increased EC_{50}) and potential interaction of the constituents at the allosteric binding site will reduce E_{max}.

3.2 Methods

3.2.1 Cannabis Extracts

The cannabis extracts for this study were selected from the collection of THC-dominant extracts characterized in Chapter 2. Initial dried inflorescence material was obtained from a licenced producer (Flowr, BC), where cannabis plants were subject to the standardized growth and harvesting conditions. Dried inflorescence was extracted in our laboratory following standard optimized protocols as described in Chapter 2. Extracts were selected to cover a range of THC:CBD ratios based on tincture phytochemistry. The selected ethanolic extracts (E19 "Alien OG", E51 "Grand Daddy Purps", E61 "Headband", E69 "Kosher Kush", E83 "Original Amnesia", E95 "Remo Chemo" and, E101 "Sour Tangie") were dried by roto-evaporation (37°C) then sub-sampled at 100 mgs and subjected to heat-activation (cannabinoid decarboxylation). A hemp extract with a low total cannabinoid content was also extracted for use as a control to reflect background phytochemistry.

3.2.2 Sample Preparation

Extracts

Cannabinoid concentrations in extracts will be different from concentrations in tinctures because of rotary evaporation and speed vacuuming, which lead to the loss of volatiles and concentrates remaining metabolites in dried extracts. Selected extracts (E19 "Alien OG", E51 "Grand Daddy Purps", E61 "Headband", E69 "Kosher Kush", E83 "Original Amnesia", E95 "Remo Chemo" and, E101 "Sour Tangie" as well as the hemp extract) were first reconstituted in 1 mg/ml HPLC grade methanol before heating to determine initial neutral and acid cannabinoid content by HPLC-DAD analysis. Extracts were sub-sampled and analyzed 3 separate times

(n=3) and average cannabinoid content collected. Heated samples were prepared by weighing 100 mg of selected dried extract from the library studied in Chapter 2. Extracts were heated at 120°C for 1 hour in glass vessels and covered with tinfoil. These were then reconstituted in HPLC grade methanol at 1 mg/ml to determine cannabinoid content using HPLC-DAD. Following decarboxylation, 20 mg of heated extracts were then reconstituted in DMSO and vortexed followed by 5-minute sonication at 30°C to generate stock solutions. All stock solutions of extracts were stored at >20 mg/ml in DMSO at 4°C for maximum 1 month.

THC and CBD (alone and mixed)

THC and CBD were confirmed by HPLC-DAD analysis to be 99% pure. THC was an amber viscous liquid which was solubilized at 100 mg/ml DMSO. CBD was a course white powder and was dissolved into THC dilutions. For a 1:1 ratio 50 µl of 100 mg/ml THC was added to 200 µl DMSO and 5 mg of CBD was added to this volume. For 10:1, 0.5 mg of CBD was added the same way and for 100:1 0.5 mg was added to 200 µl DMSO, then a 10:1 dilution was made into 200 µl new DMSO where the 50 µl of THC was then added.

Spiked Extracts

'Spiked' dilutions were created by using a quantified heated extract, extract 19, which when run in triplicate on HPLC-DAD had an average THC content of 585.01 ug/mg ± 2.87. A higher amount of extract was then dissolved in DMSO to match 20 mg/ml pure THC. This resulted in 34.36 mg of extract 19 dissolved in 1 ml DMSO. The CBD average CBD content was 2.18 ug/mg ± 0.46. Which when multiplied up to the new concentration is now 3.74 ug/mg. 100 µl of the 34.36 mg/ml dilution was added to 100 µl DMSO and again the CBD was dissolved in a separate 100 µl of DMSO minus the amount of CBD existing in extract to yield a 100:1, 10:1

and 1:1 THC/CBD ratio. Top concentrations of both THC and CBD were 18.667 µg/ml (59 µM) in 1:1 dilution assays, 18.667 µg/ml THC to 1.866 µg/ml CBD in 10:1 dilution assays and 18.667 µg/ml THC to 0.188 µg/ml CBD in 100:1 dilution assays.

3.2.3 Beas-2Bs (Human Bronchial Epithelium) Culturing

The laminar air floor hood is wiped down with 70% ethanol as well as anything that would enter the hood, gloves were sprayed every time following aseptic technique. All media is warmed to 37°C in a water bath as well as 0.05% Trypsin/ EDTA, Phosphate Buffered Saline (PBS) and experimental buffers. Thawing protocol followed ATCC guidelines.

The human broncho-epithelial cell lines (BEAS-2B) was used to model tracheobronchial cytotoxicity with exposure to pure compounds and extracts. BEAS-2B cells have been used to model 24 hr toxicity of CBD (Pagano et al., 2020) and of condensed cannabis smoke (Kim, Hyun Jung, Yeun Lee, Min Oh, & Hyuck Chung, 2013). Culturing technique followed ATCC guidelines. Cells were cultured in maintenance media consisting of DMEM/F12 (1:1) + L-Glutamine + 2.438 g/L Sodium Bicarbonate, 1% (v/v) Penicillin/Streptomycin and 5% FBS. BEAS-2B cells double at a much faster rate than CB_1-HEK293 cells and therefore media changes were required every 2 days and splitting every 4-5 days. Cells would reach confluency at 7×10^6 cells per 10 cm plate and will begin to differentiate if left at confluency for too long and as a result, would be split at reseeded at a density of 5×10^5 cells (ATCC). Cells were cultured at 37°C, 5.0% CO_2 and 70% humidity.

3.2.4 Cytotoxicity assays

WST-1 cell proliferation reagent (Millipore Sigma, REF: 11644807001) was employed to monitor cell viability and cytotoxicity BEAS-2Bs cells. WST-1 assay consists of a tetrazolium salt which is converted to formazan by a mitochondrial dehydrogenase, the amount of formazan created is proportional to number of living cells. Formazan has a peak absorbance at 440 nm which was measured in assay. Cells were dissociated using 0.05% Trypsin/EDTA and replated at 5×10^4 cells per well in a white opaque 96 well plate with optical polymer base (Fisher scientific, REF: 12-566-71). Cell suspension was added to microplate at 100µL per well using a multichannel pipettor and trough followed by visualization at 40x under a microscope. Cells were allowed 18-24h to adhere in the incubator at 37°C, 5.0% CO_2 and 70% humidity, prior to experimental manipulation. Stock solutions of extracts (20 mg/ml) and pure compounds (100 mg/ml or 20 mg/ml) were serial diluted in DMSO then added to warmed media, (diluted 1:200 into serum free media) to final concentrations of 0.781 µg/ml – 100 µg/ml in 0.5% DMSO.

After careful aspiration of maintenance media, each dilution for each extract or pure compound was administered to cells in triplicate by adding 50 µL per well for three separate wells. Assay controls consisted of a vehicle control of 0.5% DMSO and media control of only media. Assay optimization consisted of applying concentrations far beyond biologically relevant concentrations (1000 µg/ml) and visualizing cells under the microscope before reading to confirm cells in fact died.

After 24 hours of treatment, a 2X concentration of WST-1 reagent was prepared in warmed serum free media and 50 µL of this was directly to cells. WST-1 protocol calls for a 10:1 dilution of WST-1 reagent to media but, to avoid disrupting cells through aspiration of treatment media, the 2X concentration was prepared and added to existing medium. Following the 90-minute incubation (37°C, 5.0% CO_2 and 70% humidity), absorbance was measured at 440

nm using Cytation™ 3 Imaging Multi-Mode Reader (Agilent, Santa Clara, CA). Data reduction consisted of dividing well absorbance by average absorbance of vehicle control (0.5% DMSO). Data was collected in triplicate for each extract and pure compound on a minimum of 3 separate days on which resulted in >3 biological replicates per treatment (n>3). Plates were analyzed using a Gen5 Feature Detection Software where a minimum of 7 dilutions per extract were used to generate concentration-response curves. Data was exported to excel with values representing % cell viability

3.2.5 CB_1-HEK293 Culturing

The human embryonic kidney cell line (HEK-293) is a heterologous expression system in which cells express a gene that was transfected by a cDNA vector. HEK-293 cells were stably transfected with a human CB_1 receptor and were graciously donated by the Dr. Ken Mackie at the University of Indiana (Ken Mackie, 2018). The receptor is conjugated to a hemagglutinin (HA) tag which may be targeted in immunoassays. Cells were received at passage 3 and at passage 8 and 11 cells were frozen at a density of 2×10^6 cells/ml in heat inactivated FBS with 10% DMSO then injected into cryovials and stored in a -174°C cryochamber.

Cells were grown in maintenance media (DMEM + 4.5 g/L D-Glucose + 110 mg/L sodium Pyruvate), 10% FBS, 1% (v/v) Penicillin/Streptomycin and 1 mg/ml G418. Plasmids co-express an antibiotic resistance gene for G418 along with CB_1 to maintain expression over generations. Cell splitting was performed when a plate reached confluency, for HEK-239s this is around 1×10^7 cells on a 10 cm plate. Culture media is aspirated and washed with 1 ml 1X PBS applied on the side of the dish and not directly to cells PBS is removed and 2 ml of 0.05% Trypsin/EDTA was added to each plate directly to cells. Plates were rocked gently to cover its entire surface area. The reaction was carried out in the incubator for 5 mins at 37°C and 5% CO_2.

The reaction was neutralized by adding 4 ml of maintenance media followed by aspirating the media + Trypsin + cell mixture. The suspension was added to a 15 ml conical tube and centrifuged at 200 RCF for 5 minutes. The supernatant was discarded, and pellet resuspended in 1 ml maintenance media followed by trituration with a p1000 pipettor. Live cells were counted via haemocytometer and Trypan Blue. 100 µL of cell suspension was added to 100 µL 0.4% Trypan Blue and 14 µL were injected into a haemocytometer, % viable cell was calculated with a viable cell threshold set at >90%:

$$\%\, Viable\ cells = \left(1.00 - \frac{number\ of\ blue\ cells}{Total\ Number\ of\ cells} \right) x\ 100$$

Cells were plated at a density of 1 x 10^6 cells per plate (1:10). Media changes occurred every 4 days and splitting every 7 days.

3.2.6 Immunocytochemistry

Immunocytochemistry was employed to confirm receptor expressions in CB_1- HEK293 cells. Monitoring membrane receptor levels is integral in measurement of receptor activity in functional assays. Cells were fixed with 4% paraformaldehyde (PFA). Cells were suspended following the CB_1-HEK293 splitting protocol and plated at 5 x 10^5 cells per well with 3 ml final volume. Cells were left to adhere to the plate for 48hrs at 37°C, 5% CO_2 and 70% relative humidity. On day 2 cell media is aspirated and cells are fixed with 4% PFA solution for 10 minutes at room temperature followed by a cold PBS wash 3 times. 'Blocking' was performed using the serum of the animal that the secondary antibody was grown up in, serum was left on cells for 45 minutes. The primary antibody was an HA Tag Recombinant Rabbit Monoclonal Antibody (RM305) at 1mg/ ml (Invitrogen, REF: MA5-27914). This is diluted to 1:1000 in

PBST + 0.1% BSA solution following the manual protocol for immunocytochemistry and left exposed to cells for 24hrs at 4°C.

Primary antibody is then aspirated, and plates were washed each well 3X with PBST. The secondary antibody is a donkey polyclonal antibody to rabbit IgG conjugated to an Alexa Fluor 594 at 2mg/ml (abcam, REF: 150076). This was diluted in PBST at 1:200 as per the manual protocol for immunocytochemistry. Secondary antibodies were left on cells for 1 hour at room temperature. Cells were counterstained with DAPI for 90 seconds to visualize nuclear material. DAPI was kept at -20°C in 14.3 mM aliquots and were diluted to 1:1000 PBS. Coverslips were mounted on to glass slides using 100 µl Polyvinyl alcohol mounting medium with DABCO®, antifading. The backside of a pair of tweezers were used to gently push down on the slide to remove air bubbles. The resulting fluorescence was visualized using a Zeiss Axioport upright Microscope

3.2.7 Intracellular cAMP assay

Data was generated by an *in vitro* assay which uses free adenosine triphosphate (ATP) and free luciferin + O_2 to react with a luciferase to create luminescence (cAMP-Glo™ Max, Promega). Since ATP concentration is effectively the inverse of cAMP then luminescence represents an inverse cAMP concentration in cell. Prior to experimentation, cells were passaged, centrifuged, resuspended in serum free media, and counted using a haemocytometer. Cells were plated at a density of 75 000 cells per well on a on white opaque 96 well plate with optical polymer base (Fisher scientific, REF: 12-566-71). After 48 hours, cells were visualized under the microscope to confirm density and adhesion. Extracts, pure compounds, and standard curves were prepared on a separate plate where stock concentrations at 20 mg/ml were diluted to 3.76 mg/ml in DMSO and further diluted 1:3 for 7 dilutions. Standard curve was prepared following

assay protocol. These dilutions were then further diluted 1:200 to stimulation buffer consisting of PBS + 30 mM $MgCl_2$ + 5µM forskolin. Forskolin stocks solutions were stored at 2.43 mM in ethanol at 4°C. Assays were run in triplicate (3 wells per dilution per sample) along with wells containing just forskolin and just 0.5% DMSO in stimulation buffer. Media was carefully aspirated using a multichannel pipettor and 40µl of stimulation buffer were added to each well alongside cAMP standard curve. In total cells are exposed to test compounds for 1 hour.Extracts and pure compounds were incubated for 25 minutes at 37°C, 5.0% CO_2 and 70-90% humidity. Plates were removed from incubation for the addition of Protein Kinase A solution and Kinase-Glo reagent following assay protocol. Once completed the plates were read for luminescence by Cytation™ 3 Imaging Multi-Mode Reader and data synthesized using Gen5 Feature Detection Software.

3.2.8 Statistical Analysis

The collection of replicates (n=3-4) were added together into one spreadsheet and parameters were collected from the data summary i.e. for each of the 7 concentrations, there were 9 data points. Standard error and confidence intervals (mean ± 2SE) were gathered for each parameter and summarized.

Cytotoxicity Data

Data was exported to excel, and values were standardized to % viability relative to control (0.5% DMSO) wells and diving all well absorbance values by this value. Data was then imported into R 4.0.3 to be visualized before and after x-axis log transformation to confirm the data structure appeared sigmoidal by plotting response in relation to extract concentration in

µg/ml. The model *drm* was applied from the *drc* package (Ritz & Strebig, 2016) and the fct = LL.4 was employed, a log-logistic model with 4 parameters (*b,c,d,*e):

$$f(x,(b,c,d,e)) = c + \frac{d-c}{1+\exp\left(b(\log(x)-\log(e))\right)}$$

The parameter 'e' represents 50% the maximal response in model and is the TC_{50} value for these data. 'c' and 'd' represent the lower and upper limit of the function respectively and 'b' represents the functions slope. To be included in the data set, individual replicates needed to yield a significant TC_{50} or EC_{50} value ($p<0.05$) for both cytotoxicity and efficacy experiments.

An optimization step consisting of running a wide range of pure THC which falling higher and lower than the active range for the compound was completed on 3 different densities of cells in triplicate (12500, 25000 and 50000 cells per well). From this it was found that a top concentration of 100 µg/ml in well was shown to be effective in generating a minimum absorbance in assay that appeared asymptotic. Heated extracts in this assay were reconstituted in DMSO to the same µg/ml concentration as pure THC. Data appeared to approach a maximum and minimum asymptotically and deemed appropriate to fit a concentration-response curve. *summary(.LL4)* yielded the coefficients and the unadjusted p values and the function *ED(THCopt.LL4, c=(10,20,50), interval = 'delta')* provide the corresponding TC10, TC20 and TC50 values respectively.

Intracellular cAMP Accumulation Data

Data is transformed via standard curve to yield a response of nM cAMP. Data was exported to excel and presented as a % control response by taking the average of the forskolin only control wells and dividing all well values by this. These were then multiplied by 100 to

represent a % control response value. Data was imported into R version 4.0.3 and visualized before and after x-axis log transformation to confirm the data structure appeared sigmoidal by plotting response in relation to extract concentration in µg/ml. The same log-logistic model as employed in cytotoxicity experiments was applied to these data to provide the corresponding EC_{50} values as well as the lower limit of the function or parameter 'c' which corresponds to the maximum affect of the compounds/ extract or the E_{max}. The same inclusion criteria were used where replicates needed to generate statistically significant parameters to be included in the larger data set. Parameter values along with the standard area are summarized and CI calculated (mean ± 2SE).

3.3 Results

3.3.1 Cannabinoid Content of Heat Activated Extracts

THC, CBD and their acid forms in heat-activated extracts were quantitatively profiled by HPLC-DAD to confirm THC-dominance and decarboxylation of acid cannabinoids to their neutral forms, and to determine THC:CBD ratios. **Figure 3-1** shows a representative chromatogram of an extract (Sample 51) which illustrates both the dominant THC peak as well as smaller peaks representing minor cannabinoids. THC concentrations ranged from 318-585 µg/mg extract (32-59% w/w) (**Table 3-1**). The executed heat-activated resulted >90% decarboxylation as neutral forms predominated the heated extracts. CBD levels ranged from 0.1-12.4 µg/mg extract (0.01-1.24% w/w). Six extracts (E51, E61, E69. E83, E95, E101) and the hemp sample were selected for comparative analysis of bioactivity. The seventh extract (E19) was reserved for spiking experiments to test how increasing CBD levels (decreasing THC:CBD ratio) impacts bioactivity in a consistent chemical background.

Table 3-1: THC, CBD content (µg/ml) of selected heated cannabis extracts as determined by HPLC-DAD analysis and the resulting THC:CBD ratio. Analysis consisted of 3 biological replicates (n=3) and mean content calculated

Extract	THC (µg/mg)	CBD (µg/mg)	Ratio (THC:CBD)
E19*	585	2	268
E51	564	6	85
E61	318	2	142
E69	370	5	71
E83	474	12	38
E95	467	2	206
E101	447	0	5311
Hemp	0	0	0

*E19 was subsequentially spiked with CBD to generate 100:1, 10:1 and 1:1 THC/CBD ratio extracts

** Hemp sample was used as a negative control in CB_1-HEK293 cell experiments

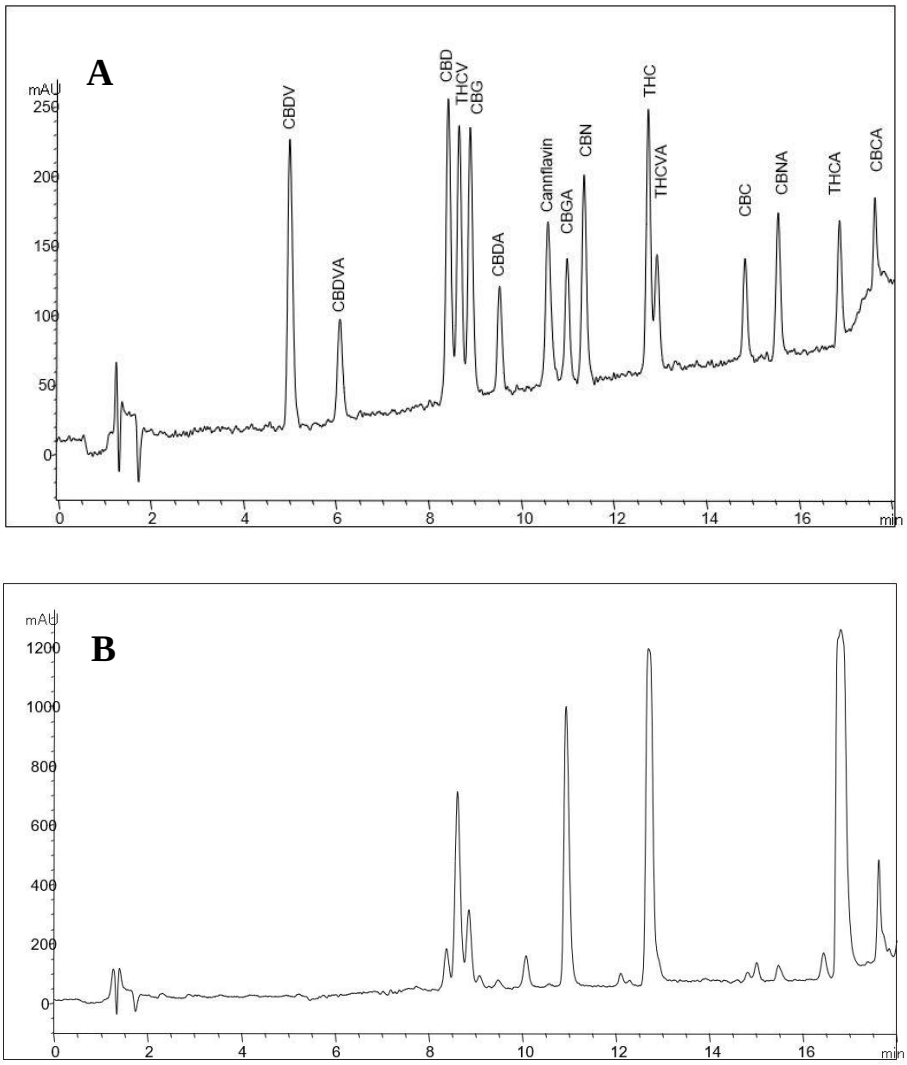


Figure 3-1: (A) Chromatograms of peak intensity (mAU) over time (min) of cannabinoid standard mix (THC/A, CBD/A, CBG/A, CBC/A, CBN/A, CBDV/ and THCVA) and **(B)** extract 51. Targeted compounds separated by HPLC and read by DAD at 210 nm. Upper panel shows standard mix of 15 cannabinoids with labelled peaks. Lower panel shows a replicate from sample 51 injected at 1 mg/ml

3.3.2 Cytotoxicity – Comparison by extract weight

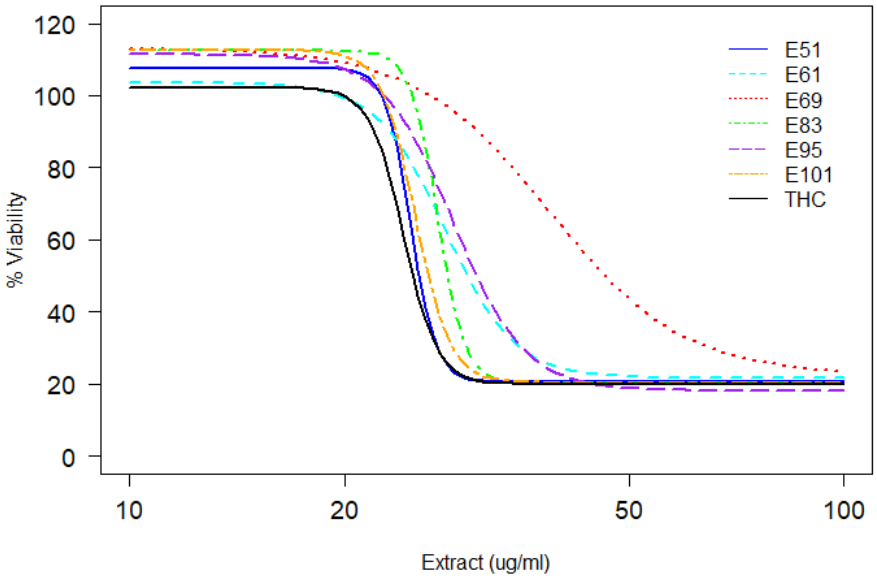
To gauge and compare the cytotoxicity of cannabis samples in a model of pulmonary administration, heat-activated extracts, as well as pure THC and CBD, were administered to BEAS-2B cells in varying concentrations (0.783-100 µg/ml). Median toxic concentrations (TC_{50}) ranged from 24.49 - 38.78 µg/ml for extracts and that of THC was 23.15 µg/ml (**Table 3-2, Figure 3-2A)**. One-way ANOVA revealed no significant difference in TC_{50} based on extract identity ($p= 0.0534$) and the only significant difference identified by Tukey's HSD test was between extract E69, which had the highest TC_{50}, and both E101 and pure THC (**Figure 3-2A, Table 3-2**). On a weight-by-weight basis, TC_{50} values of all extracts were higher than that of THC, as expected, but insignificantly.

Pure CBD toxicity was evaluated in 24 hour toxicity as well, it was found that CBD had a TC_{50} of 12.4 µg/ml ($p <0.001$) (**Figure 3-3**). CBD's TC_{50} was nearly half that of THC (23.15 µg/ml) in assay and also significantly more toxic than all extracts.

3.3.3 Cytotoxicity – Comparison by THC Content of Extracts

To identify if extract toxicity is based entirely or predominantly on THC content, concentration-response curves were expressed relative to the THC content of each extract (e.g.

for E51 with 564 µg THC/mg extract, 10 µg/ml extract = 5.64 µg THC). The resulting extract-response curves were shifted to the right (with no change to pure THC) (**Figure 3-2B**). ANOVA followed by Tukey HSD analysis revealed that TC_{50} values for all extracts were significantly lower than that of pure THC ($p<0.001$), with significant differences among extracts as the TC_{50}'s of E61 and E101 were significantly lower than those of the others (E51, E69, E83, E95) ($p<0.05$). General linear regression of TC_{50} as a function of THC content revealed a weak negative, relationship $TC_{50} = -8.029\ln(\text{THC}) + 77.907$, $R^2 = 0.3169$.



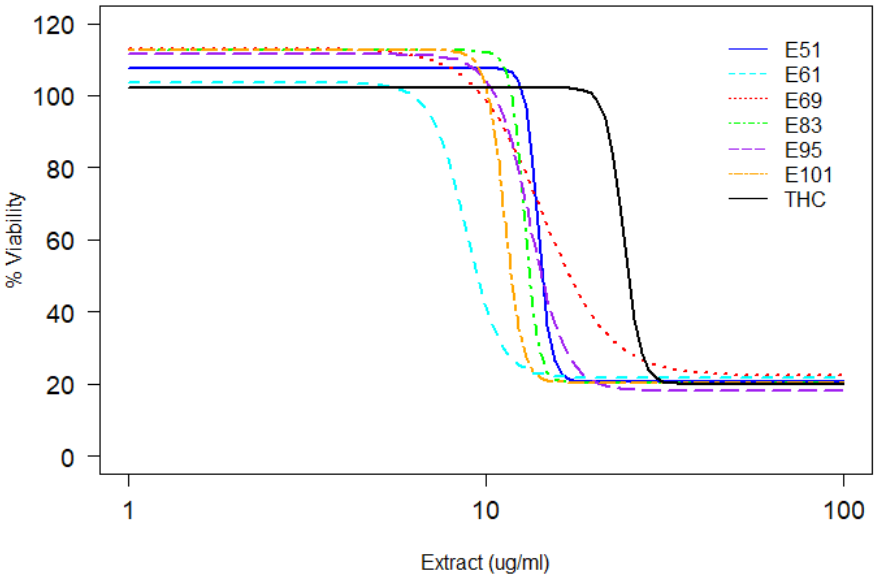


Figure 3-2: (A) Cell Viability of BEAS-2-B cells exposed to varying concentrations of cannabis extracts and pure THC, expressed relative to vehicle control (0.5% DMSO) based on extract weight (µg extract/ml). (B) Cell Viability of BEAS-2-B cells exposed to varying concentrations of cannabis extracts and pure THC, expressed relative to vehicle control (0.5% DMSO) based on THC content of each extract (refer to Table 1) (µg THC/ml).Percent cellular viability was determined via WST tetrazolium salt assay following 24 hours at a density of 50 000 cells per well. Experiments were completed in triplicate for each of the 7 dilutions on three separate occasions (n=3).

Table 3-2: Median toxic concentration (TC_{50}) values of extracts and pure THC as determined by WST assay following 24-hour exposure to varying concentrations of extracts, THC or vehicle to BEAS-2B cells. Data was collected from n=3 trials and 3 replicates per trial to yield TC_{50}. Confidence intervals represent ± 2SE.

Extract	TC_{50} (µg/ml)*	SE	CI (95%)
E51	24.49^{ab}	0.65	23.2-25.78
E61	27.18^{ab}	4.5	18.18-36.18
E69	38.78^{a}	3.64	31.5-46.06
E83	30.55^{ab}	4.68	21.19-39.91
E95	27.66^{ab}	3.61	20.45-34.88
E101	25.68^{b}	0.55	24.58-26.78
THC	23.15^{b}	2.39	18.37-27.93

*Values with shared letters (a,b) are not significantly different and values with different letters are significantly different ($p<0.05$) as identified by ANOVA with Tukey's HSD post hoc test

Table 3-3: Median toxic concentration (TC_{50}) values of extracts and pure THC as determined by WST assay following 24-hour exposure to varying concentrations of extracts, THC, or vehicle to BEAS-2B cells. Data was collected from n=3 trials with 3 replicates per trial to yield TC_{50} based on THC content of each extract (µg THC /ml). Confidence intervals represent ± 2SE.

Extract	%THC TC_{50} (ug/ml)*	SE	CI (95%)
E51	13.82^{a}	0.36	13.1-14.54
E61	8.63^{c}	1.43	5.77-11.49
E69	14.33^{a}	1.35	11.63-17.03
E83	14.49^{a}	2.22	10.05-18.93
E95	12.91^{a}	1.68	9.55-16.27
E101	11.29^{c}	0.25	10.79-11.79
THC	23.15^{b}	2.39	18.37-27.93

*Values with shared letters (a,b,c) are not significantly different and values with different letters are significantly different ($p<0.05$) as identified by ANOVA with Tukey's HSD post hoc test

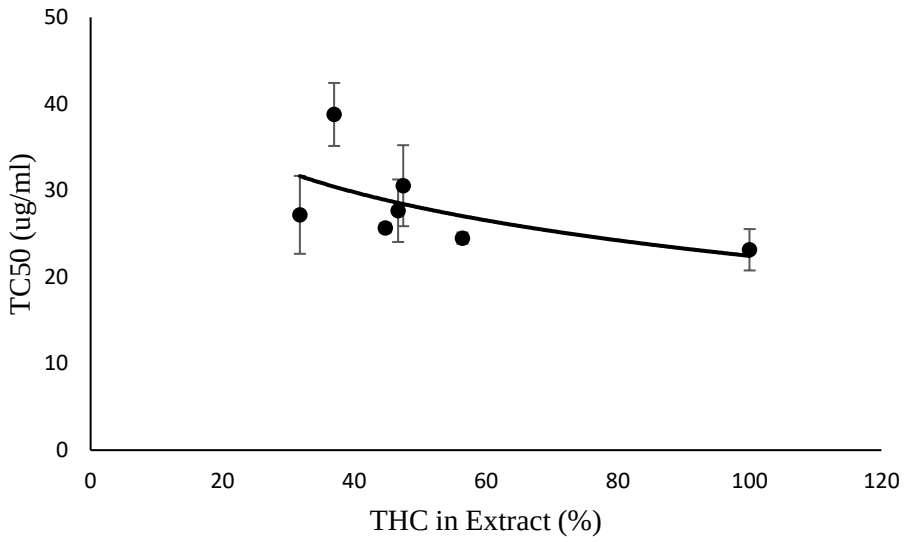


Figure 3-3: The relationship between % THC in extracts (and pure THC, 100%) and observed TC_{50} values. Error bars represent ± SE and each data point consists of 4 replicates (n=4). Fitted line represented by $TC_{50} = -8.029\ln(THC) + 77.907$, $R^2 = 0.3169$

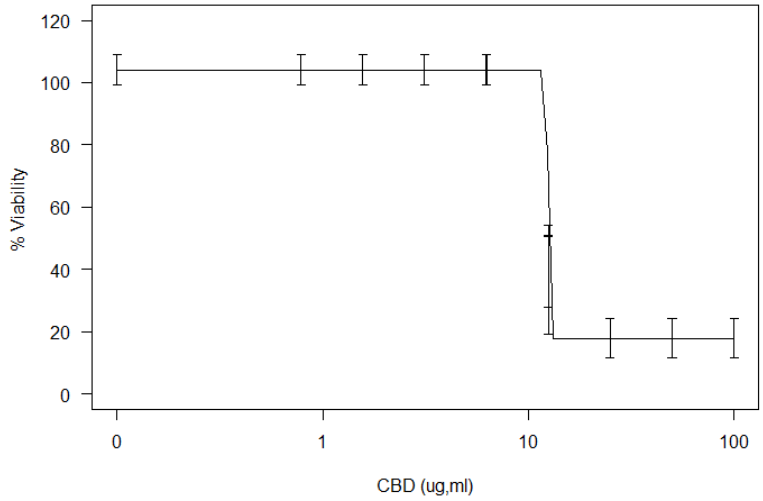


Figure 3-4: The resulting cytotoxicity of CBD after 24hr exposure to BEAS-2-Beas cells in culture. Cells were seeded at a density of 50 000 cells per well and WST-01 reagent was added for 90 minutes after exposure. Data was collected in triplicate on the plate and repeated 4 times (n=4). TC_{50} of CBD is 12.4 µg/ml ($p<0.001$)

3.3.4 CB_1 activation - Immunocytochemistry and Assay Validation

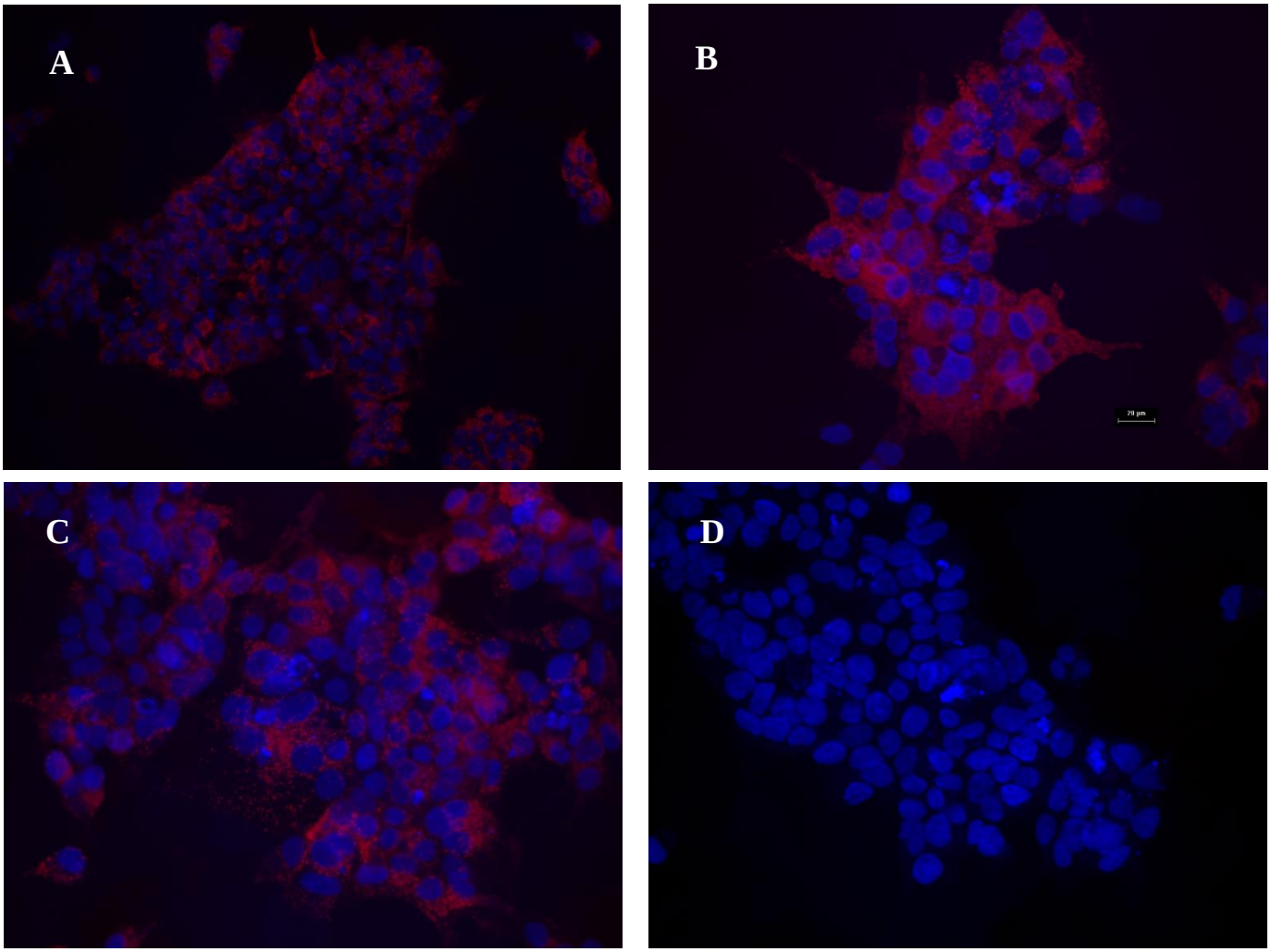


Figure 3-5: Immunocytochemistry of CB_1-HEK293 cells. Images were captured using a Zeiss Axioport upright microscope with fluorescence microscopy at 20x (A) and 40x (B-D). Cells were stained with an HA-Tag Recombinant Rabbit Monoclonal Antibody (RM305) with a secondary donkey anti rabbit polyclonal antibody conjugated to an Alexa Fluor 594. Cells were counterstained with DAPI. Cells with primary omission treatment (D).

CB_1-HEK293 cells were thawed at passage 11 (p11) and cultured to a sufficient density for experimentation. All intracellular cAMP assays were performed on cells that were between p13 and p18. Cells were imaged at p13 using immunocytochemical detection and localization of

CB_1 to confirm receptor expression. Receptors appeared to be localized to cell perimeter (**Figure 3-5**) and no staining occurred in primary occlusion immunostains. Optimization of forskolin concentration for experimental use found forskolin concentrations greater than 3.385 µM were sufficient in stimulating intracellular cAMP accumulation in cells. 5 µM of forskolin was therefore used in stimulation buffer for all of experiments, which corroborated with results from previous studies using forskolin to stimulate AC as well (Iwamura et al., 2001; Lunn et al., 2006; Slipetz et al., 1995).

3.3.5 CB_1 Activation – Comparison by Extract Weight

CB_1 receptor activation was measured by monitoring forskolin induced accumulation of intracellular cAMP in CB_1-HEK293 cells using the cAMP Glo-MAX™ assay. As expected, pure THC reduced forskolin-stimulated accumulation of cAMP in a concentration dependent manner (median effective concentration, EC_{50} = 107 ng.ml, 340 nM) (**Table3-4**). Pure CBD had no impact on cAMP levels at lower concentrations but increased cAMP accumulation >1 µg/ml and, serving as a 'cannabinoid-free' control, a heat-activated hemp extract elicited no response at the tested concentrations (**Figure 3-6**) The six heat-activated high-THC extracts, co-administered to cells in the presence of forskolin, elicited concentration-dependent decreases in cAMP (**Figure 3-8A**) with EC_{50} values ranging from 144-414 ng/ml f (**Table 3-4**). One-way ANOVA with tukey HSD *post hoc* test revealed that the EC_{50} of E69 was significantly lower ($p<0.05$) than those of E83 and E95. THC's EC_{50} was significantly lower than those of E95 and E83 ($p<0.05$) but statistically similar to other extracts. No other significant differences were observed. As observed for cytotoxicity (TC_{50}), all extract EC_{50} values were higher than that of THC's (nominally if not significantly)

Using maximum reduction in cAMP as an indicator of efficacy (E_{max}), the lower limit of each concentration-response curve was curated and compared. THC was most effective in reducing cAMP (36% relative to forskolin + vehicle control), significantly greater than the maximum effects of E51, E61, E83 and E95 but not of E69 or E101. The observed maxima of extracts were statistically similar (**Table 3-4, Figure 3-8**)

3.3.6 CB_1 Activation – Comparison by THC Content of Extracts

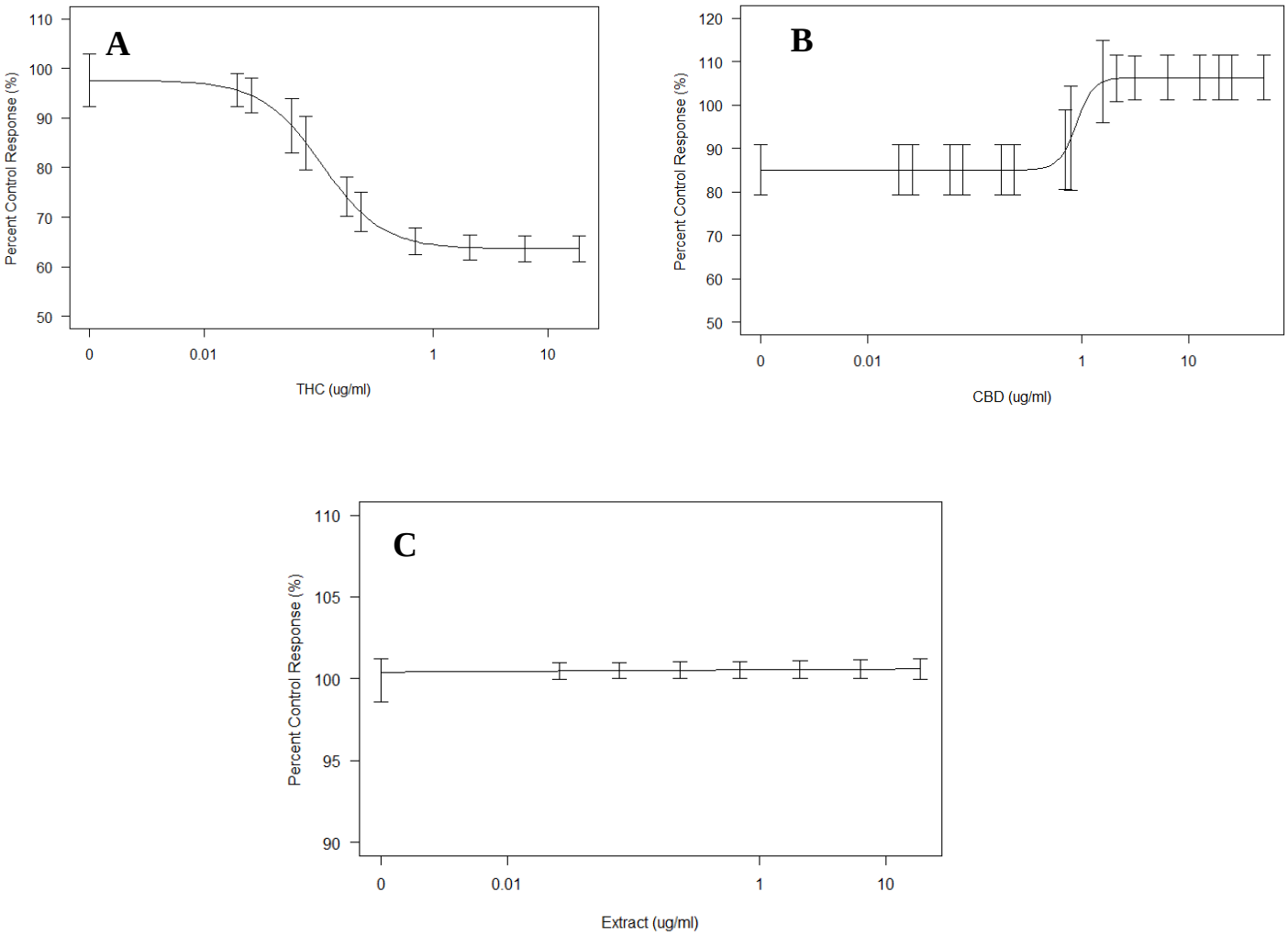


Figure 3-7: Intracellular cAMP accumulation in CB_1-HEK293 cells exposed to (A) THC (EC_{50} = 0.107 µg/ml $p<0.05$), (B) CBD (EC_{50}= 0.882 µg/ml $p<0.05$), and (C) heated hemp extract as determined by cAMP Glo-MAX™ assay following 25-minute administration. Data transformed by standard curve and expressed relative to vehicle control (0.5% DMSO). Values were collected in triplicate in assay for each of the 7 dilutions per extract and repeated 4x (n=4).

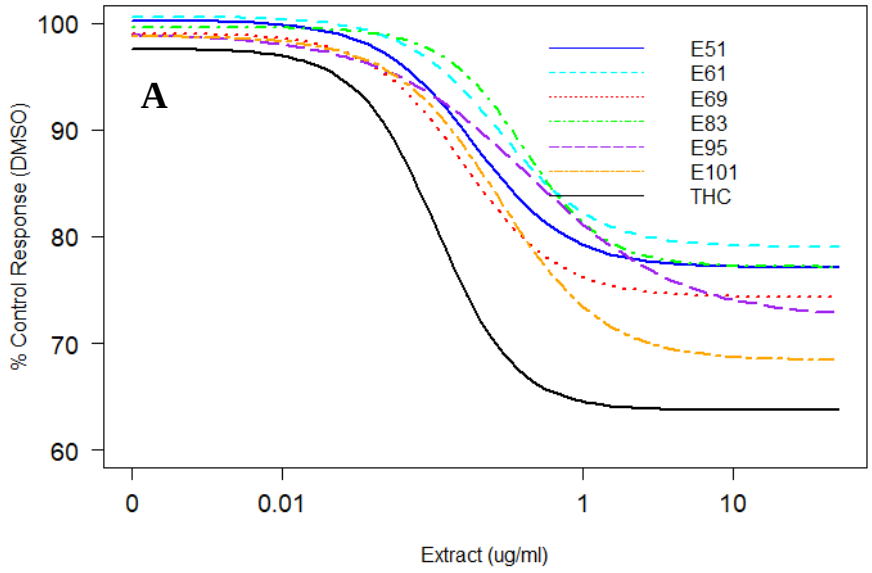
Upon converting extract concentrations to corresponding THC concentrations (**Table 3-5**), all extract response curves shifted to the left, closer to THCs activity curve (**Figure 3-8B**). Only extract 69 had a significantly lower EC_{50} than that of Extract 83 but no difference was detected between THC and extracts. Post-hoc simple linear regression analysis revealed no

significant relationship between THC content and EC_{50} ($p = 0.403$) **Figure 3-9A** with the following predictive model: $EC_{50} = -0.123\ln(THC) + 1.0191$, $R^2 = 0.2104$ (n.s).

When compared based on THC content, cannabis extracts appeared to activate CB_1 receptors with comparable EC_{50} values but, in most cases, significantly reduced maximum effects (**Table 3-4**).

The same replicates were analyzed based on percent cAMP reduction (E_{max}), and the lower limit of concentration-response curves were curated. THC was most effective in reducing intracellular cAMP which was significant compared to E51, E61, E83 and E95 however no significant difference between E69 and E101 in one way ANOVA with Tukey's HSD *post hoc* test ($p< 0.05$). The same linear regression analysis with % cAMP reduction as a function of THC was carried out including pure THC (**Figure 3-9B**). The resulting predictive model is as follows: $E_{max} = 10.417\ln(THC) + 4.6631$, $R^2 = 0.5122$ ($p<0.001$). Comparing only extracts (without pure THC), this relationship is insignificant.

This limited impact on EC_{50}, yet reduced E_{max} compared to pure THC suggest non-competitive antagonism or other negative allosteric interactions mediated by non-THC components in the extracts. Since CBD is a known NAM of THC at CB_1 (R. B. Laprairie et al., 2015) we investigated whether THC:CBD content contributed to cAMP responses.


A
E51
E61
E69
E83
E95
E101
THC
% Control Response (DMSO)
100
90
80
70
60
0
0.01
1
10
Extract (ug/ml)

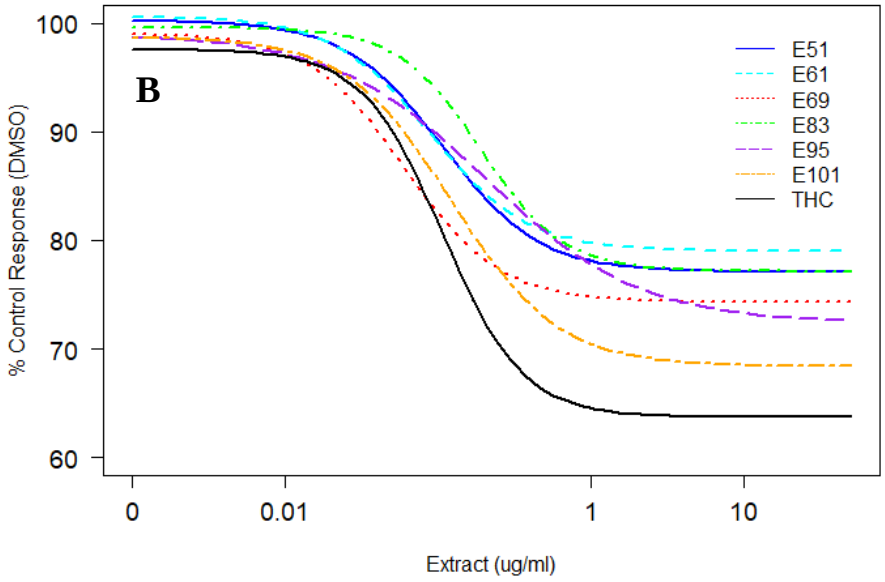

B
E51
E61
E69
E83
E95
E101
THC
% Control Response (DMSO)
100
90
80
70
60
0
0.01
1
10
Extract (ug/ml)

Figure 3-8 (A): Intracellular cAMP accumulation in CB_1-HEK293 cells exposed to varying concentrations of cannabis extracts and pure THC, data transformed by standard curve and expressed relative to vehicle control (0.5% DMSO) based on extract weight (µg extract/ml). **(B):** Intracellular cAMP accumulation in CB_1-HEK293 cells exposed to varying concentrations of cannabis extracts and pure THC, data transformed by standard curve and expressed relative to vehicle control (0.5% DMSO) based on THC content of each extract weight (µg THC /ml). Percent cellular viability was determined via cAMP Glo-MAX™ assay following 25 minutes at a density of 75 000 cells per well. Experiments were completed in triplicate Values were collected in triplicate in assay for each of the 7 dilutions per extract and repeated 4x (n=4).

Table 3-4: EC_{50} values and % cAMP reduction of extracts and pure THC as determined by intracellular cAMP Glo MAX™ assay following 25-minute administration of extracts, THC, or vehicle to CB_1-HEK293 cells. Data was collected from n=4 trials and 3 replicates per trial to yield EC_{50}) based on extract weight (µg extract/ml). All trials yielded significant EC_{50} parameters to be included.

Extract ID	**EC_{50} µg/ml** (mean ±SE)*	**% cAMP Reduction** (mean ±SE)
E51	0.187 ± 0.10^{ab}	22.919 ± 2.461^{a}
E61	0.279 ± 0.11^{ab}	20.995 ± 2.037^{a}
E69	0.144 ± 0.05^{b}	30.948 ± 2.118^{b}
E83	0.398 ± 0.06^{a}	22.853 ± 1.074^{a}
E95	0.414 ± 0.12^{a}	27.244 ± 1.74^{a}
E101	0.274 ± 0.06^{ab}	31.604 ± 1.69^{b}
THC	0.107 ± 0.02^{b}	36.294 ± 1.184^{b}

*Values with shared letters (a,b) are not significantly different and values with different letters are significantly different ($p<0.05$) as identified by ANOVA with Tukey's HSD post hoc test

Table 3-5: EC_{50} values of extracts and pure THC as determined by intracellular cAMP Glo MAX™ assay following 1 hour administration of extracts, THC, or vehicle to CB_1-HEK293 cells. Data was collected from n=4 trials and 3 replicates per trial to yield EC_{50}) based on extract weight (µg extract/ml). All trials yielded significant EC_{50} parameters to be included.

Extract	%THC EC_{50} (µg/ml)	SE	CI (95%)
E51	0.106^{ab}	0.056	-0.006-0.218
E61	0.088^{ab}	0.035	0.018-0.158
E69	0.053^{a}	0.02	0.013-0.093
E83	0.189^{b}	0.031	0.127-0.251
E95	0.193^{ab}	0.057	0.079-0.307
E101	0.123^{ab}	0.029	0.065-0.181
THC	0.107^{ab}	0.021	0.065-0.149

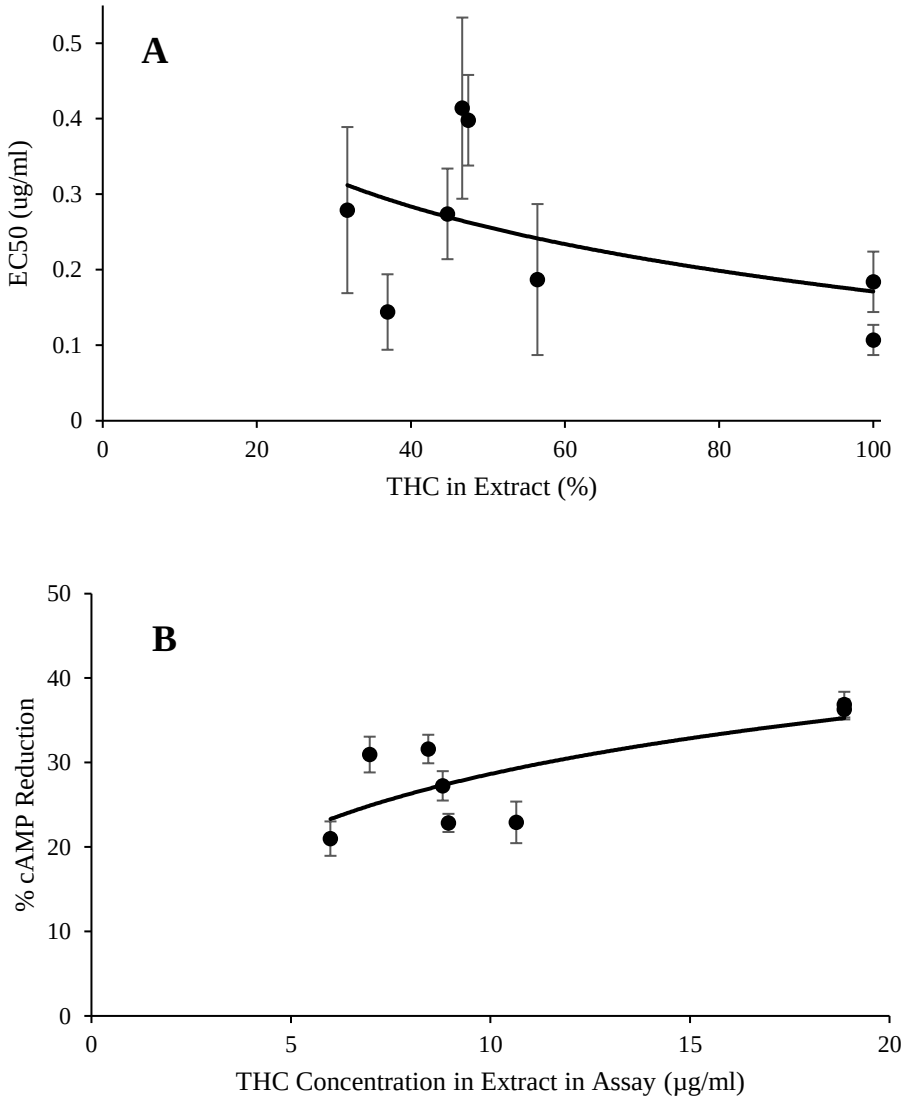


Figure 3-9 (A): The relationship between % THC in extract and pure THC on EC_{50}, fitted line represented by y = -0.123ln(x) + 1.0191, R^2 = 0.2104 and **(B)** % cAMP reduction, Fitted line represented by y = 10.417ln(x) + 4.6631, R^2 = 0.5122. Values determined by intracellular cAMP accumulation assay on forskolin stimulated CB_1-HEK293 cells as determined by cAMP Glo-MAX™ assay following 25-minute administration at a density of 75 000 cells per well. Error bars represent ± SE and each data point consists of 4 replicates (n=4).

3.3.7 Efficacy of Pure Compounds and 'Spiked' Extracts at CB_1 Receptors

The comparable impact on EC_{50} yet reduced E_{max} of extracts compared to pure THC suggests non-competitive antagonism or other negative allosteric interactions mediated by non-THC components in extracts. The NAM CBD on the effects of THC at CB_1 (R. B. Laprairie et al., 2015) were investigated by analyzing differences in THC:CBD content and evaluating cAMP responses. To do so, a heat-activated cannabis extract (E19) with a high THC:CBD ratio (268:1) was sub-sampled and spiked with different amounts of CBD to yield a 100:1, 10:1, and 1:1 THC/CBD ratio with otherwise identical phytochemical complement. The spiked extracts were then tested alongside mixtures of pure THC and CBD at the same ratios. For both extracts and pure cannabinoid mixtures, the maximum concentrations of both THC and CBD were 18.667 µg/ml (59.34 µM) in 1:1 assays, testing serial dilutions to generate concentration-response curves **(Figure 3-10)**.

For pure cannabinoid mixtures, although response curves appeared to shift to the right compared to pure THC, no significant difference was observed in EC_{50} between any THC:CBD ratio rand THC. However, when % cAMP reduction was compared, a 1:1 ratio of THC/CBD was significantly less effective in reducing cAMP when compared to pure THC (**Figure 3-10A, Table 3-6**). EC_{50} was observed in concentration-response curves between the un-spiked (E19) and spiked extracts **(Figure 3-10B)**. However, when % cAMP reduction was compared to un-spiked E19 (298:1 THC:CBD), both samples spiked to 1:1 and 10:1 THC:CBD were significantly less effective in reducing intracellular cAMP. Curves from pure compounds were then compared to those curves of 'spiked' heated extracts. EC_{50} did not differ between heated extracts and pure compounds of the same ratios. The 1:1 THC/CBD mixture reduced intracellular cAMP more effectively than 1:1 in heated extract. 10:1 THC/CBD did not differ

significantly between pure compounds and heated extracts. However, 100:1 THC/CBD in heated extract was more effective in reducing intracellular cAMP. Pure THC and pure heated extract curves of the same THC content appeared remarkably similar.

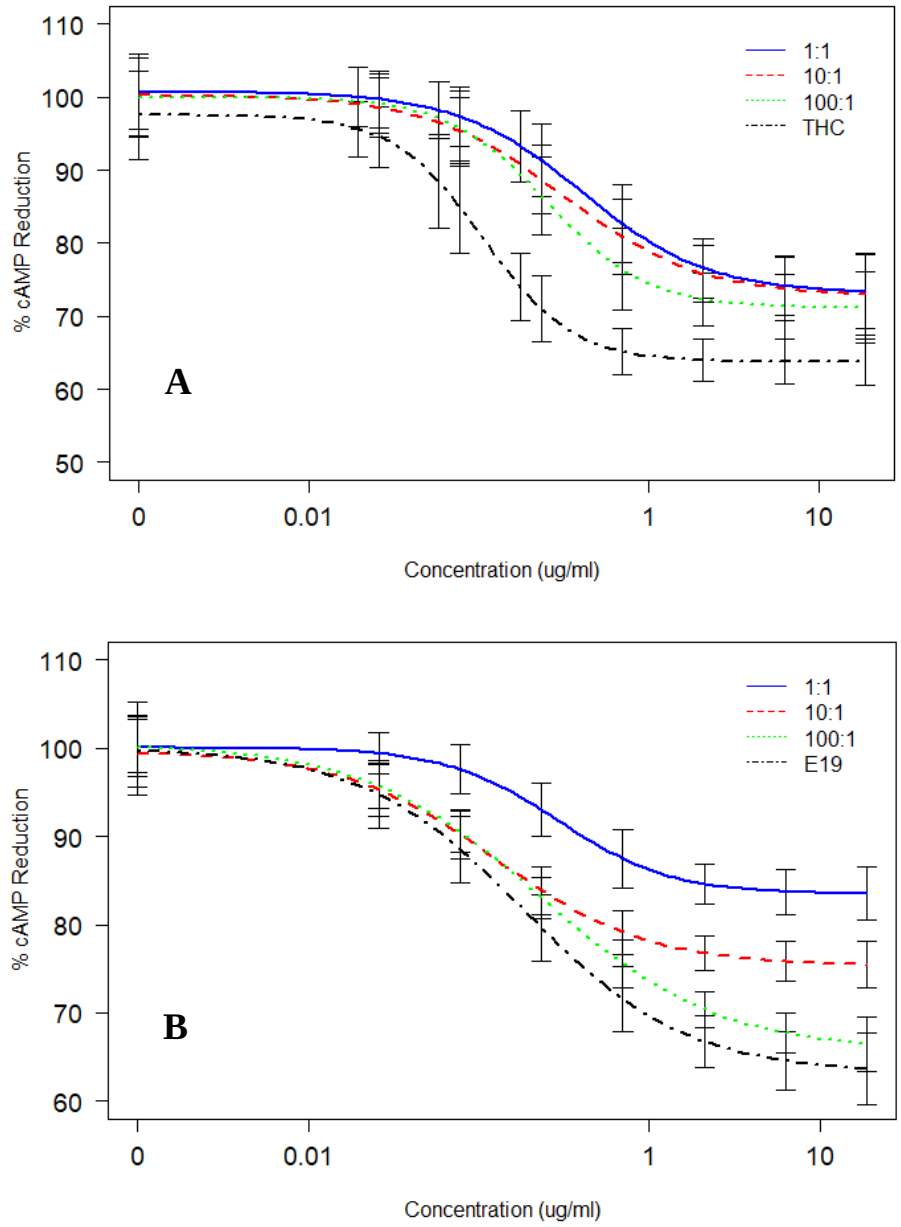


Figure 3-10 (A): Intracellular cAMP accumulation in CB_1-HEK293 cells exposed to sample preparations consisting of 100:1, 10:1, and 1:1 THC/CBD and **(B)** Intracellular cAMP accumulation in CB_1-HEK293 cells exposed extract preparations consisting of 100:1, 10:1, and 1:1 THC/CBD as determined by intracellular cAMP Glo MAX™ assay following 25-minute administration at a density of 75 000 cells per well. Data transformed by standard curve and expressed relative to vehicle control (0.5% DMSO) based on extract weight (µg extract/ml). Experiments were completed in triplicate Values were collected in triplicate in assay for each of the 7 dilutions per extract and repeated 3 times (n=3).

Table 3-6: EC_{50} values and % cAMP reduction elicited by mixtures of pure THC and CBD at ratios of 100:1, 10:1, and 1:1 THC/CBD as determined by intracellular cAMP Glo MAX™ assay following 1 hour administration of mixtures, THC, or vehicle to CB_1-HEK293 cells. Data was collected from n=3 trials and 3 replicates per trial to yield EC_{50}) based on extract weight (µg extract/ml).

Compound Ratio (THC:CBD)	**EC_{50} µg/ml** (mean ± SE)*	**% cAMP Reduction** (mean ± SE)
1:1	0.41 ± 0.19^{a}	26.91 ± 3.00^{b}
10:1	0.32 ± 0.16^{a}	27.30 ± 3.38^{a}
100:1	0.25 ± 0.09^{a}	22.51 ± 2.59^{a}
THC Alone	0.11 ± 0.03^{a}	36.29 ± 1.61^{a}

*Values with shared letters (a,b) are insignificantly different and values with different letters are significantly different ($p<0.05$) as identified by ANOVA with Tukey's HSD post hoc test

Table 3-7: EC_{50} values and % cAMP reduction of extract preparations consisting of 100:1, 10:1, and 1:1 THC/CBD as determined by intracellular cAMP Glo MAX™ assay following 1 hour administration of extracts, THC, or vehicle to CB_1-HEK293 cells. Data was collected from n=3 trials and 3 replicates per trial to yield EC_{50}) based on extract weight (µg extract/ml). All trials yielded significant EC_{50} parameters to be included.

Compound Ratio In Extract (THC:CBD)	**EC_{50} µg/ml** (mean ±SE)	**% cAMP Reduction** (mean ±SE)
1:1	0.294 ± 0.09^{a}	12.42 ± 1.56^{b}
10:1	0.125 ± 0.04^{a}	24.76 ± 1.57^{b}
100:1	0.233 ± 0.07^{a}	34.38 ± 3.10^{a}
E19 Alone	0.184 ± 0.04^{a}	36.86 ± 1.52^{a}

*Values with shared letters (a,b) are insignificantly different and values with different letters are significantly different ($p<0.05$) as identified by ANOVA with Tukey's HSD post hoc test

3.4 Discussion and Conclusion

In this chapter, a collection of heat-activated high-THC cannabis extracts were tested *in vitro* for cytotoxicityand cannabimimetic activity to investigate i) whether bioactivity differs (or is the same) among THC-dominant yet phytochemically distinct extracts, and ii) whether bioactivity is predicted by THC content.

3.4.1 Cytotoxicity

Assessed in human broncho-epithelial cell line as a model of pulmonary exposure, the cytotoxicity of different cannabis extracts varied (TC_{50}: 24.5 – 38.8 µg/ml) but, overall, were statistically similar not only to each other but to pure THC (**Table 3-2**). Despite significant differences in extract THC (and CBD) content, the only significant difference was between the most toxic extract (E51), which had the highest THC content, and least toxic extract (E69) which had the 2nd lowest THC content (**Table 3-1, 3-2**). However, when considering TC_{50} relative to THC content for all tested extracts, only a weak positive relationship was observed (**Figure 3-3**), suggesting that THC is not the only compound contributing to cytotoxicity.

When plotting cell viability based on THC concentration rather than extract weight, curves for all extracts shifted to the left with significantly lower TC_{50} values than that of pure THC, again suggestive that there are other components in extracts causing cytotoxicity and that THC alone is not dictating toxicity.

In a recent similar study Janatova *et al.* (2022) assessed the cytotoxicity of six THC-dominant cannabis extracts in five cell lines and observed that Δ^9-THC and CBD appeared to drive toxicity *in vitro* but that differences in selectively suggested potential synergies with the other substances. CBD, which has proven more cytotoxic than THC in endometrial, glioma, and

breast cancer cell lines (Fonseca, 2018; Ligresti et al., 2006; Marcu et al., 2010), was also more toxic than THC to BEAS-2B cells (12.4 vs, 23.3 µg/ml). This finding was contrary to my initial hypothesis in that THC would be more toxic in assay. With CBD concentrations in extracts (0.2 – 1.2%), however, the highest CBD content was 12.35 µg/ml in solution and cells were exposed to a maximum of 1.235 µg/ml of CBD, which is an order of magnitude lower than the observed TC_{50} for CBD. Since extracts with the highest or lowest THC:CBD ratio were not the most or lest toxic, and no relationship was observed between TC_{50} and either CBD content or THC:CBD ratio of extracts, the cytotoxicity of cannabis extracts to BEAS-2B cells cannot be explained by these two cannabinoids alone. Since concentrations of CBD in these experiments remained too low, testing the toxicity of balanced and high-CBD cannabis varieties may be able to further evaluate the role of CBD and potential interactions with THC.

Janatova *et al.* (2022) reported that terpene content influenced cytotoxicity. In the previous chapter, Extract 61 is a "Headband" sample, extract 101 is a "Sour Tangie" sample, extract 83 is "Original Amnesia" and extract 95 is a "Remo Chemo" sample. Extract 61 has a terpene profile reflecting "Other" as well as extract 101 and 83. Multivariate analysis revealed these strains differ in cannabinoid and terpene content by location on PC1 and PC2 (**Appendix Figure 3**). Metabolomic data shows Extract 61, 83 and 95 clustering together in PC1 (0.5) and PC2 (0.5). Differences in toxicity between extracts and THC may be due to compounds that when heated become more cytotoxic at biologically relevant concentrations and may be revealed by studying heated and non heated extracts in cytotoxicity assays.

Bioactivity data shows that heated extracts are more cytotoxic to BEAS-2Bs cells than just their THC content. Peak annotation of cannabis metabolites could shed light on potential toxic compounds found in heated high THC extracts and lead to defining a class of cannabis

which may be correlated to higher toxicity. Toxic concentrations of THC, CBD and extracts can be used in future in other cell models of the upper and lower respiratory tract such as HFL-1 (Lung epithelium), THP-1 (monocytic/ macrophage cells) and HUVEC (vein endothelial) (Muthumalage & Rahman, 2019; Pagano et al., 2020). As vaping and smoking of cannabis extracts and inflorescences becomes me ubiquitous and better research in human populations it may be revealed that some cannabis components may be contributing to higher levels of respiratory cellular toxicity just as this has been identified with E-cigarette flavourings (Morris et al., 2021; Rickard, Ho, Tiley, Jaspers, & Brouwer, 2021) and diluents such as vitamin E acetate (Huanhuan Jiang et al., 2020).

3.4.2 CB_1 Efficacy

It was found that THC had an EC_{50} of 0.107 µg/ml (340 nM) and CBDs EC_{50} = 0.882 (2804 nM) µg/ml. CBDs curve however had a positive slope as exposure appeared to increase intracellular cAMP alone. Hemp extract containing no detectable THC or CBD had no effect in assay.

Extracts for the most part had similar EC_{50} values to THC and other extracts. The only significant differences were E83 and E95 when compared to THC. E83 and E95 have 474.21 ug/mg and 466.65 ug/mg THC content but different THC/CBD ratios with E83 being 38:1 and E95 206:1. Considering this aspect and the lack of significant relationship in general linear regression CBD in THC dominant strains does not affect EC_{50} at CB_1 in this context.

When extract concentration-response curves were considered relative to their respective THC content, curves shifted left and appeared more like that of THC. No significant difference

between THC and extract multiplied by % THC content is evidence that THC proportion in extract is indicative of changes in EC_{50} in THC-dominant extracts. However, amount of THC in extract does not appear to dictate EC_{50} between extracts.

A weak relationship in generalized linear regression of EC_{50} as a function of THC content would contradict this (EC_{50} = -0.123ln(THC) + 1.0191, R^2 = 0.2104). Most likely utilizing a larger sample size of many extracts with a range of low to high THC content would increase the predictive power of linear or generalized linear regression models. A collection of 47 different extracts were also unable to create a predictive model for THC and CBD content for EC_{50} at CB_1 receptors (Yang et al., 2020). The authors quantified major cannabinoids (THC, CBD , THCA and CBDA) in heated extracts of cannabis and measured their activity (EC_{50}) at CB_1 and CB_2. No major cannabinoids could be correlated with CB_1 activity based on a partial least sum of squares model; however, a significance was found at CB_2 using THC and CBD as parameters in a generalized linear model. Modulation at an allosteric site or non-competitive inhibition/ activation would change E_{max} in concentrations response data, as seen with CBD and THC at CB_1 (R. B. Laprairie et al., 2015). This led us to a focus on % cAMP reduction in assay or E_{max} by highest concentrations of extracts and pure compounds in data analysis.

Curve morphology of extracts compared to pure THC alluded potential difference in their respective E_{max} (**Figure 3-8**). % cAMP reduction varied among extracts and THC and generalized linear regression revealed a significant relationship between % cAMP reduction and THC in a CB_1 *in vitro* model (E_{max} = 10.417ln(THC) + 4.6631, R^2 = 0.5122). Incorporation of CBD content into the model was insignificant. E101 and E69 were insignificantly different when compared to pure THC and most effective in reducing intracellular cAMP when compared to other extracts. E101 has the highest THC:CBD ratio and E69 has the second lowest ratio,

both extracts had mid-level THC content. CBDs concentration in heated extract did not significantly effect EC_{50} or % cAMP reduction suggesting the extract content besides THC had effects which resemble NAM. This may be attributed to cannabinoid acids that were not fully decarboxylated in heating or potentially the additive effects of individual or all minor cannabinoids in extract (Rosenthaler et al., 2014).

3.4.3 Pure Compounds vs Heated Extracts

When EC_{50} and % cAMP reduction was compared in pure compounds vs heated 'spiked' extract data, only 1:1 pure THC/CBD was more effective in reducing intracellular cAMP when compared to pure THC, but heated extract 1:1 *and* 10:1 was more effective when compared to pure extract. 1:1 pure THC/CBD was more effective in reducing cAMP compared to extracts of 1:1 THC/CBD, however this relationship flips where 100:1 THC/CBD heated extract was more effective than 100:1 pure compound. Considering this variability in the data set compounds in extracts may be contributing to NAM at CB_1. Heated extract and pure compound concentration-response curves differ insignificantly but EC_{50} does appear to increase as CBD in samples increases. This effect was missing in extract and THC data but was achieved when CBD concentrations were much higher in extract from spiking. Adding more biological replicates in cAMP accumulation assays may reveal significant differences between extracts in future. Also further evaluating the metabolome of heated cannabis and increasing extract sample size may shed some light on what may be causing this disparity between heated extracts and pure compounds.

The apparent interaction of cannabis extract components with THC at the receptor corroborated with other studies in the field of cannabis pharmacology and suggests that a more complete labelling system of cannabis products may be necessary in future. Cannabis inflorescences are often labelled with their dominant terpene(s) and sometimes prevalence of minor cannabinoids like CBG, THCV and CBC. However a more robust phytochemical breakdown such as cannabis 'chemovars', would apply a 'fingerprint' for cultivars of cannabis by the use of metabolomic analysis (A Hazekamp & Fischedick, 2012). More consistent chemistry in strains may lead to more consistent and desired effects for cannabis users which is beneficial to both regulators and industry (Fischedick et al., 2010)

More representative extracts from the 3 established terpene profiles to evaluate CB_1 efficacy is needed to parse out potential differences directly related to phytochemistry especially between high myrcene-pinene and high caryophyllene-limonene groups due to their difference in THC:CBD ratios. β-caryophyllene has been identified as a CB_2 selective agonist (Gertsch et al., 2008) and other terpenes have been connected to THC and CBD entourage effects (E. B. Russo, 2011), however more recent research has begun to refute claims of cannabis terpene entourage (Finlay et al., 2020; Santiago, Sachdev, Arnold, Mcgregor, & Connor, 2019). In future it would be interesting to incorporate TC_{50}, EC_{50} and E_{max} values into the multivariate analysis to determine if some strains cluster out around any of these parameters and are therefore correlated with higher toxicity or efficacy.

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Chapter 4: Final Discussion and Conclusion

4.1 Evaluation of Hypotheses

The first objective in this study was to establish groups of THC-dominant cannabis by applying traditional *indica*, *sativa* and *hybrid* designations or a more modern means of discerning cannabis chemotypes based on terpene profiles. Here I used quantitative phytochemical methods coupled with multivariate and univariate analyses to define cannabis groupings based on cannabinoid, terpene and untargeted metabolomic data. The *indica, sativa* and *hybrid* designations did in fact fall short, as hypothesized, in being able describe variability in cannabinoid, terpene and metabolomic data, meaning that these groups were phytochemically indistinguishable based on our results, as reported previously (Fischedick J, 2015; Sawler et al., 2015).

Two recent studies analyzing large phytochemical datasets of THC-dominant cannabis samples independently identified three major groups based on dominant terpenes (Jikomes et al., 2021; Orser et al., 2018; Reimann-Philipp et al., 2020). Introducing this terpene-based classification system consisting of "High β-Myrcene/Pinene", "High β-Caryophyllene/Limonene" and "High Terpinolene Menthol" was effective in capturing variability in our cannabis collection. Interestingly, these groups were discernable using not only terpene data but cannabinoid-terpene data, but only by the use of pareto scaled data to reduce the impact of abundant THCA content in data. Terpene based clustering, however, was lost at the untargeted level with poor separation of these groups observed in metabolomic data. This observation may be explained by the loss of terpenes during extract drying (for metabolomics), reducing their concentrations and statistical weight in modelling. Retaining these terpenes and repeating the untargeted analyses on freshly made tinctures would help clarify whether the three

terpene “chemotypes” are distinguishable at the metabolomic level. Other factors that may have contributed to the lack of terpene-metabolome relationships were a) too much variability in THC dominant cannabis metabolomes for any groupings to be identified, or b) that there are compounds coupled to unknown mass features beyond cannabinoids and terpenes which are driving separation in the data set. Hazekamp *et al.,* 2016 found that strains labelled either *sativa* or *indica* separate in supervised OPLS-DA which contradicts the findings in this study. THC did not differ between *indica* and *sativa* however CBC and CBG were higher in *sativa* strains and hydroxylated terpenes (guaiol, fenchol, alpha-terpineol, linalool, geraniol, and camphor) were higher in *indica* varieties. Differences in major cannabinoids (THC, CBD and CBG) is very inconsistent across THC-dominant groupings which has led to chemotyping methods that involve a more exhaustive analysis of cannabinoids (acid precursors, -varin side chain derivatives and isomers) to potentially reveal subgroups based on cannabinoid chemistry alone (Cerrato et al., 2021).

I hypothesized that strain samples would exhibit low fidelity to strain name however, some strains appeared to have confident groupings with samples of the same strain name while others seemed to vary based on metabolomic data (**Figure 2-10**). “Girl Scout Cookie”, “Grapefruit GOD”, “Headband”, “Remochemo” and, “White Russian” displayed confident groupings with their own strain while “Kosher Kush”, “Sour Tangie”, “Tahoe OG” and “Violator Kush” displayed high variability. Fischedick *et al.,* 2010 also found that THC-dominant strains do in fact separate based on cannabinoid and terpene content however some strains do display higher variance within strains in PCA and other strains overlap extensively based on phytochemistry. Hazekamp *et al.,* 2012 also found that common ‘coffee shop’ pharmacy samples had tight groupings in PCA, and differing genotypes, but often clustered

within confidence intervals of other cannabis varieties in PCA. Although challenging to achieve, strains with consistent and reliable chemistry should be the ideal endpoint in cannabis breeding and product development since consistent chemistry will yield more consistent bioactivity – not only in research assays but in terms of therapeutic and toxic effects.

β-Myrcene/pinene groups possessed significantly higher CBD content and a significantly lower THC:CBD ratio when compared to β-caryophyllene/ limonene and other terpene groups. This was corroborated by findings by Jikomes *et al.,* 2020 as well as myrcene being correlated to more balanced varieties of cannabis (Arno Hazekamp, Tejkalová, & Papadimitriou, 2016)

Connecting cannabis chemistry to bioactivity THC-dominant heated extracts revealed extracts possess a high proportion of THC, however, extracts are complex and therefore the activity of the remainder of extracts were analyzed. Significant differences in TC_{50} were revealed when extracts were compared based on proportion of THC in extract (µg THC/mg). A mild negative relationship between THC and TC_{50} was found (TC_{50} = -8.029ln(THC) + 77.907, R^2 = 0.3169). More extracts representing balanced and high CBD strains may bolster this relationship as samples remained low and between 390-540 µg/mg THC. The three most toxic extracts E61, E101 and E83 all belonged to the "Other" dominant terpene group. Further investigation into dominant terpenes within this group and MS/MS analysis of mass features correlated to these strains may help identify potential noxious compounds. High variability in EC_{50} led to insignificant differences in EC_{50} between extracts and pure THC besides E69, E101 and THC being significantly lower than E83 and E95. E83 has the highest lowest THC:CBD ratio and very similar THC content to E95. A larger sample size with a wider range of THC:CBD ratios may be needed to determine if ratio does truly effect EC_{50} or not. In this context it can be assumed that CBD and other minor cannabinoid and phytochemical content is

not interacting significantly with the orthosteric site of CB_1. This is bolstered when extract EC_{50} values were calculated based on their THC content and all curves fell around the THC curve and differed non significantly. It would appear when observing pure compounds and spiked extract data that even at high CBD concentrations, EC_{50} remains unaffected. However, ratios of 1:1 and 10:1 THC/CBD do in fact significantly reduce E_{max} suggestive of NAM. % cAMP reduction or E_{max} in this context showed lower intra strain variability and a confident general linear model $E_{max} = 10.417\ln(THC) + 4.6631$, $R^2 = 0.5122$ ($p<0.001$). Again, CBD concentrations in extracts appears to be too low as adding a CBD term to the regression was found nonsignificant. Considering the lower E_{max} of extracts E51, E61, E95 and E83 with no correlation to THC:CBD ratio, it could be concluded that components of heated extracts are contributing to NAM at CB_1 receptors.

4.2 Real World Application

Studying complex plant extract chemistry and relating chemistry to bioactivity (biochemometrics) is a necessary model to understand not only cannabis but natural health products in general. Metabolomics fit perfectly into the study of cannabis as there exists many metabolites with known or unknown interaction with THC/CBD and receptors (Aliferis & Bernard-Perron, 2020; E. B. Russo, 2011). With increasing knowledge of cannabis chemistry, a more modern classification systems such as terpene profiling will become the standard in recreational and medicinal cannabis use, moving away from – or at least complementing – popular *indica*, *sativa* and *hybrid* designations.

More studies contributing to toxicity of extracts is required to flag potentially noxious pyrolytic compounds which may then be related to strains that more readily accumulate these compounds. Inhalation of vaporized and smoked cannabis is still the most popular mode of

consumption (Rotermann, 2019). Smoked or vaporized THC and CBD have similar bioavailability (10-35%) and dosing depends on number of inhales, duration, interval of puffs, breath hold time and inhalation volume (Solowij, Broyd, van Hell, & Hazekamp, 2014).

Growers and vendors as well as recreational consumers would benefit from enhanced evidence of which cannabinoids or compounds truly create a more effective therapeutic or pleasing psychoactive experience. CBD seems to modulate some of the effects of THC and users of high CBD strains appear to lower appetitive effects for drug and food stimuli (Morgan, Freeman, Schafer, & Curran, 2010). Preference for varieties based on user experience is still mainly influenced by *indica* and *sativa* designations still as well as terms like 'Haze', 'Cheese' and 'Kush' (Mason, Sami, Notley, & Bhattacharyya, 2021). Users tend to choose based on THC:CBD ratio as well depending on the desired effects such as pain or sleep regulation (Babson, Sottile, & Morabito, 2017). More recently cannabis edibles have started to gain more popularity in since legalization in Canada. Edible cannabis circumvents the direct access to the blood stream where it is subjected to first pass metabolism by CYP p450 liver enzymes (Lucas et al., 2018a). THC is metabolized to 11-hydroxy THC and CBD to 7-hydroxy CBD. 11-hydroxy THC has been identified as having psychoactive activity, but little is known about the metabolites of CBD. Metabolized plant extract efficacy at CB_1 and CB_2 would therefore be relevant to study *in vitro*.

4.3 Future Considerations

4.3.1 Allosteric Modulation

Well established *in vitro* methodology has been employed to study cannabinoid type 1 and 2 receptor signalling. These systems consist of an immortalized cell lines meaning they will

grow indefinitely following ATCC guidelines. CB_1 and CB_2 recruit G proteins upon stimulation where the alpha subunit, either $G\alpha_{i/o}$, $G\alpha_s$ or $G\alpha_q$, may be liberated from the complex to activate downstream cellular pathways. CB activation also recruits β-arrestin1 or β-arrestin2 which may lead to gene transcription and internalization/ desensitization respectively (Wouters et al., 2019). CB_1 is pertussis toxin (PTx) sensitive meaning signalling through $G\alpha_{i/o}$ subunit can be blocked by the addition of PTx to experimental media. Similarly, signalling through $G\alpha_s$ is blocked by Cholera toxin (CTx). A control at the orthosteric site of CB_1 could be a synthetic antagonist such as O-2050. If bioactivity is not blocked by a CB_1 antagonist, it could be speculated that signalling is being mediated through a different receptor. Allosteric modulation of receptors may be negative or positive by decreasing or increasing the affinity of the agonist for the orthosteric site. Negative allosteric modulation by CBD has been identified but potential positive modulation by minor cannabinoids may enhance the effects or THC or endocannabinoids (AEA and 2-AG). Recently an operational model of allosteric modulation was proposed by Jakubík, Randáková, Chetverikov, & Fakahany, 2020):

$$Response = \frac{E_{MAX}\tau_A[A](K_B + \alpha\beta[B])}{[A]K_B + K_AK_B + [B]K_A + \alpha[A][B] + \tau_A[A](K_B + \alpha\beta[B])}$$

Where [A] is the concentration of the agonist and [B] is the concentration of the allosteric modulator. E_{MAX} is the maximal response of the system, K_A and K_B are the dissociation constants for agonist-receptor and allosteric modulator-receptor complex, respectively and τ_A is the operational factor of efficacy of the agonist. α represents the factor of binding cooperativity of the agonist enzyme complex and β represents the binding cooperativity of the enzyme-agonist-modulator complex. More advanced modeling of allosterism is difficult with plant extracts as there are many compounds with potential activity at orthosteric and allosteric sites,

identifying dissociation constants for all compounds would require radioligand displacement assays and cell sorting which is beyond the scope of the lab and this study. However, this technique is still possible, and this model can be fit with modulation data in future.

4.3.2 Natural Expression Systems and Signalling Bias

Cells that endogenously express CB_1 and CB_2 receptors are natural expression systems which is one step closer to modelling an *in vivo* system. It is common practice to study a heterologous expression system alongside a natural expression system. Common preparations include rat whole brain membranes (cerebellar, striatal or hippocampal), neuroblastoma (N18TG2) (Dalton & Howlett, 2012) or ST*Hdh*$^{Q7/Q7}$ cells (R. B. Laprairie et al., 2015; Robert B. Laprairie et al., 2014). An important consideration for any natural expression system are existing enzymes that synthesize and hydrolyze endocannabinoids. The controls for these enzymes is Phenylmethylsulphonyl fluoride (PMSF), a non-specific fatty acid amide hydrolase FAAH inhibitor, will inhibit the degradation of anandamide and 2-AG, as well as other potential phytocannabinoids (Mcpartland, Duncan, Marzo, & Pertwee, 2015). Secondly IBMX is a selective phosphodiesterase inhibitor that prevents the degradation of cAMP in cell culture, which is also necessary when using an endogenous expression system (Dhopeshwarkar & Mackie, 2016; Soethoudt et al., 2017; Vrecl et al., 2009).

The endocannabinoids AEA and 2-AG are degraded by fatty acid amide hydrolase FAAH and monoacylglycerol lipase (MAGL) respectively (Di Marzo, 2006). FAAH degradation occurs by the hydrolysis of the amide bond in AEA and 2-AG to produce arachidonic acid and ethanolamine (Cravatt et al., 2001). As a result, *faah* knockout mice and FAAH inhibitors have been studied to increase endogenous AEA levels (Fegley et al., 2005; Kathuria et al., 2003) which proved effective in mediating inflammation and anxiety in a mouse model. CBD has been

shown to inhibit FAAH synthesis (Watanabe et al., 1998) which could modulate downstream bioactivity.

More forward, it has been established that cannabinoid binding at CB_1 receptors decrease Ca^{2+} conductance (K. Mackie & Hille, 1992) and enhances inward rectifying K+ channel conductance (K. Mackie, Lai, Westenbroek, & Mitchell, 1995). Along with this, inhibition of adenylate cyclase (AC) has been demonstrated in cultured cells expressing CB_1 (Matsuda et al., 1990) as well as CB_2 (Slipetz et al., 1995). Intracellular cAMP mediates the increase in voltage-dependent potassium A-current I_A in rat hippocampal cells (Deadwyler, Hampson, Mu, Whyte, & Childers, 1995). This as a result lowers EPSPs generated in the terminals of central and peripheral neurons (Childers & Deadwyler, 1996; Pertwee, 2008). Studying changes in Ca^{2+} conductance involves real-time measurements and can be performed using the Fura-2-AM assay kit (ThermoFisher).

4.4 Conclusion

Terpene profiles based on dominant terpenes in cannabis inflorescence was sufficient in discerning groups in THC-dominant cannabis varieties however these profiles were insufficient in discerning groups in metabolomic data using multivariate analysis. In terms of strain sample fidelity to strain name based on chemistry, some strains exhibited very similar chemistry and clustered tightly with other samples of the same name while others displayed large overlapping confidence intervals in PCA. We conclude that the strains “grapefruit GOD”, “headband” and “White Russian” are examples of ‘good chemovars’ based on multivariate analysis of metabolomic data.

Here we show that toxicity is not completely dictated by THC content and in fact other extract components are contributing to toxicity in BEAS-2Bs. CBD is found to be nearly twice as toxic as THC however high THC (drug-type) cannabis heated extracts appear to have levels of CBD which are insufficient in effecting TC_{50}. THC within extracts appeared to drive efficacy at CB_1 receptors by reducing intracellular cAMP accumulation but did not dictate variation in EC_{50}. THC in extract also appeared to increase percent cAMP reduction in cells regardless of low CBD content but a 1:1 balanced THC/CBD extract revealed reduced percent cAMP reduction. Pure compounds compared to extracts of the same THC/CBD ratio performed very similarly at CB_1 receptors besides 1:1 extract preparation having reduced % cAMP reduction compared to 1:1 pure compounds suggestive of NAM by extract components. These results suggest that not all high THC varieties of cannabis are the same both in chemistry but also as evidenced by extract performance in cytotoxicity and CB_1 activation assays.

One year after cannabis legalization still 40.1% of Canadians acquire their cannabis illegally and therefore free of regulation (Rotermann, 2020). A better understanding of the variability in cannabis and dosing by QA/QC will ensure safe cannabis use as more of the population purchase cannabis products from licensed vendors. Growers would benefit from having more confidence in strain fidelity to established strain chemistry by opting for plants that appear to have similar and reliable chemistry. Biochemometrics and continued monitoring of cytotoxicity and efficacy of plant extracts will contribute to the working database of TC_{50} and EC_{50} values that will in turn benefit both regulators and consumers. A more robust understanding of cannabis compound activity *in vitro* and more rigorous effort to identify strains or ‘chemovars’ that maintain consistent phytochemistry will lead to safe and effective products.

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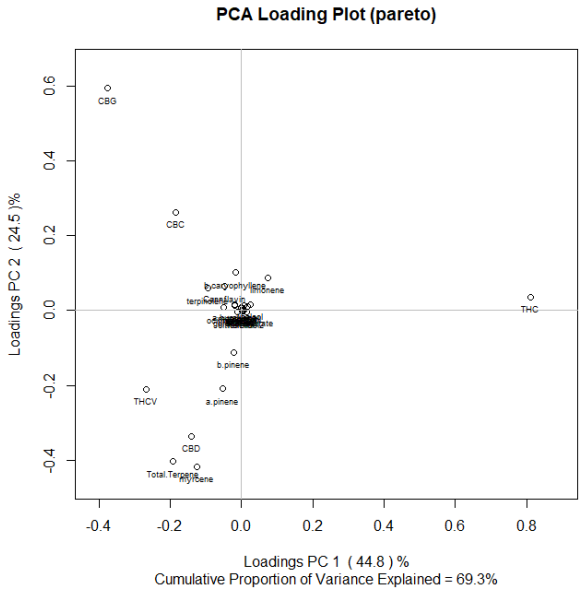
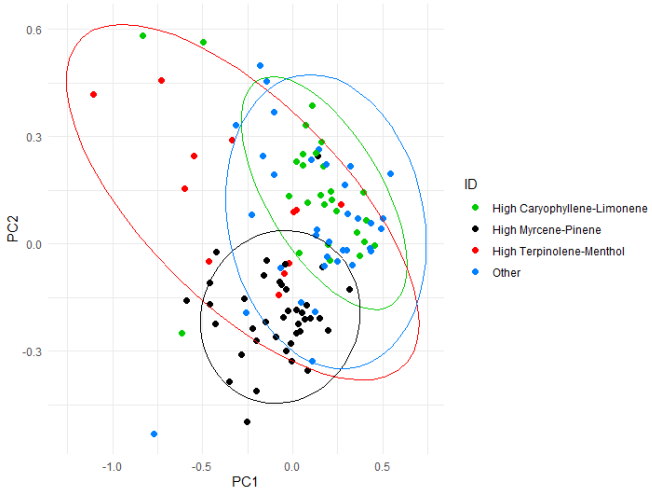
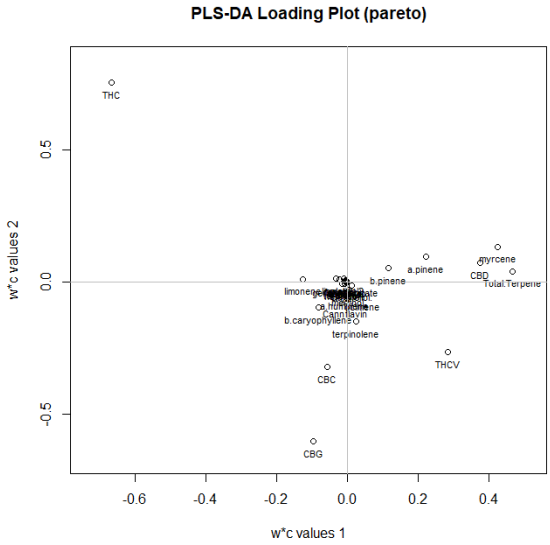
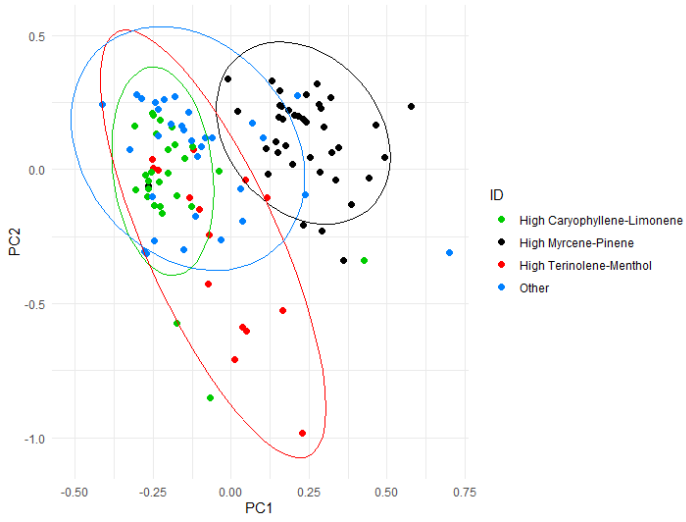
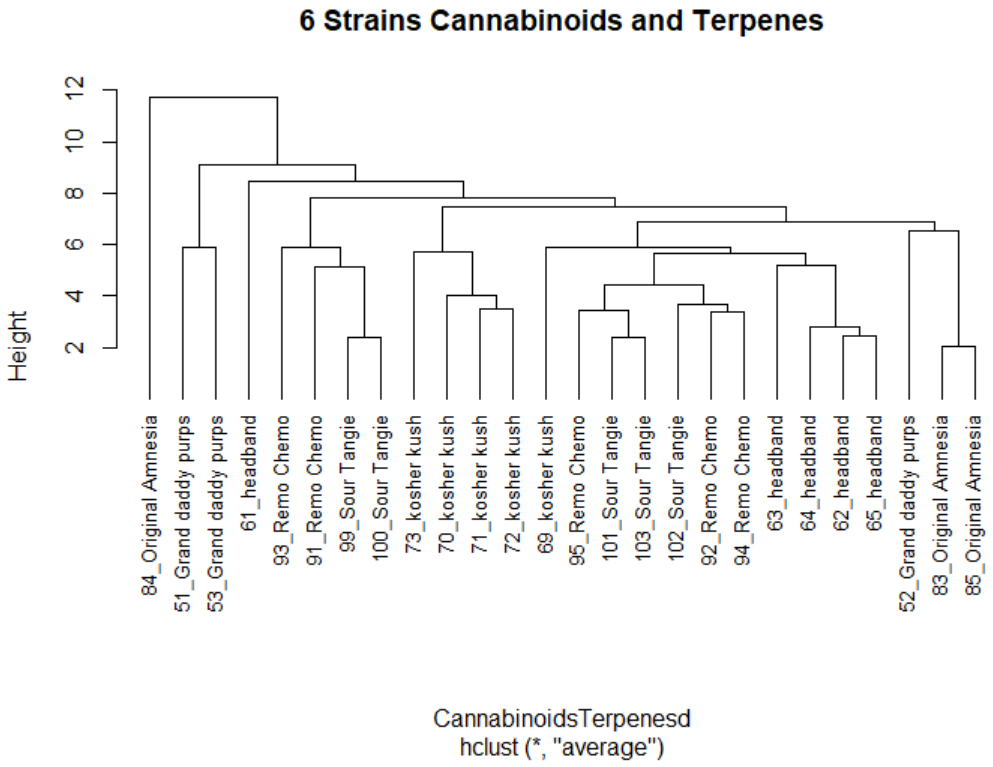
Appendices


PCA Loading Plot (pareto)
Loadings PC 2 (24.5)%
Loadings PC 1 (44.8) %
Cumulative Proportion of Variance Explained = 69.3%
CBG
CBC
THC
THCV
CBD
b.pinene
a.pinene
Total.Terpene
myrcene
limonene
terpinolene

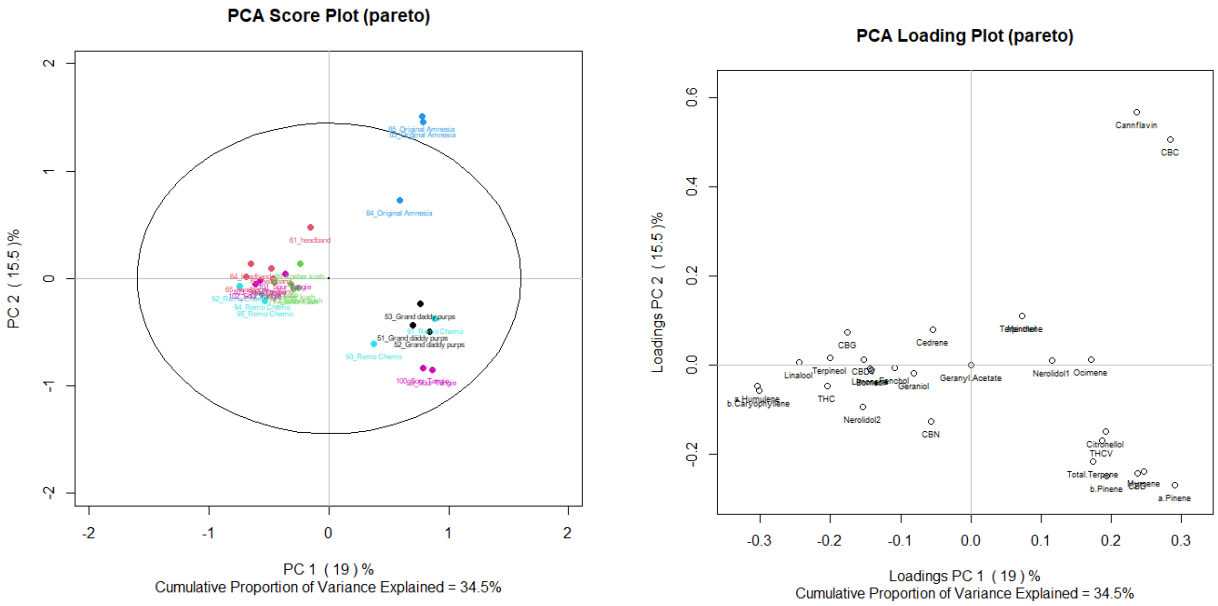

PC2
PC1
ID
High Caryophyllene-Limonene
High Myrcene-Pinene
High Terpinolene-Menthol
Other


PLS-DA Loading Plot (pareto)
w*c values 2
w*c values 1
THC
CBG
CBC
THCV
CBD
a.pinene
b.pinene
myrcene
Total.Terpene
terpinolene
b.caryophyllene


PC2
PC1
ID
High Caryophyllene-Limonene
High Myrcene-Pinene
High Terinolene-Menthol
Other



Appendix Figure 2: Hierarchical cluster analysis of strain metabolomes using unweighted pair group method with arithmetic mean (UPGMA) or average linkage clustering. Strains represented as their ID_strainname. Each ID represents the average of 3 replicates (n=3)



Appendix Figure 3: Score and loadings plot of 6 strains selected with 3-5 replicates per strain name as represented by colour (n=3-5) for analysis in bioactivity assays. Cannabinoid and terpene loading data was pareto scaled in R and unsupervised clustering performed.

Appendix Table 1: Tukey's HSD *post hoc* analysis results of differences in extract TC_{50} when compared based on THC content to pure THC.

Extract	diff	p adj
E51	-8.56	0.01
E61	-13.37	0.0001
E69	-7.71	0.013
E83	-8.80	0.00437
E95	-8.84	0.011
E101	-11.11	0.004

Appendix Table 2: Monoisotopic masses and major mass adducts from UPLC-HRMS analysis of cannabis standards

Standard	Monoisotopic mass	M+H	M+H-H2O	Retention Time
46_THCV_100ugml_MeOH	286.1926	287.199876	269.192676	5.57
47_CBDV_100ugml_MeOH	286.1926	287.199876	269.192676	5.91
48_CBN_100ugml_MeOH	310.1926	311.199876	293.192676	6.14
49_CBG_100ugml_MeOH	316.2394	317.246676	299.239476	5.88
50_CBD_100ugml_MeOH	314.2238	315.231076	297.223876	5.91
51_D9_THC_100ugml_MeOH	314.2238	315.231076	297.223876	6.29
52_CBC_100ugml_MeOH	314.2238	315.231076	297.223876	6.45
53_CBDVA_100ugml_ACN	330.1824	331.189676	313.182476	5.52
54_THCVA_100ugml_ACN	330.1824	331.189676	313.182476	6.12
55_CBGA_100ugml_ACN	360.2292	361.236476	343.229276	5.87
56_CBCA_100ugml_ACN	358.2136	359.220876	341.213676	6.51
57_CBNA_100ugml_ACN	354.1824	355.189676	337.182476	6.34
58_CBDA_100ugml_ACN	358.2136	359.220876	341.213676	5.79
59_D9_THCA_100ugml_ACN	358.2136	359.220876	341.213676	6.49