The bioactivity of cannabis phytochemicals for antimicrobial activity, gastrointestinal and urological protection

Submitted by

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ABBREVIATIONS AND ACRONYMS

2',7'-Dichlorodihydrofluorescein diacetate	DCFDA
40,6-diamidino-2-phenylindole	DAPI
Acinetobacter baumannii	*A. baumannii*
Adenosine triphosphate	ATP
American Type Culture Collection	ATCC
Analysis of variance	ANOVA
Antibiotic-resistant microbial infection	ARMI
Antimicrobial resistance	AMR
Bicinchoninic acid	BCA
Biofilm killed	BK
Bovine serum albumin	BSA
Cannabichromene	CBC
Cannabidiol	CBD
Cannabigerol	CBG
Cannabinoid receptor 1	CB1-R
Cannabinoid receptor 2	CB2-R
Cannabinol	CBN
Cannabis sativa	Cannabis

Carbon dioxide	CO_2
Cation-adjusted MHB	CAMHB
Clinical isolate	CI
Cyclooxygenase	COX (COX-1 and COX-2)
Dimethyl sulfoxide	DMSO
Dulbecco's Modified Eagle Medium	DMEM
Endocannabinoid system	ECS
Enterococcus faecium	*E. faecium*
Escherichia coli	*E. coli*
Ethylenediaminetetraacetic acid	EDTA
Fluorescence of CBD derivative treatment	F_T
Fluorescence of untreated control	F_C
Foetal bovine serum	FBS
Fractional inhibitory concentration index	FICI
Gastrointestinal	GI
Gastrointestinal mucositis	GIM
Glutathione	GSH
G-Protein coupled receptor	GPCR (GPR55 and GPR119)
Heat shock factor 1	HSF1
Human bladder carcinoma cell lines	RT4 and T24

Human colonic carcinoma cells	Caco-2
Humulus lupulus	Hops
Inflammatory bowel disease	IBD
Irritable bowel syndrome	IBS
Inhibitor of NF-κB alpha	IκBα
Interleukin 1-β	IL-1β
Klebsiella pneumoniae	*K. pneumoniae*
Lipopolysaccharide	LPS
Luria-Bertani broth	LB
Methicillin-resistant *Staphylococcus aureus*	MRSA
MIC of antibiotic in the presence CBD derivative	MIC_{A+B}
MIC of antibiotic treatment alone	MIC_{A}
MIC of CBD derivative in the presence of antibiotic	MIC_{B+A}
MIC of CBD derivative treatment alone	MIC_{B}
Minimum bactericidal concentration	MBC
Minimum inhibitory concentration	MIC
Mitogen-activated protein kinase	MAPK
Mueller-Hinton broth	MHB
N-acetylcysteine	NAC
Non-essential amino acids	NEAA

Nonsteroidal anti-inflammatory drugs	NSAID
Nuclear factor erythroid 2-related factor 2	Nrf2
Nuclear factor-kappa B	NF-κB
Optical Density 600 nm	OD_{600}
Optical Density of growth control sample	OD_{GC}
Optical Density of treatment sample	OD_{T}
Peroxisome proliferator-activated receptors	PPAR
Phosphate buffered saline	PBS
Phytocannabinoids	pCBs
Polyethylene Terephthalate	PET
Polyvinylidene fluoride	PVDF
Pseudomonas aeruginosa	*P. aeruginosa*
Radioimmunoprecipitation buffer	RIPA
Reactive oxygen species	ROS
Standard error of the mean	SEM
Staphylococcus aureus	*S. aureus*
Staphylococcus epidermidis	*S. epidermidis*
Structure-activity relationship	SAR
tert-butyl hydroperoxide	t-BHP
Thiazolyl blue tetrazolium bromide	MTT

Topoisomerase II	TOP2
Trans-Epithelial Electrical Resistance	TEER
Transient receptor potential of the vanilloid type-1	TRPV1
Transient receptor potential of the vanilloid type-4	TRPV4
Tryptic soy agar	TSA
Tumour necrosis factor-α	TNF-α
X-linked Inhibitor of Apoptosis Protein	XIAP
Δ^9-tetrahydrocannabinol	THC

ABSTRACT

Antimicrobial resistance, inflammatory gastrointestinal disorders and bladder disease are significant global health challenges, each placing a substantial burden on healthcare systems with rising incidence and treatment limitations. Although often studied independently, these challenges share overlapping features, including chronic inflammation, immune dysregulation and impaired cellular integrity which can increase susceptibility to infection and complicate treatment. Conventional treatments are frequently associated with toxicity, resistance or limited efficacy highlighting the need for alternative therapeutic options. Phytochemicals from *Cannabis sativa L.* including cannabinoids, terpenes and their derivatives have emerged as promising candidates due to their diverse and multifunctional pharmacological activities.

This thesis investigated the i). Antimicrobial potential of cannabidiol (CBD) and CBD derivatives against antibiotic resistant bacteria, ii). Gastroprotective bioactivity of terpenes in Caco-2 epithelial cells and iii). Cytotoxicity of novel cannabidiol hydroxyquinone (CBDHQ) in bladder urothelial cancer cells.

The first Chapter screened 42 CBD derivatives against ESKAPE pathogens using standard microbiological assays, including Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), checkerboard synergy and AlamarBlue biofilm viability. Several derivatives demonstrated bactericidal activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* and *Enterococcus faecium*, with two compounds demonstrating synergy with daptomycin. No activity was observed against Gram-negative species. Growth curve analysis confirmed rapid bactericidal action within 6 hours, and minimal cytotoxicity was observed at active concentrations supporting further investigation into CBD-based antimicrobial development.

The second Chapter evaluated the cytotoxic and protective effects of 16 terpenes in Caco-2 colonic epithelial monolayers and cells using Thiazolyl Blue Tetrazolium Bromide (MTT) viability assays, Trans-Epithelial Electrical Resistance (TEER) for barrier function effects and Western blot analysis of selected tight junction proteins. Most terpenes demonstrated low intrinsic toxicity at ≤200 μM, with α-bisabolol, linalool, and citronellol enhancing Caco-2 cell viability. Notably, linalool preserved TEER under inflammatory conditions; however, there was no upregulation of tight junction proteins claudin-1 or occludin, suggesting alternative mechanisms such as cytoskeletal stabilisation or redox modulation for the barrier-augmenting effect of this terpene.

The third Chapter assessed the cytotoxic effects of CBD and CBDHQ in malignant RT4 and T24 bladder urothelial cancer cells using the MTT cell viability and 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA) reactive oxygen species (ROS) assays. Both compounds reduced cell viability in a concentration-dependent manner, with heightened sensitivity in the more highly proliferative T24 cells. CBDHQ exhibited thiol-sensitive activity without significantly elevating ROS, indicating a non-redox associated mechanism of action for cytotoxicity.

Together, these findings demonstrate the broad and selective bioactivity of Cannabis-derived compounds and their derivatives. However, further research and development is required to explore their potential as adjunctive or standalone therapies for infectious diseases, gastrointestinal inflammation and urothelial cancer. Future research should consider individual variability, prioritise *in vivo* validation, pharmacokinetic profiling and formulation development to enable clinical translation.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree. The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time. I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

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Date signed: 31/07/2025

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CONFERENCE ATTENDANCE AND POSTER PRESENTATIONS

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AIMS AND OBJECTIVES

This thesis aimed to investigate the therapeutic potential of cannabis-derived phytochemicals across three domains of human health: antimicrobial resistance, gastrointestinal inflammation, and bladder cancer. The first study evaluated the antimicrobial activity of cannabidiol (CBD) and its derivatives against antibiotic-resistant pathogens. The second assessed the bioactivity of cannabis-prevalent terpenes on intestinal epithelial cells and associated barrier function under normal and inflammatory conditions. The third examined the cytotoxic effects of CBD and its metabolite cannabidiol hydroxyquinone (CBDHQ) in bladder cancer cell lines. Collectively, these studies explore the pharmacological potential of cannabis-derived phytochemicals in key clinical areas of unmet need, affording insights into the bioactivity of cannabis components contemporarily to the emerging use of medicinal cannabis in Australia and globally.

CHAPTER 1

LITERATURE REVIEW

1.0 INTRODUCTION

Cannabis sativa (cannabis) is well known for its diverse biochemical composition, comprised of a mixture of phytochemicals such as phytocannabinoids (pCBs), terpenes/terpenoids (monoterpenes, diterpenes and sesquiterpenes) and flavonoids. Thus far, more than 120 pCBs, 400 terpenes/terpenoids and 20 flavonoids have been identified (Rice and Koziel, 2015; Hanuš *et al.,* 2016; Booth and Bohlmann, 2019; Sommano *et al.,* 2020; Bautista *et al.,* 2021). These compounds have demonstrated potential therapeutic effects in conditions such as gastrointestinal disease and urinary disorders, as well as in the treatment of antibiotic-resistant microbial infection (ARMI) (Whiting *et al.,* 2015; Ahmed and Katz, 2016; Pagano *et al.,* 2016; Fontes *et al.,* 2017; Abrams, 2018; Couch *et al.,* 2018; Millar *et al.,* 2018; Naftali, 2020; dos Reid Rosa Franco *et al.,* 2020).

However, the majority of research has focused on two pCBs in particular; Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) leaving a large proportion of cannabis-derived phytochemicals, novel pCBs, terpenes/terpenoids and flavonoids that warrant further investigation. This gap in research presents an opportunity to improve our understanding of the safety profiles and potential side effects of cannabis phytochemicals, as well as the synergistic interactions referred to as the "entourage effect" which remain poorly defined. (Ben-Shabat *et al.,* 1998; Russo, 2011; Iffland and Grotenhermen, 2017; Russo, 2019). As such, studies investigating novel pCBs, derivatives and other cannabis-derived phytochemicals are valuable for informing and developing cannabis-based therapeutics.

1.1 PHYTOCANNABINOIDS

Phytocannabinoids are naturally occurring C21 terpenophenolic compounds common to all species of cannabis (ElSohly *et al.,* 2017; Berman *et al.,* 2018). They share similar

structural features comprised of three moieties: the isoprenyl residue **(A)**, the resorcinol nucleus **(B)** and the alkyl side chain **(C)** (Figure 1-1) (Hanuš *et al.,* 2016) The structure of pCBs varies greatly and so they are divided into sub-classes based on their structure (Figure 1-2) (Marsh and Smid, 2021). This structure can be manipulated to form derivatives ultimately influencing their activity and directly influencing their biological and pharmacological effects (Compton *et al.,* 1993; Stern and Lambert, 2007; Chung *et al.,* 2021). This bioactivity including antioxidant and anti-inflammatory action contributes to protective effects mediated through multiple biochemical pathways. Given their chemical and biological diversity, pCBs represent promising candidates for the development of novel therapeutics.

CBD in particular has gained interest due to its demonstrated anticancer activity in preclinical models. Studies across a range of cancer types have shown that CBD impairs cell viability, promotes apoptosis, disrupts mitochondrial function and increases intracellular reactive oxygen species (ROS) (Ruiz, Miguel and Díaz-Laviada 1999; Carracedo *et al.,* 2006; Greenhough *et al.,* 2007; Aviello *et al.,* 2012; Hernán Pérez de la Ossa *et al.,* 2013; Massi *et al.,* 2012; De Petrocellis *et al.,* 2013; Kosgodage *et al.,* 2018; Honarmand *et al.,* 2019; Simmerman *et al.,* 2019).

A key oxidative metabolite of CBD, cannabidiol hydroxyquinone (CBDHQ or HU-331), has also shown therapeutic potential demonstrating cytotoxic effects through mechanisms such as topoisomerase II inhibition, resulting in cell cycle arrest independent of ROS production (Kogan *et al.,* 2007; Regal, Mercer and Deweese 2014; Wilson *et al.,* 2018). Notably, CBDHQ appears to selectively target cancer cells making it a promising therapeutic candidate (Massi *et al.,* 2012; Jiang, Tashiro and Ikejima 2020; Trac, Keck and Deweese 2021). However, further research is needed to evaluate its efficacy and safety, particularly in preclinical animal models.

Additionally, CBD has demonstrated antimicrobial activity against a wide range of more than 20 Gram-positive bacteria, including highly resistant strains of *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Clostridioides difficile*. This activity extends to a small subset of Gram-negative bacteria including pathogens of concern such as *Neisseria gonorrhoeae.* With low propensity to induce resistance and excellent activity against biofilms (an accumulation of bacterial culture/s encased in a protective film), CBD has the potential to become a new class of antimicrobial agent. However, to improve on its antibiotic properties further research is required to develop derivatives with systemic *in vivo* activity (Blaskovich *et al.,* 2021), including overcoming limitations in the oral bioavailability of CBD.

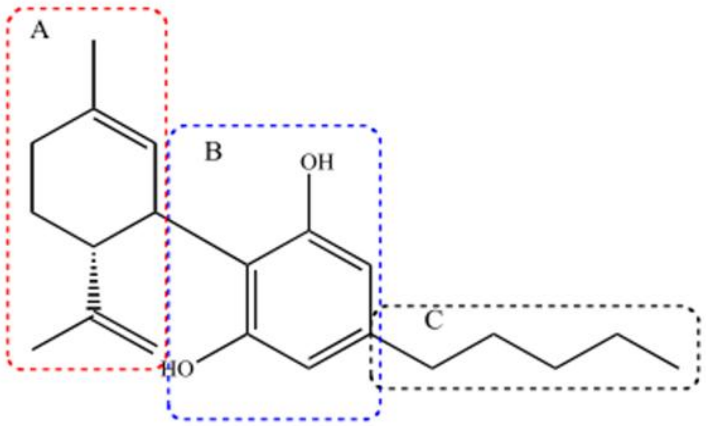

Figure 1-1. Schematic general structure of a phytocannabinoid skeleton, with the three variable structural fragments: the isoprenyl residue (A), the resorcinol nucleus (B) and the alkyl side chain (C).

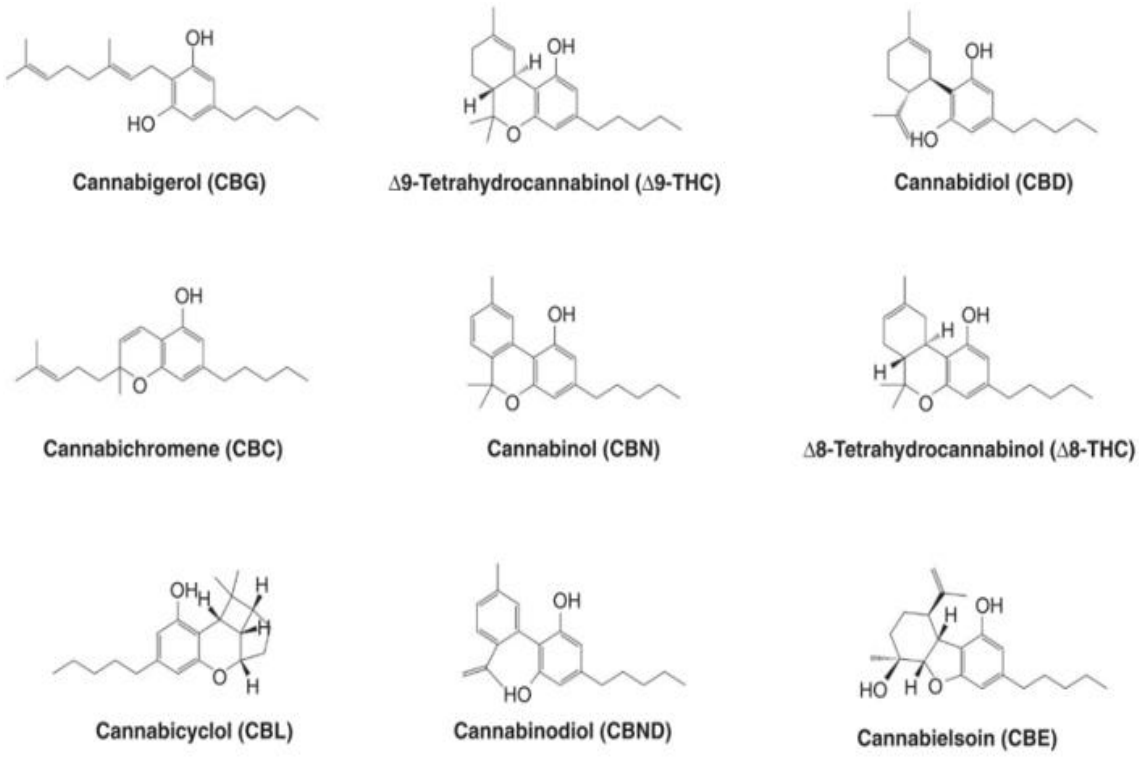

Figure 1-2. Structures of the main phytocannabinoids

1.2 TERPENES/TERPENOIDS

Terpenes and terpenoids are a naturally occurring class of aromatic hydrocarbons produced by a wide variety of plants and some animals often functioning as attractants or repellents. They are commonly found in essential oils and are responsible for the typical fragrance of many plants, protecting against pathogens such as mould, fungus and bacteria, and can attract pollinating insects or repel herbivores (Gershenzon and Dudareva, 2007; Chen *et al.,* 2011). Terpenoids differ from terpenes as they contain an oxygen moiety and additional structural rearrangements that can influence bioactivity (Boncan *et al.,* 2020; Zielińska-Błajet and Feder-Kubis, 2020).

In comparison, *Humulus lupulus* (hops) belong to the same plant family as cannabis; *Cannabaceae*. They both produce and accumulate terpene/terpenoid-rich glandular trichomes most abundant on the surface of the female inflorescence. Although hops are

devoid of pCBs they contain many of the same terpenes/terpenoids found in cannabis (Bernotienë *et al.,* 2004; Bedini *et al.,* 2016). Terpenes/terpenoids from cannabis, hops and other essential oils exhibit a wide range of biological activities with potential applications in both medicinal and pharmaceutical contexts. Historically, hops have been used as a sedative and for the treatment of insomnia, depressive symptoms, anxiety and digestive disorders. These compounds have demonstrated anxiolytic, antibacterial, anti-inflammatory, anti-spasmodic, anti-diabetic and sedative effects. Accordingly, this therapeutic potential could be applied in the treatment of pain, inflammation, depression, anxiety, addiction, epilepsy, cancer, fungal and bacterial infections (Russo, 2011; Nuutinen, 2018; Zielińska-Błajet and Feder-Kubis, 2020).

Greater antimicrobial activity was observed from terpenes/terpenoids containing hydroxyl groups (phenolic and alcohol compounds), supporting the theory that additional structural arrangements may influence bioactivity. Eugenol, terpineol, carveol, citronellol and geraniol from essential oils have demonstrated the ability to reduce cell membrane integrity and function in bacterial cultures of *Salmonella enterica*, *Staphylococcus aureus* and *Escherichia coli* (Guimarães *et al.,* 2019). Some of the predominant terpenes present in cannabis include myrcene, terpineol, carveol, citronellol, β-caryophyllene, caryophyllene oxide and humulene (Figure 1-3). However, the presence and quantity of terpenes will be influenced by a variety of factors including chemovar genetics, environmental conditions, processing and storage. For example, β-caryophyllene comprises 64% of the terpene content for the chemotype “gorilla glue” but is almost absent (1.2 ± 0.2%) in other cannabis chemotypes (Nuutinen, 2018).

Today, terpenes and terpenoids from a variety of plant-derived sources are used in industry as food additives and traditional medicines such as gums/mints, beverages, healthcare products and cosmetics. They are well tolerated without side effects and therapeutic effects are acquired with a wide margin to toxic and lethal doses (Nuutinen, 2018).

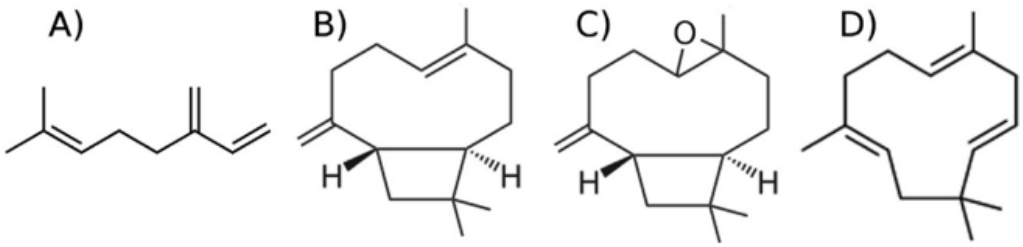

Figure 1-3. Structures of myrcene (A), β-caryophyllene (B), caryophyllene oxide (C) and humulene (D).

1.3 FLAVONOIDS (CANNFLAVINS)

Alongside pCBs, terpenes and terpenoids are 20 different flavonoids as well as 3 cannflavins, cannflavin A, B and C that have been identified so far. The latter are prenylated (C5) and geranylated (C10) flavones that are relatively unique to cannabis (Figure 1-4) (Radwan *et al.,* 2008; Flores-Sanchez and Verpoorte, 2008; Bautista *et al.,* 2021). These cannflavins have demonstrated a variety of therapeutic properties including anti-inflammatory, anti-oxidative, anti-cancer and anti-parasitic bioactivity via a variety of mechanisms (Fairbairn and Pickens, 1981; Barrett *et al.,* 1985; Brunelli *et al.,* 2009; Ogungbe *et al.,* 2014; Werz *et al.,* 2014; Moreau *et al.,* 2019).

One notable advantage of cannflavin A is weak inhibition of cyclooxygenases (COX) COX-1 and COX-2, therefore circumventing adverse side effects such as gastrointestinal erosion exhibited by non-steroidal anti-inflammatory drugs, whereby mode of action is COX inhibition (Bautista *et al.,* 2021). Although these compounds are in low concentrations under normal growth conditions, there are ways to induce over-expression to potentially increase their accumulation. While the cannflavins were not a primary focus in this thesis, their novel structures and relative exclusivity in cannabis warrants further investigations into bioactivity.

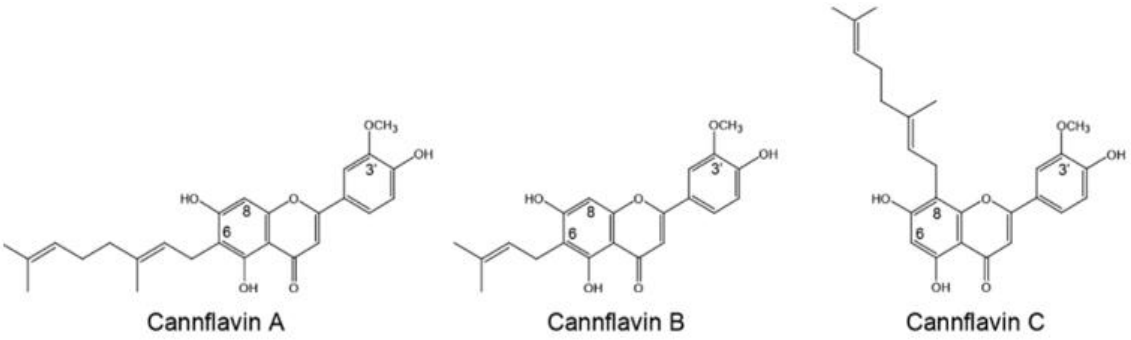

Figure 1-4. Structures of cannflavin A, B and C.

1.4 THE ENDOCANNABINOID SYSTEM

The endocannabinoid system (ECS) is a complex lipid signalling network that plays a critical role in maintaining physiological homeostasis in response to internal and external stressors. It comprises cannabinoid receptors, endogenous ligands (endocannabinoids) and associated biosynthetic and degradative enzymes (Iannotti *et al.,* 2013; Iannotti *et al.,* 2014). The two principal receptors, cannabinoid receptor 1 (CB1-R) and cannabinoid receptor 2 (CB2-R), are G-protein-coupled receptors (GPCRs) predominantly expressed in the central nervous and immune systems respectively, but are also found throughout the gastrointestinal epithelium and bladder urothelium (Andersson 2002; Fowler, 2002; Hayn *et al.,* 2008; Grundy, Johnson and Greenwood-Van Meerveld, 2017). The differential distribution of CB1-R and CB2-R receptors allows for potentially distinct functional roles in modulating gut and bladder physiology.

Endocannabinoids such as anandamide and 2-arachidonoylglycerol are produced on demand from membrane phospholipids and act as neuromodulators. Within the enteric nervous system these ligands contribute to the regulation of gastrointestinal motility, secretion and inflammatory responses. By modulating both presynaptic and postsynaptic neurotransmission they influence the activity of enteric neurons and glial cells (Cani *et al.,*

2007). This interplay is essential for maintaining gut homeostasis and responding to inflammatory or harmful stimuli (Sharkey and Wiley, 2016).

THC acts as a partial agonist at both CB1-R and CB2-R receptors, with its psychoactive effects mediated by high affinity binding to CB1-R (Pertwee, 2008). In contrast, CBD exhibits low direct binding affinity for CB1-R receptors and CB2-R receptors, but can antagonise their activation by THC through allosteric modulation (Thomas *et al.,* 2007). Specifically, CBD functions as a non-competitive negative allosteric modulator of CB1-R receptor, decreasing the efficacy and potency of both THC and anandamide (Laprairie *et al.,* 2015; Millar *et al.,* 2018). Notably, the terpene β-caryophyllene acts as a selective agonist at CB2-R receptors (Gertsch *et al.,* 2008). Although the precise mechanisms remain to be fully understood, this complementary activity among cannabinoids and terpenes may contribute to the so-called entourage effect non-phytocannabinoid cannabis components, whereby combined cannabis-derived phytochemicals produce synergistic biological effects.

Increasing research into the ECS has led to the identification of additional receptors, endogenous ligands and novel physiological roles, particularly within the gut. This broader signalling network, now referred to as the "endocannabinoidome," encompasses bioactive lipids such as N-acylethanolamines and monoacylglycerols, which act on non-canonical cannabinoid receptors including GPR55, GPR119, peroxisome proliferator-activated receptors (PPARs), and the transient receptor potential vanilloid type-1 (TRPV1) channels (Maione *et al.,* 2013; Di Marzo and Piscitelli, 2015; Lian *et al.,* 2022; Piñeiro and Falasca, 2012; Rahman *et al.,* 2021). These receptors are increasingly recognised as critical mediators of gut function and metabolic homeostasis. For instance, PPARs regulate lipid metabolism, adipogenesis, energy balance, and inflammatory responses (Decara *et al.,* 2020; Lago-Fernandez *et al.,* 2021).

Similarly, various receptors expressed in the bladder urothelium including CB1-R and CB2-R, muscarinic acetylcholine receptors, transient receptor potential vanilloid type-4 (TRPV4) channels and calcium-activated potassium channels play key roles in regulating bladder function and contribute to the pathophysiology of detrusor overactivity (Andersson 2002; Fowler, 2002; Hayn *et al.,* 2008; Merriam, *et al.,* 2008; Cho *et al.,* 2014; Kim *et al.,* 2015; Kim and Yang 2016; Kim *et al.,* 2017). Given the endocannabinoidome's role in regulating gut motility, the enteroendocrine system, bladder function and general homeostasis, further investigation of cannabinoid and cannabinoid-like receptors may present novel therapeutic targets for gastrointestinal and urinary disorders such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), overactive bladder and interstitial cystitis.

1.5 GASTROINTESTINAL DISEASE

Gastrointestinal disease is a global disease with the highest reported prevalence in Europe, North America and Oceania and developing countries also seeing increasing incidence (Ng *et al.,* 2017; Laing *et al.,* 2019). In 2015, in the United States, annual health care expenditures for gastrointestinal diseases totalled more than $135.9 billion. Per annum there are approximately 266,600 gastrointestinal related cancers diagnosed and 144,300 cancer deaths (Peery *et al.,* 2019). Prevalence of gastrointestinal disease is expected to increase, further burdening health systems at detriment to patient's quality of life.

The intestinal epithelium functions as a barrier whilst regulating the absorption of nutrients and secretion of waste products. This barrier relies on the integrity of tight junction proteins, which can form both an absolute barrier and selective barrier via paracellular ion channels (Turner, 2009). For example, tight junction proteins, claudin-2 and claudin-15 can form paracellular pores for the permeability of water and ions in enterocytes (Günzel and

Yu, 2013; Raju *et al.,* 2020). Defects in these tight junction proteins increase permeability, causing leaks and pore pathways to form compromising the intestinal barrier and its function. This can lead to a positive-feedback cycle of immune-associated barrier dysfunction. A combination of genetic factors, environmental factors, a malfunctioning immune system and the gut microbiota have all been identified as important contributing factors for the pathogenesis and progression of gastrointestinal diseases (Khor *et al.,* 2011; Caruso *et al.,* 2020).

Gastrointestinal disease such as IBD or Gastrointestinal mucositis (GIM) refers to diseases causing bowel inflammation or reoccurring chronic inflammation (Kaser *et al.,* 2010). This inflammation negatively impacts the structure and function of the intestinal epithelium. Two conditions commonly associated with IBD are Crohn's disease and ulcerative colitis. Symptoms including bloody diarrhoea, abdominal pain, increased fatigue and malabsorption are common but extraintestinal manifestations can impact nearly all organ systems (Rogler *et al.,* 2021). Additionally, given the prevalence of cancer it is noteworthy to consider the impact of conventional chemotherapeutic agents such as irinotecan, which cause side effects including diarrhoea, rectal bleeding and infection. Irinotecan has been implicated in the development of GIM, delaying chemotherapeutic treatment due to the requirement for treatment dose reductions (Gibson *et al.,* 2016; Dahlgren *et al* 2021; Sougiannis *et al.,* 2021)

Irinotecan treatment results in stimulation of nuclear factor kappa B (NF-κB), leading to increased expression of numerous pro-inflammatory cytokines including interleukin 1-β (IL-1β) and tumour necrosis factor-α (TNF-α). These pro-inflammatory cytokines have been linked to significant increases in intestinal permeability *in vitro* and *in vivo* (Logan *et al.,* 2008; Al-Sadi *et al.,* 2009; Chelakkot *et al.,* 2018). Increased levels of intestinal TNF-α and IL-1β are associated with increased disease severity and are involved in the

progression of both irinotecan-induced GIM and IBD, (Sands and Kaplan, 2007; Al-Sadi *et al.,* 2008; Melo *et al.,* 2008; Mao *et al.,* 2018; Arifa *et al.,* 2014).

Underlying causative factors such as ROS generation and oxidative stress promote the progression of IBD and GIM. Upstream ROS generation may activate nuclear factor kB (NF-κB) resulting in increased transcription of genes such as those coding for TNFα and IL-1β, resulting in upregulation of these cytokines (Sonis, 2004; Lingappan, 2018). This upregulation may activate caspase pathways generating a feedback loop with NF-κB resulting in an exacerbated inflammatory response (Cinausero *et al.,* 2017). Therefore, compounds with the ability to inhibit ROS generation and provide mucoprotective properties by mitigating the inflammatory response may be beneficial for the treatment and management of IBD and GIM, especially in those undertaking irinotecan treatment (Wardill *et al.,* 2023).

Conventional treatment of IBD and GIM ranges from corticosteroids to anti-inflammatory drugs. Anti-inflammatory drugs such as aminosalicylates have the best safety profile of all medicine therapies currently used for IBD and have proven therapeutic benefit for treatment of Crohn's disease and ulcerative colitis acutely. However, minor side effects including rash, diarrhoea, indigestion, flatulence, headache and nausea are common, and in rare cases serious side effects such as pleuritis, pericarditis, myocarditis, pancreatitis and cholestatic hepatitis can occur (Williams *et al.,* 2011).

Interestingly, GPCRs of the ECS have been identified to regulate gastrointestinal inflammation and the pathophysiology of colitis in animal models. The protective role of CB1-R is supported by studies demonstrating that CB1-R knockout mice exhibit exacerbated experimental colitis compared to wild type mice (Massa *et al.,* 2004; Pacher and Mechoulam, 2011). Similarly, activation of CB2-R has been shown to reduce both

inflammatory damage and the accelerated gastrointestinal transit associated with experimental colitis in CB2-R knockout mice (Storr *et al.,* 2009).

In a human colonic explant model, Harvey *et al.,* (2013) reported that selective activation of CB2-R by anandamide and the synthetic CB2-R selective agonist JWH-015, significantly reduced cytokine-induced mucosal damage and lymphocyte infiltration. However, despite these anti-inflammatory effects, epithelial barrier dysfunction indicated by increased permeability, remained unaltered, suggesting that CB2-R activation alone may be insufficient to restore epithelial integrity under inflammatory conditions.

Although inhaled cannabis has been associated with improved quality of life and reduced disease activity for a small group of IBD patients (Lahat *et al.,* 2012), the pharmacological evidence supporting the efficacy of cannabis-derived pCBs in the human colon has yet to be fully understood (Kienzl *et al.,* 2020). CBD has shown promise, demonstrating the ability to inhibit ROS production and restore epithelial permeability during inflammation and oxidative stress conditions, suggesting its possible application as an adjuvant in IBD management (Cocetta *et al.,* 2021).

Importantly, the actions of endocannabinoids in the gut extend beyond CB1-R and CB2-R activation, encompassing non-cannabinoid receptor-mediated mechanisms that contribute to intestinal homeostasis, immune regulation, barrier integrity, and microbial balance (Cuddihey, MacNaughton and Sharkey, 2022). Together, these findings underscore the multifaceted role of the ECS in regulating intestinal inflammation. While CB1-R and CB2-R activation offer protective effects in preclinical colitis models, these actions alone may be insufficient to fully restore epithelial barrier function under inflammatory conditions. The ECS also engages broader regulatory networks involved in oxidative stress, immune signalling and host-microbiota interactions, all of which are critical to intestinal homeostasis (Cuddihey, MacNaughton and Sharkey, 2022). Further investigation is

warranted to define the therapeutic potential of cannabis-derived pCBs in human IBD, and to understand the specific mechanisms by which they influence disease progression.

1.6 BLADDER DISORDERS/DISEASE

Bladder disorders encompass a broad spectrum of conditions ranging from functional abnormalities, such as overactive bladder and interstitial cystitis/bladder pain syndrome, to diseases such as bladder cancer. Many of these conditions, particularly chronic inflammatory disorders, have complex and multifactorial pathophysiologies involving immune dysregulation, epithelial and urothelial barrier dysfunction and altered neurochemical signalling (Stewart *et al.,* 2003; Hanno *et al.,* 2011; Marcu, Campian and Tu 2018; Lenis, Lec and Chamine 2020). Overactive bladder, for instance, is often characterised by detrusor muscle overactivity, which may be mediated by abnormal activation of receptors such as CB1-R, CB2-R, TRPV1 and muscarinic acetylcholine receptors expressed in the urothelium (Abrams *et al.,* 2000; Stewart *et al.,* 2003). Similarly, bladder pain syndrome is increasingly recognised as an inflammatory condition involving urothelial permeability defects, mast cell activation and sensory nerve hypersensitivity (Hanno *et al.,* 2011; Marcu, Campian and Tu 2018). Current treatments including antimuscarinics, β3-agonists and intravesical therapies offer limited efficacy and are frequently associated with side effects or inadequate symptom control (Thiagamoorthy, Cardozo, Robinson 2016; Visco *et al.,* 2016; Kwon *et al.,* 2024).

Bladder cancer is one of the ten most commonly diagnosed cancers worldwide, with urothelial carcinoma comprising over 90% of all cases (Lenis, Lec and Chamine 2020). It typically manifests as either low-grade, non-invasive tumours or high-grade, muscle-invasive disease. While low-grade tumours have a high recurrence rate, high-grade tumours are associated with poor prognosis and increased mortality. Despite improvements

in surgical techniques and chemotherapeutic interventions, recurrence and treatment resistance continue to pose significant clinical challenges. These therapeutic limitations, coupled with increasing prevalence in ageing populations highlight a pressing need for alternative and more targeted approaches. The emerging understanding of the ECS and its role in modulating bladder function and inflammation presents a compelling rationale for the development of cannabinoid-based therapies in managing bladder disorders and disease.

Endocannabinoids and cannabinoid derivatives have been shown to decrease bladder motility under both normal and inflammatory conditions, suggesting that cannabinoid receptors exert functional regulatory effects on bladder activity (Jagger, Sellaturay and Rice; 1998; Dimitrieva and Berkley 2002; Hiragata *et al.,* 2007). Notably, hyperalgesia associated with turpentine-induced acute bladder inflammation was prevented by administration of a cannabinoid agonist (Facci *et al.,* 1995). These mechanisms suggest that modulation of the endocannabidiome may support urothelial barrier function, mitigate chronic inflammation and offer cytostatic or cytotoxic actions in bladder disorders or disease.

1.7 ANTIBIOTIC RESISTANCE

Possibly one of the greatest global health challenges is the increasing prevalence of antibiotic resistance amongst microorganisms. Antibiotics are one of our best weapons against life-threatening infections, however with extensive use arises the potential for the further development of antimicrobial resistance. Most concerning is the fact that in some cases this resistance can be passed onto other microorganisms without them being exposed to the antibiotic (Zhen *et al.,* 2021). It is estimated that in 2019, 1.27 million people died

worldwide due to antibiotic-resistant microbial infections (ARMI). A further 4.95 million deaths identified ARMI as a significant contributing factor (Murray *et al.,* 2022).

Our first line of defence against severe infections are fluoroquinolones and beta-lactam antibiotics; however, resistance to these primary antibiotics is estimated to account for more than 70% of deaths caused by ARMI in 2019 (Murray *et al.,* 2022). Antibiotic resistance is a global concern with the potential to affect anyone at any point in life. However, there is increased risk in people receiving healthcare with potentially increased exposure and those that are immunocompromised. Concerningly, young children were also found to be at higher risk, with approximately one in five deaths attributable to ARMI occurring in children under the age of five (Murray *et al.,* 2022). Additionally, procedures that we heavily rely on today such as joint replacements, organ transplants and cancer therapy increase the risk of infection and exposure to ARMI (Zhen *et al.,* 2021).

Antibiotic resistance is more prevalent in low- and middle-income countries, although higher income countries are also seeing alarmingly high levels of antibiotic resistance. Previous estimates predicted the impact of ARMI would contribute to 10 million deaths annually by 2050 (Murray *et al.,* 2022). Currently, the prevalence of ARMI is greater than previously estimated, emphasising the importance of implementing controls and developing antibiotics with reduced propensity to induce resistance. Unfortunately, there are few new therapeutics in development due to the unfavourable economics of antibiotic development (Renwick and Mossialos, 2018; Roope *et al.,* 2019).

Antibiotic resistance is a One Health challenge – health of the people is connected to the health of animals and the environment. Antibiotic-resistant microorganisms can move quickly across different settings such as communities, food supply, healthcare facilities and the environment affecting animals including pets, zoo animals and livestock increasing the spread and burden (Zhen *et al.,* 2021). Bacterial infections are the leading cause of sepsis,

estimated at over 30 million cases and 5.3 million deaths annually (Fleischmann *et al.,* 2016). A cascading release of immunomodulatory mediators induced by microbiological products leads to an exacerbated immune response in hosts that are susceptible due to genetic or other factors. This can affect the immune system, procoagulant and antifibrinolytic pathways, cellular metabolism and signalling in vital organs (O'Brien *et al.,* 2007).

According to the National Institutes of Health, bacterial biofilms are responsible for approximately 80% of microbial infections in the human body (Davies, 2003). Bacterial biofilms are aggregates of bacteria in a self-assembled, protective matrix. They can contain/consist of one or multiple bacteria and are more resistant to antibiotics, establishing multi-drug resistance and evading the immune response (Hall-Stoodley *et al.,* 2004; Van Acker and Coenye, 2016). As new types of resistance mechanisms and antibiotic-resistant bacteria continue to emerge and spread globally, the need for new antibiotics has never been greater. Therefore, alternative options such as the use of novel cannabis phytochemicals are currently being assessed for antimicrobial activity against high priority antibiotic-resistant pathogens identified by the World Health Organisation.

Interestingly, CBD has demonstrated promising antimicrobial activity against a range of antibiotic-resistant pathogens. This includes the high priority pathogens *Enterococcus faecium* and Methicillin-resistant *Staphylococcus aureus* (MRSA) as well as a large set of clinical isolates (Blaskovich *et al.,* 2021). Importantly, repeated exposure to CBD do not appear to induce resistance. Mechanisms of action currently point towards disruption of cell membranes and critical synthesis pathways. However, further research is required to understand how variations in outer membrane composition of microorganisms affect CBD antimicrobial activity (Blaskovich *et al.,* 2021). In this setting, CBD derivatives may provide unique mechanisms of action and improve systematic microbicidal activity against selected antibiotic-resistant microorganisms.

As our understanding of the ECS, microbiome and endocannabinoidome continues to evolve, we are seeing that these critical processes operate synergistically to maintain health and homeostasis. Therefore, the use of narrow spectrum antibiotics is becoming increasingly important so as not to upset the natural microbiome by killing off beneficial commensal bacteria or mammalian cells (Melander *et al.,* 2018). Promisingly, low cytotoxicity to mammalian cells screened thus far has suggested collectively that CBD may be safe to use as an antibiotic, although a more comprehensive screening should be undertaken (Blaskovich *et al.,* 2021), including further development at overcoming bioavailability limitations with CBD.

1.8 CONCLUSION

The ECS plays an important role in regulating inflammation and visceral organ motility throughout the body, mainly via CB1-R and CB2-R receptor activation. Given the ECS works closely with other systems such as the enteric nervous system and the immune system, it is plausible that changes in one system can influence the others. This interaction highlights the potential of therapeutic compounds that act synergistically with endogenous pathways to maximise therapeutic outcomes. As a result, there is increasing interest in targeting the ECS using cannabis-derived compounds such as pCBs, their derivatives and other component phytochemicals such as terpenes/terpenoids and flavonoids. These compounds are structurally diverse and show a range of bioactivities, supporting their potential use in a wide range of health conditions. Furthermore, manupulation of structure can influence their bioactivity suggesting that formulations could be developed to target specific therapeutic outcomes.

Although promising, critical knowledge gaps remain. Placebo-controlled trials of cannabinoid therapies are limited, with most evidence acquired from small or observational

studies using non-standardised formulations. Dose–response relationships remain poorly defined and mechanistic understanding beyond CB1-R/CB2-R receptor activation, particularly involving targets like TRP channels, GPCRs/orphan GPRs and PPARs is incomplete. The therapeutic role of minor cannabinoids, terpenes and the proposed entourage effect lacks thorough investigation and long-term safety data, particularly in vulnerable populations (e.g. elderly, pregnant women), is limited, as are studies assessing drug interactions.

To address some of these research gaps, this study includes assessment of cannabis-derived phytochemicals, including cannabidiol and its derivatives, cannabidiol hydroxyquinone (CBDHQ) and terpenes/terpenoids, with the aims to:

- Evaluate the structure–activity relationships of CBD derivatives against resistant bacterial pathogens.
- Assess the cytotoxic and protective properties of terpenes on intestinal epithelial cells under inflammatory conditions.
- Investigate the selective cytotoxicity of CBD and CBDHQ in bladder cancer cells and define a potential role for redox-mediated mechanisms.

Outcomes of these studies will aid to inform both the development of new medicines and their application in the treatment of gastrointestinal disease, bladder disease and antibiotic-resistant microbial infections.

CHAPTER 2

CANNABIDIOL AND ITS DERIVATIVES POSSESS BACTERICIDAL ACTIVITY AGAINST GRAM-POSITIVE, METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS, ENTEROCOCCUS FAECIUM AND STAPHYLOCOCCUS EPIDERMIDIS

Statement of Authorship

Title of Paper	CANNABIDIOL AND ITS DETIVATIVES POSSESS BACTERICIDAL ACTIVITY AGAINST GRAM-POSITIVE, METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS, ENTEROCOCCUS FAECIUM AND STAPHYLOCOCCUS EPIDERMIDIS
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Principal Author

Name of Principal Author (Candidate)	Luke Wooley		
Contribution to the Paper	Experimental design, performed the experiments, performed the analysis and wrote the manuscript.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	09/07/2025

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr Adrian Abdo		
Contribution to the Paper	Provided technical support and provided critical review of the manuscript.		
Signature		Date	09/07/2025
Name of Co-Author	Dr Scott Smid		
Contribution to the Paper	Conceived the project, acquired the cannabidiol derivatives and provided critical review of the manuscript.		
Signature		Date	24/07/2025
Name of Co-Author	Associate Professor Katharina Richter		
Contribution to the Paper	Project conceptualisation, design of methodology, provision of resources and funding, supervision, critical review and editing of the manuscript.		
Signature		Date	09/07/2025

PREFACE

This manuscript was written to the specifications of the University of Adelaide. Essential formatting rules were followed in order to satisfy the thesis guidelines for the University of Adelaide's School of Biomedicine Doctor of Philosophy in Pharmacology program:

- 12pt Times New Roman font
- Double spacing
- Block justified
- Left margin 3.5cm; top, bottom and right margins 2cm
- Figures and tables are embedded in the appropriate places within the text, rather than at the end of the document or in a separate document
- Highlight section is not included.
- References are not a numbered style.

The word count for the manuscript (excluding references, acknowledgements and supplementary material) is 5158.

TITLE

Cannabidiol and its derivatives possess bactericidal activity against Gram-positive, methicillin-resistant *Staphylococcus aureus*, *Enterococcus faecium* and *Staphylococcus epidermidis*

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2.0 ABSTRACT

This study assessed the antimicrobial activity of 42 cannabidiol (CBD) derivatives against bacteria from the ESKAPE pathogen group to identify potential candidates for antimicrobial therapy. Standard microbiological assessment protocols, Minimum Inhibitory Concentration Minimum Bactericidal Concentration, growth curve analysis, synergy assessment by checkerboard assay and AlamarBlue biofilm viability assay were performed to evaluate the efficacy of each derivative. Cytotoxicity assessment was conducted using human intestinal Caco-2 cells to determine concentration-dependent epithelial cell toxicity. Five CBD derivatives demonstrated bactericidal activity against three Gram-positive bacteria: methicillin-resistant *Staphylococcus aureus*, *Enterococcus faecium*, and *Staphylococcus epidermidis*. Growth curve analysis indicated rapid antimicrobial action within the first six hours of treatment exposure. Two CBD derivatives demonstrated synergistic action when combined with daptomycin against one clinical isolate of *E. faecium*. No antibacterial activity was observed against Gram-negative bacteria. These findings highlight the potential of CBD derivatives as promising candidates for combating antimicrobial resistant bacteria, particularly Gram-positive bacteria. Leveraging the antimicrobial activity of cannabidiol and its derivatives may inform further drug development and provide novel therapeutic strategies against antibiotic-resistant infections.

Keywords: Cannabidiol derivatives, ESKAPE pathogens, Antimicrobial resistance, Gram-positive bacteria, Biofilm viability, Minimum Inhibitory Concentration, Checkerboard assay.

2.1 INTRODUCTION

Antimicrobial resistance (AMR) among pathogenic microorganisms poses a significant global health threat (Solomon and Oliver 2014; McClure and Day 2014). This escalating threat is becoming increasingly difficult to overcome, fuelled by the complex interplay of factors such as antibiotic misuse in both clinical practice and agriculture (Bell 2014; Manyi-Loh *et al.,* 2018; Malik and Bhattacharyya 2019). Furthermore, the economics of antibiotic development present a formidable barrier to the creation of novel therapeutic solutions in this era of muti-drug resistant and extensively-drug resistant bacteria (Basak, Singh and Rajurkar 2016; Renwick and Mossialos 2018; Roope *et al.,* 2019).

In general, antibacterial treatments target an essential protein or process (either directly or indirectly), inhibiting protein biosynthesis, DNA replication, cell wall or membrane integrity, or folic acid metabolism (Kapoor, Saigal and Elongavan 2017). Antibiotics with a single target protein have been favoured by healthcare providers due to their high target specificity and reduced risk of side effects. However, having a single target means that resistance mechanisms may develop faster, therefore, combination therapy has been a useful approach to overcome AMR and is even more effective when combining multi-target antibiotics (Oldfield and Feng 2014).

As AMR continues to rise, there is a need for innovative and effective solutions. Naturally occurring compounds could provide an alternative novel approach, with phytochemicals from *Cannabis sativa L.* gaining interest for their antimicrobial activity. Cannabidiol (CBD) is one of hundreds of different phytochemicals that can be produced by the cannabis plant, and many of these compounds have demonstrated biological activity (ElSohly and Slade 2005).

CBD is a non-psychoactive phytocannabinoid with a chemical structure consisting of a pentyl-substituted bis-phenol aromatic group (the resorcinol moiety) linked to an alkyl-

substituted cyclohexene terpene ring system (Figure S2-1). Preclinical studies of CBD demonstrate effective antimicrobial activity against a range of antibiotic-resistant bacteria with a low propensity to develop resistance (Blaskovich *et al.,* 2021). This activity extends to bacteria considered as high priority threats by the World Health Organisation (World Health Organisation 2015).

Given that many simple phenolic compounds display antimicrobial properties, a plausible hypothesis is that the resorcinol moiety of cannabinoids serves as the antibacterial pharmacophore with the alkyl, terpenoid, and carboxylic appendices influencing its activity (Puupponen-Pimiä *et al.,* 2001; Nohynek *et al.,* 2006; Appendino *et al.,* 2008). Previous structure-activity relationship studies have indicated the potential for CBD derivatives to be developed with specific targets or functions for improved bioactivity or adjuvant therapeutic treatments (Blaskovich *et al.,* 2021; Appendino *et al.,* 2008). However, the mechanisms of action underpinning this antimicrobial activity are still not fully understood.

To this end, we investigated the standalone and adjunctive bactericidal and bacteriostatic activity of 34 selected CBD derivatives against ESKAPE pathogens; *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and Enterobacter species to understand whether these derivatives exhibit comparable or superior antimicrobial properties to CBD.

2.2 MATERIALS AND METHODS

AlamarBlue (resazurin), propylene oxide (CAS No. 75-56-9), Mueller-Hinton broth (MHB; Cat. No. 211443), tryptic soy broth (Cat. No. CM0989B) and Luria-Bertani broth (LB; Cat. No. 22700025) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Daptomycin (Product No D2446), colistin (Cas. No. 1264-72-8), 75 cm^2 tissue

culture flasks (Corning Life Sciences, Lowell, MA, USA), flat bottom polystyrene 96-well microtiter plates (Corning, Cat. No. 3370), flat-bottom, black-walled 96-well microtiter plates (Corning, Cat. No. CLS3603-48EA), dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT), Dulbecco's Modified Eagle Medium (DMEM), non-essential amino acids (NEAA), penicillin/streptomycin, 0.25% w/v trypsin with phenol red and ethylenediaminetetraacetic acid (EDTA), foetal bovine serum (FBS) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MI, USA). CBD was purchased from Australian Government National Measurement Institute (Lindfield, NSW, Australia). A total of 42 CBD derivatives were kindly supplied by Professor Claudio Viegas Jr at Laboratório de Pesquisa em Química Medicinal (Universidade Federal de Alfenas, Brazil) (Figure S2-1). All materials were autoclaved for sterility and all analysis was conducted under sterile conditions within a lamina flow.

2.2.1 Compound preparation

CBD and CBD derivatives were prepared as a 3.2 mg/mL stock solution in 100% DMSO and standard antibiotics (daptomycin and colistin) were prepared as a 1.6 mg/mL stock solution in 0.9% saline. Compounds were stored at -20 °C and allowed to thaw at room temperature when required. As CBD and CBD derivatives were dissolved in DMSO, a vehicle control using the same volume of DMSO was included in all experiments.

2.2.2 Bacterial strains and culture medium

The Gram-positive bacteria methicillin-resistant *S. aureus* (MRSA) USA 300, *E. faecium* ATCC 19434, *S. epidermidis* ATCC 12228, *S. epidermidis* ATCC 14990 and *S. epidermidis* ATCC 35984 as well as the Gram-negative bacteria *K. pneumoniae* ATCC 13883*, A.*

baumannii ATCC 19606, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 16692 were purchased from American Type Culture Collection (Manassas, VA, USA). MRSA Clinical Isolate (CI) 1, MRSA CI2, *E. faecium* CI1 and *E. faecium* CI2 were obtained from Adelaide Pathology Partners (Mile End, SA, Australia). The MRSA clinical isolates showed resistance against penicillin, oxacillin, amoxicillin-clavulanic acid, cephalexin, and erythromycin. Tryptic soy agar (TSA) was used to culture Gram-positive species while LB agar was used to culture Gram-negative species. Cation-adjusted MHB (CAMHB) with 50 mg/L $CaCl_2$ and 10 mg/L $MgCl_2$ was used for planktonic assays. Bacteria were cultured from pure stock maintained at -80 °C, cultured on respective agar and incubated for 20 – 24 h at 37 °C. Overnight streak plates were prepared 20 – 24 h prior to antimicrobial testing.

2.2.3 Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of 42 CBD derivatives was assessed by broth microdilution MIC assay as described by the Clinical and Laboratory Standards Institute (M07-A11 and M100) (Alshareef 2021; Abdo *et al.,* 2023). MRSA (USA300) and *E. coli* (ATCC 25922) were used as reference control strains. CBD derivatives, alongside CBD and standard antibiotics were serially diluted two-fold in CAMHB in 96-well plates. Treatment concentrations for CBD and derivatives ranged from 0.5 – 128 μg/mL. Treatment concentrations for daptomycin and colistin ranged from 0.25 – 32 μg/mL. Treatment concentrations for vehicle control (DMSO) ranged from 0.015 – 4%. Single colonies of each bacteria listed above were suspended in 0.9% saline and adjusted to 0.5 McFarland units (approximately 1×10^8 CFU/mL), then further diluted 1:20 in CAMHB (approximately 5×10^6). The bacterial suspension was then added to 96-well plates containing CBD derivatives, CBD, antibiotics, and controls, resulting in a total volume of

100 µL in each well and a cell density of approximately 5×10^5 CFU/mL. A sterile broth control, untreated bacterial control and a vehicle control were included with each plate. Plates were covered and statically incubated at 37 °C for 20 – 24 h. The MIC was determined as the lowest compound concentration showing no visible growth.

2.2.4 Minimum Bactericidal Concentration (MBC)

The MBC of selected CBD derivatives (247, 290, 291, 302 and 303) was determined for MRSA USA300, MRSA CI1, MRSA CI2, *E. faecium* ATCC 19434, *E. faecium* CI1, *E. faecium* CI2, *S. epidermidis* ATCC 12228, *S. epidermidis* ATCC 14990 and *S. epidermidis* ATCC 35984 cultures by inoculating 10 µL of each microdilution from MIC assays onto TSA and incubated for 20 – 24 h at 37 °C. The MBC was determined as the lowest concentration killing 99.9% of the inoculum. These five CBD derivatives were selected for MBC assessment based on their ability to inhibit bacterial growth, as determined by MIC screening. Only compounds demonstrating measurable MIC activity were progressed to MBC analysis, as the determination of bactericidal capacity is only relevant for agents that exhibit antimicrobial potential. Compounds lacking MIC activity were not evaluated further, as they are unlikely to possess meaningful bactericidal effects.

2.2.5 Growth Curve analysis

The effect of different concentrations of the compounds and different exposure times on Optical Density at 600 nm (OD_{600}) as a measure of bacterial growth was determined by growth curve analysis (Abdo *et al.,* 2023). MRSA USA300 and *S. epidermidis* (ATCC 12228) cultures were tested, and bacteria were prepared as previously described in the MIC assay. OD_{600} measurements were taken using a FLUOstar OPTIMA plate reader and

corrected for background fluorescence using the respective sterile medium. Plates were incubated for 24 h at 37 °C with measurements taken at 0, 2, 4, 8, 12 and 24 h exposure times.

2.2.6 Synergy testing by Checkerboard assay

Synergy between two different compounds was determined by the checkerboard assay with modifications (Kaul *et al.,* 2022). MRSA USA300, *E. faecium* CI1 and *S. epidermidis* ATCC 12228 cultures were tested, and bacteria were prepared as previously described in the MIC assay. Briefly, CBD or CBD derivatives (302 or 303) were tested in combination with daptomycin. These two derivatives were selected based on their MIC and MBC activity across the three different cultures as they were the only two derivatives that demonstrated activity across all three cultures tested. Each plate contained 6 concentrations of daptomycin ranging from 0.25 – 8 μg/mL and 6 concentrations of CBD or a CBD derivative ranging from 0.25 – 8 μg/mL and 2 – 64 μg/mL, respectively. Each plate contained a sterile broth control, untreated bacterial growth control, and MICs of daptomycin and CBD/CBD derivatives alone. The percentage relative growth between treated wells and the growth control was calculated using Equation 1:

$$Growth\ (\%) = \frac{OD_T}{OD_{GC}} \times 100 \quad (1)$$

Where OD_T (treatment) refers to the OD_{600} for bacteria treated with antibiotics and CBD or its derivates alone or in combination, and OD_{GC} (growth control) refers to the OD_{600} for untreated wells. OD_{600} measurements were taken using a FLUOstar OPTIMA plate reader and corrected for background fluorescence using the respective sterile medium.

The MIC was defined as the lowest drug concentration reducing bacterial growth by more than 80%. The synergistic effect was calculated using Equation 2:

$$FICI = \frac{MIC_{A+B}}{MIC_A} + \frac{MIC_{B+A}}{MIC_B} \quad (2)$$

Where the FICI is the fractional inhibitory concentration index, MIC_A and MIC_B refer to the MIC of antibiotic alone and CBD derivative alone, respectively, MIC_{A+B} refers to the MIC of antibiotic in the presence CBD derivative and MIC_{B+A} refers to MIC of CBD derivative in the presence of antibiotic. Synergy was defined as a FICI ≤0.5, additive effects by an FICI >0.5 – 1, indifference with FICI >1 to <2 and antagonistic effect with FICI ≥2 (European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases, 2000).

2.2.7 AlamarBlue biofilm viability assay

The metabolic activity of biofilms after treatment exposure was determined by the AlamarBlue (resazurin) viability assay with modifications for selected CBD derivatives (247, 290, 291, 302 and 303) (Kim *et al.,* 2010; Macia, Rojo-Molinero and Oliver 2014; Di Bonaventura and Pompilio 2022; Kaul *et al.,* 2023). Briefly, single colonies of MRSA USA300, *E. faecium* CI1 and *S. epidermidis* ATCC 12228 were suspended in 0.9% saline and adjusted to 0.5 McFarland units then further diluted 1:20 in CAMHB. Black 96-well plates were then inoculated with 100 µL of the bacterial suspension, covered and incubated for 20 – 24 h at 37 °C on a rotating platform (3D Gyratory Mixer, Ratek Instruments, Boronia, Australia) at 70 rpm. A sterile broth control, untreated bacterial control and vehicle control were included in each plate.

Biofilms were rinsed with 0.9% saline to remove planktonic cells, followed by exposure to treatment for 20 – 24 h. Concentrations of CBD derivatives ranged from 0.25 – 256 µg/mL, CBD ranged from 0.0625 – 64 µg/mL and daptomycin ranged from 0.125 – 64 µg/mL. DMSO was included as vehicle control with a treatment range of 0.25 – 8%. Plates

were covered and incubated for 20 – 24 h at 37 °C on a rotating platform at 70 rpm. Biofilms were rinsed with 0.9% saline to remove planktonic cells, followed by the addition of 100 µL of a 10% resazurin dilution in CAMHB. Plates were covered and incubated at 37 °C on a rotating platform at 70 rpm. Fluorescence was measured half-hourly on a FLUOstar OPTIMA plate reader using bottom optics with excitation and emission bands of 530 ± 5 nm and 590 ± 5 nm, respectively, and gain of 1100. Maximum fluorescence was typically reached after 2 h for *S. epidermidis* 12228 and *E. faecium* CI1 and 4 h for MRSA USA300.

Results were quantified according to Equation 3.

$$BK\ (\%) = \frac{F_C - F_T}{F_C} \times 100 \quad (3)$$

Where BK represented percentage biofilm killed, and F_C and F_T represent the fluorescence of untreated control and CBD derivative-treated bacteria, respectively.

2.2.8 Cytotoxicity assessment

The Caco-2 human colorectal adenocarcinoma cell line was used as an *in vitro* intestinal epithelial cell model to assess the cytotoxicity of CBD and selected CBD derivatives (247, 290, 291, 302 and 303). Treatment concentrations were selected based on MIC results and ranged from 32 ng/mL to 128 ug/mL for CBD and CBD derivatives, and 0.001 to 4% for vehicle control DMSO. Each plate contained an untreated control.

Cell viability was measured as previously described (Marsh and Smid 2022). Briefly, Caco-2 cells were cultured in 75 cm^2 tissue culture flasks with complete DMEM containing 10% v/v FBS, 1% v/v NEAA and 1% v/v penicillin/streptomycin and maintained at 37 °C with 5% CO_2. For cell viability measurements, Caco-2 cells were

seeded in 96-well plates at a density of 2×10^4 cells per well in complete DMEM, then incubated at 37 °C with 5% CO_2 for 24 h to adhere prior to treatment.

Cells were treated with either CBD or a CBD derivative then incubated for 24 h at 37 °C with 5% CO_2, after which time cell viability was measured using the MTT assay. Culture media was aspirated and replaced with serum-free media containing 0.25 mg/mL MTT then incubated for 2 h at 37 °C, 5% CO_2. MTT solution was then aspirated, and cells lysed with DMSO. Absorbance was measured at 570 nm using a Synergy MX microplate reader (Bio-Tek, Bedfordshire, UK). Cell viability was calculated using Equation 4:

$$Cell\ Viability\ (\%) = \frac{Absorbance\ of\ control}{Absorbance\ of\ treated\ sample} \times 100 \quad (4)$$

2.2.9 Statistical analysis

The MTT assay was analysed by two-way analysis of variance (ANOVA) with Dunnett's multiple comparisons used to determine significance versus vehicle control for cell viability ($p < 0.05$) within each treatment group. All experiments were conducted in triplicate except for growth curve analysis and cytotoxicity assessments which were conducted in quadruplicate. GraphPad Prism 10.0 (GraphPad Software, Boston, MA, USA) was used for data analysis and graphical representation.

2.3 RESULTS

2.3.1 Minimum Inhibitory Concentration (MIC) – Broad screen

An initial screening of the 42 CBD derivatives was performed to determine their antimicrobial activity against ESKAPE pathogens. The MIC values were determined for each CBD derivative against the selected bacteria alongside CBD and control antibiotics

daptomycin and colistin. Five derivatives exhibited antimicrobial activity against Gram-positive bacteria. The MIC values for CBD derivatives ranged from 32 to 128 μg/mL (Table 2-1a). No activity was observed against Gram-negative bacteria (Table 2-1b).

Table 2-1. Minimum Inhibitory Concentrations (MIC) of CBD and derivatives against Gram-positive and Gram-negative bacteria compared to daptomycin and colistin, respectively. Clinical Isolate (CI).

a) Gram-positive bacteria

	Bacteria strain and species		
Compound	*S. aureus* (MRSA USA300)	*E. faecium* (CI1)	*S. epidermidis* (ATCC 12228)
242 to 246	-	-	-
247	64	-	64
248 to 249	-	-	-
273 to 276	-	-	-
290	64	-	64
291	128	-	128
292 to 295	-	-	-
300 to 301	-	-	-
302	32	64	128
303	32	64	128
304 to 309	-	-	-
337 to 350	-	-	-
CBD	4	2	4
daptomycin	2	8 to 16	4

b) Gram-negative bacteria

	Bacteria strain and species			
Compound	*K. pneumoniae* (ATCC 13883)	*A. baumannii* (ATCC 19606)	*E. coli* (ATCC 25922)	*P. aeruginosa* (ATCC 16692)
CBD and CBD derivatives	no activity			
colistin	0.125 to 0.25	0.5 to 4	0.125 to 0.25	0.25 to 2

Values are in μg/mL; - indicates growth with all concentrations and no MIC was determinable.

2.3.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Initial MIC results against Gram-positive bacteria prompted a deeper investigation into the specificity and potential variability of this antimicrobial activity. To achieve this, a secondary MIC screening was carried out against an expanded panel of Gram-positive strains using the five CBD derivatives that displayed antimicrobial activity (Table 2-2a, b, c). The chemical structure of CBD and the five selected CBD derivatives (247, 290, 291, 302 and 303) are displayed in Figure 2-1. Secondary screening confirmed CBD derivatives maintained their antimicrobial activity against previously tested strains and MIC values were consistent with initial findings. This was followed by MBC screening, which showed that MBC values aligned closely with the MIC values, indicating bactericidal activity.

Notably, while some CBD derivatives (247, 290, 302 and 303) demonstrated consistent antimicrobial activity against different strains within a species, others demonstrated variable effects (291). This strain-specific behaviour was evident for MRSA, *E. faecium* and *S. epidermidis* with some strains were susceptible to CBD derivatives, while others displayed reduced sensitivity. However, this was not the case for CBD, which displayed consistent activity across most strains.

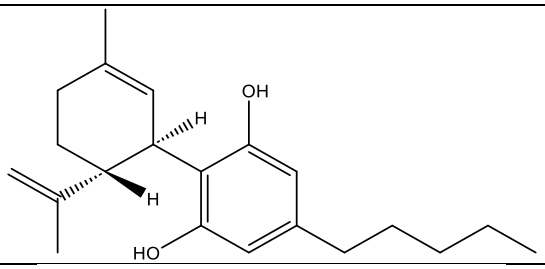
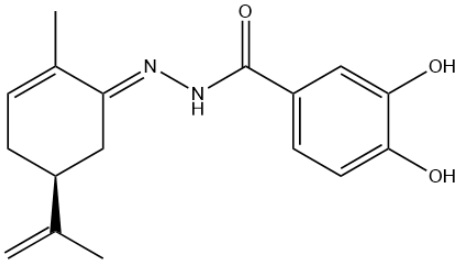
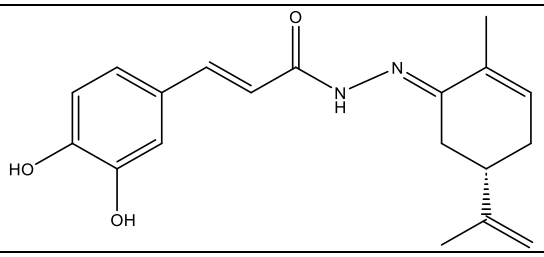
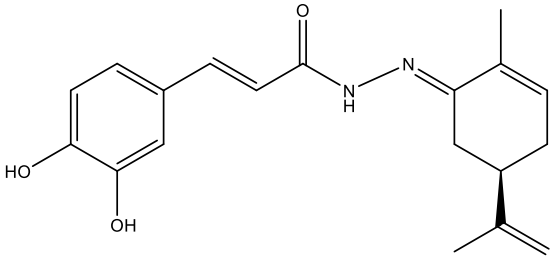
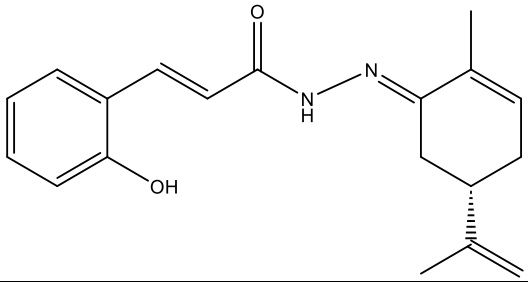
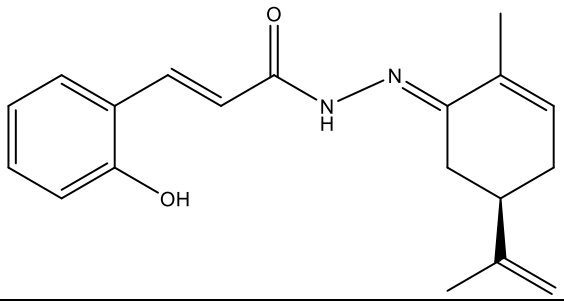
Compound ID	Nomenclature	Compound Structure
Cannabidiol (CBD)	2-[(1R,6R)-6-Isopropenyl-3-methyl-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol	
247	(S,E)-3,4-dihydroxy-N'-(2-mehtyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)benzohydrazide	
290	(E)-3-(3,4-dihydroxyphenyl)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
291	(E)-3-(3,4-dihydroxyphenyl)-N'-((R,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
302	(E)-3(2-hydroxyphenyl)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
303	(E)-3(2-hydroxyphenyl)-N'-((r,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	

Figure 2-1. Structures of CBD and CBD derivatives (247, 290, 291, 302 and 303) showing MIC activity in preliminary screening assay.

Table 2-2. a-c) Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of CBD and derivatives against Gram-positive and Gram-negative bacteria compared to daptomycin and colistin, respectively. Clinical Isolate (CI).

a) methicillin-resistant *S. aureus* (MRSA)

	MIC			**MBC**		
	MRSA strain			**MRSA strain**		
Compound	USA300	CI1	CI2	USA300	CI1	CI2
247	64	64	-	64	64	-
290	64	64	-	64 to 128	64	-
291	128	-	-	128	-	-
302	32	64	-	32 to 64	64	-
303	32	32 to 64	-	32 to 64	64	-
CBD	4	4	4	4 to 8	4	4
daptomycin	2	2	2	2 to 4	2	2

b) *E. faecium*

	MIC			**MBC**		
	***E. faecium* strain**			***E. faecium* strain**		
Compound	ATCC *19434*	CI1	CI2	ATCC *19434*	CI1	CI2
247	-	-	-	-	-	-
290	-	-	-	-	-	-
291	-	-	-	-	-	-
302	-	64	-	-	64	-
303	-	64	-	-	64	-
CBD	2	2	4	2	2	4
daptomycin	32	8 to 16	4	32	8 to 16	4

c) *S. epidermidis*

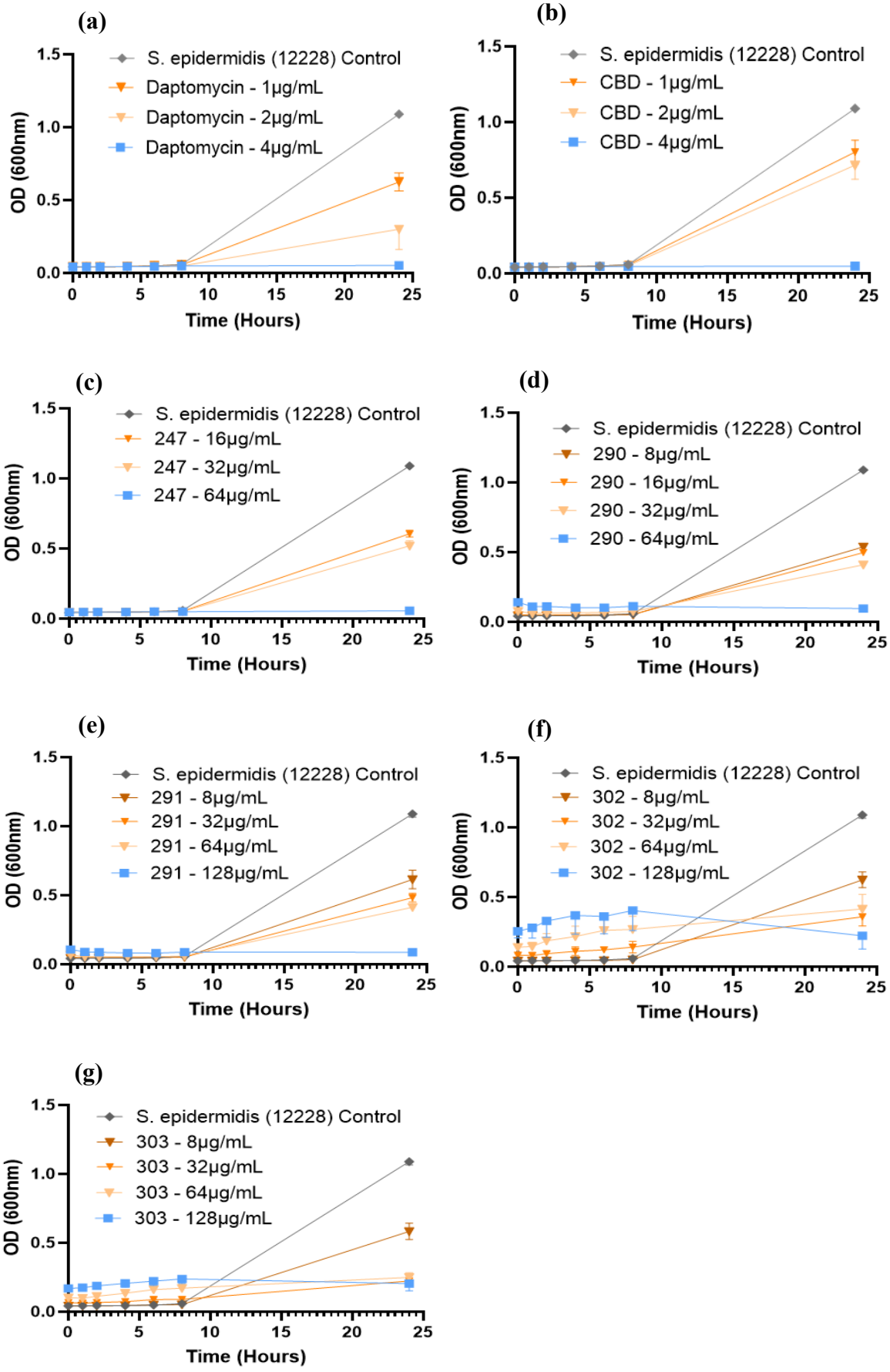
	MIC			MBC		
	***S. epidermidis* strain**			***S. epidermidis* strain**		
Compound	ATCC 12228	ATCC 14990	ATCC 35984	ATCC 12228	ATCC 14990	ATCC 35984
247	64	-	-	64 to 128	-	-
290	64	-	64	64 to 128	-	64
291	128	-	-	128	-	-
302	128	-	-	128	-	-
303	128	-	-	128	-	-
CBD	4	4	4	4 to 8	4	4
daptomycin	4	2	2	4 to 8	2	2

Values are in μg/mL; - indicates growth with all concentrations and no MIC was determinable.

2.3.3 Growth Curve analysis

Growth curve analysis was used to assess the dynamics of antimicrobial activity of CBD, five CBD derivatives (247, 290, 291, 302 and 303) and daptomycin against *S. epidermidis* (ATCC 12228) and MRSA USA300. The analysis revealed an early onset of activity within 4-6 h of exposure. Notably, the antimicrobial activity displayed a clear positive concentration-dependent response to inhibiting bacterial growth compared to the growth control (Figure 2-2). Furthermore, this effect was sustained over the 24 h incubation period, suggesting the potential for prolonged activity. Variations in OD_{600} readings warrant clarification. Some CBD derivatives exhibited higher OD_{600} readings in the initial 8 h of incubation. This initial increase in OD_{600} values is most likely attributed to solubility issues at concentrations >32 μg/mL. This may be due to the formation of aggregates or precipitates, leading to increased turbidity and subsequently higher OD_{600} readings. However, it is important to emphasise that these elevated OD_{600} values were transient and not indicative of bacterial growth or activity. After 8 h, a gradual decline in OD_{600} readings

was observed. Importantly, it is unlikely that this solubility issue impacted the MIC analysis, as MIC analysis was conducted over a 24 h incubation period, and comparative wells were indicative of the concentration thresholds at which CBD derivatives effectively inhibited bacterial growth. Growth curve analysis was performed using MRSA and *S. epidermidis* as representative Gram-positive species because these strains showed the greatest susceptibility in the initial MIC and MBC screening.


(a)
S. epidermidis (12228) Control
Daptomycin - 1µg/mL
Daptomycin - 2µg/mL
Daptomycin - 4µg/mL
OD (600nm)
Time (Hours)
(b)
S. epidermidis (12228) Control
CBD - 1µg/mL
CBD - 2µg/mL
CBD - 4µg/mL
OD (600nm)
Time (Hours)
(c)
S. epidermidis (12228) Control
247 - 16µg/mL
247 - 32µg/mL
247 - 64µg/mL
OD (600nm)
Time (Hours)
(d)
S. epidermidis (12228) Control
290 - 8µg/mL
290 - 16µg/mL
290 - 32µg/mL
290 - 64µg/mL
OD (600nm)
Time (Hours)
(e)
S. epidermidis (12228) Control
291 - 8µg/mL
291 - 32µg/mL
291 - 64µg/mL
291 - 128µg/mL
OD (600nm)
Time (Hours)
(f)
S. epidermidis (12228) Control
302 - 8µg/mL
302 - 32µg/mL
302 - 64µg/mL
302 - 128µg/mL
OD (600nm)
Time (Hours)
(g)
S. epidermidis (12228) Control
303 - 8µg/mL
303 - 32µg/mL
303 - 64µg/mL
303 - 128µg/mL
OD (600nm)
Time (Hours)

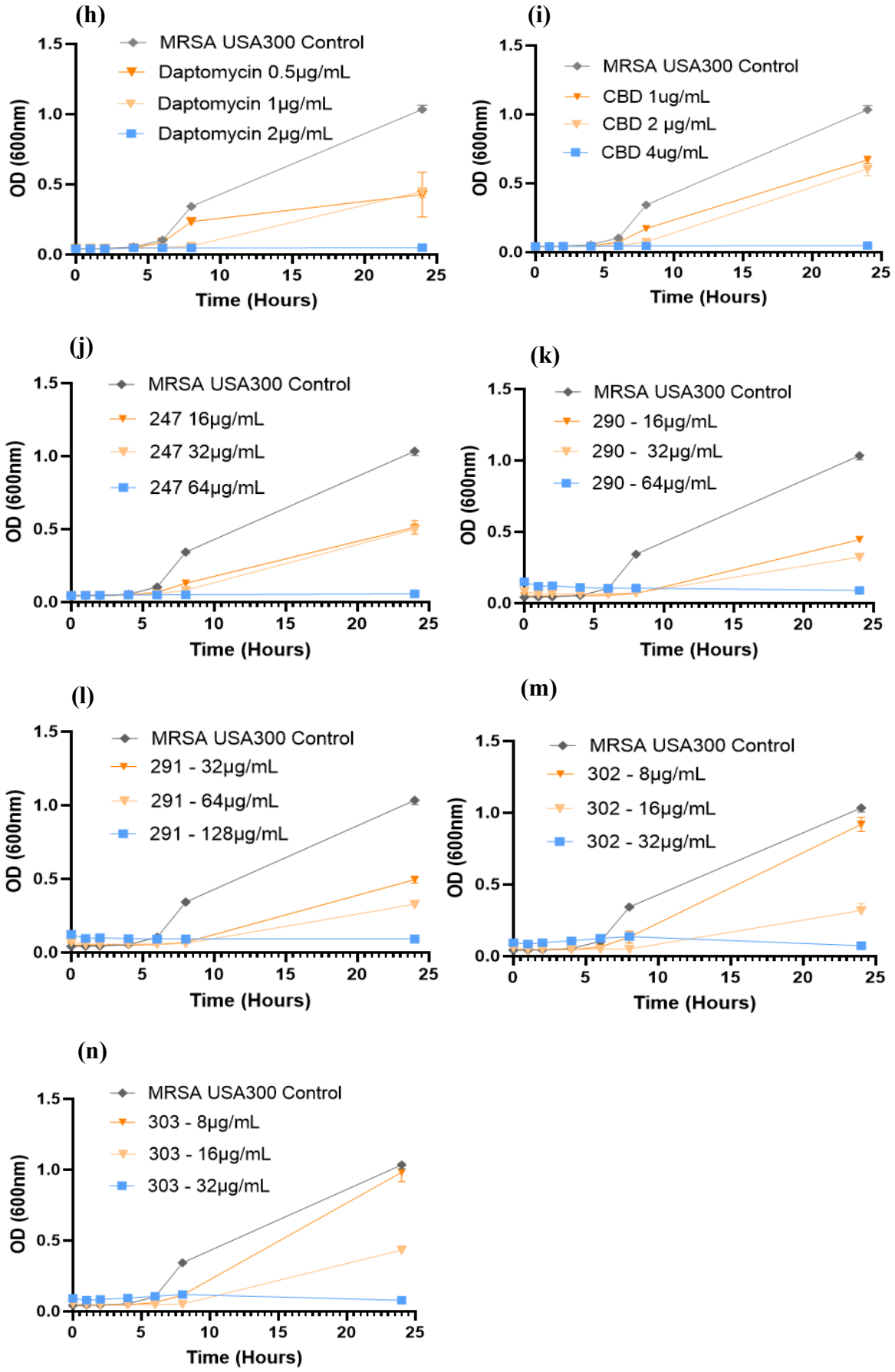

(h)
MRSA USA300 Control
Daptomycin 0.5µg/mL
Daptomycin 1µg/mL
Daptomycin 2µg/mL
OD (600nm)
Time (Hours)
(i)
MRSA USA300 Control
CBD 1ug/mL
CBD 2 µg/mL
CBD 4ug/mL
OD (600nm)
Time (Hours)
(j)
MRSA USA300 Control
247 16µg/mL
247 32µg/mL
247 64µg/mL
OD (600nm)
Time (Hours)
(k)
MRSA USA300 Control
290 - 16µg/mL
290 - 32µg/mL
290 - 64µg/mL
OD (600nm)
Time (Hours)
(l)
MRSA USA300 Control
291 - 32µg/mL
291 - 64µg/mL
291 - 128µg/mL
OD (600nm)
Time (Hours)
(m)
MRSA USA300 Control
302 - 8µg/mL
302 - 16µg/mL
302 - 32µg/mL
OD (600nm)
Time (Hours)
(n)
MRSA USA300 Control
303 - 8µg/mL
303 - 16µg/mL
303 - 32µg/mL
OD (600nm)
Time (Hours)

Figure 2-2. a-n) Growth curve analysis of *S. epidermidis* (12228) (a-g) and MRSA USA300 (h-n) in the presence of daptomycin, CBD or CBD derivatives (247, 290, 291, 302 and 303). Bacterial growth was measured as optical density at 600 nm (OD_{600}) over time (h). Each curve represents the growth profile of bacteria under different treatment concentrations, represented by different colours. Notably, the growth curves demonstrated an early onset of activity within 4-6 h, with a dose-dependent response. Each data point represents the mean OD_{600} ± standard error of the mean (SEM) of four independent experiments. Bacterial growth controls were included in each assessment.

2.3.4 Synergy assessment by Checkerboard assay

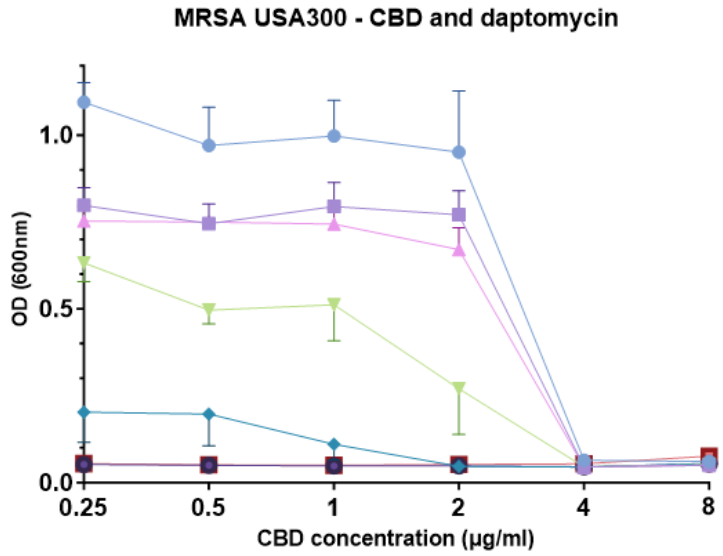
A combined approach involving daptomycin and CBD derivatives was undertaken to explore potential synergy that could enhance antimicrobial activity. Combining daptomycin with selected CBD derivatives (302 or 303) resulted in varying degrees of antibacterial activity with MIC values ranging from 2 to 16 μg/mL. Synergy was quantified using the fractional inhibitory concentration index (FICI) (Table 2-3) and dose-response curves (Figure 2-3) were generated to visualise the concentration dependent bacterial killing and supplement FICI values. As daptomycin concentrations increase, a leftward shift in the dose-response curve indicated synergistic action, while a rightward shift indicated antagonism, while lower bacterial growth at the same CBD/derivative concentration indicated additive interactions. Both CBD derivatives exhibited a distinct synergistic effect when paired with daptomycin against *E. faecium* (Table 2-3) and (Figure 2-2a).

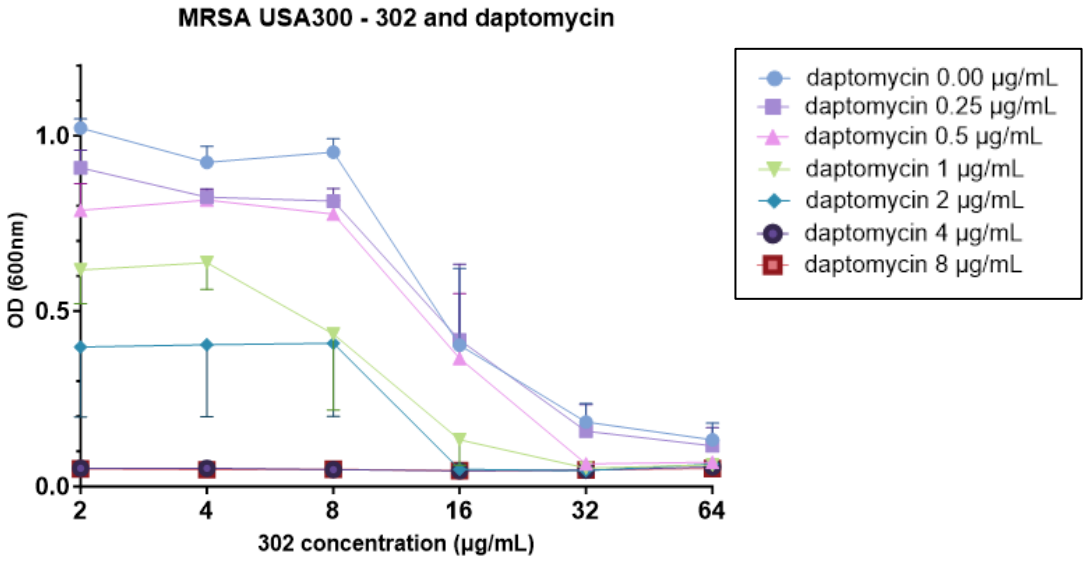
Table 2-3. Fractional inhibitory concentration index (FICI) values (and interpretation) of CBD, derivative 302 and 303 with daptomycin against Gram-positive bacteria. Clinical isolate (CI).

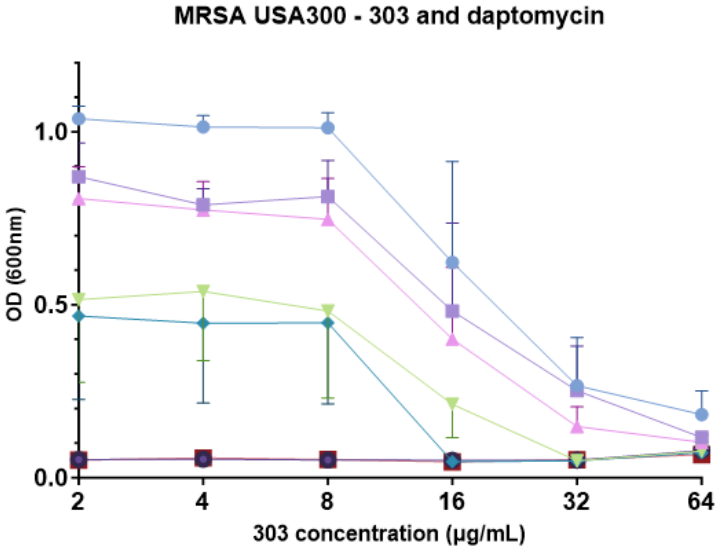
Bacteria strain (species)			
Compounds	MRSA (USA300)	*E. faecium* (CI1)	*S. epidermidis* (ATCC 12228)
302/daptomycin	1 (I)	0.375 (S)	2 (I)
303/daptomycin	1.25 (I)	0.375 (S)	1.5 (I)
CBD/daptomycin	1.25 (I)	0.625 (A)	1.5 (I)

I- indifferent; S- synergistic; A- additive

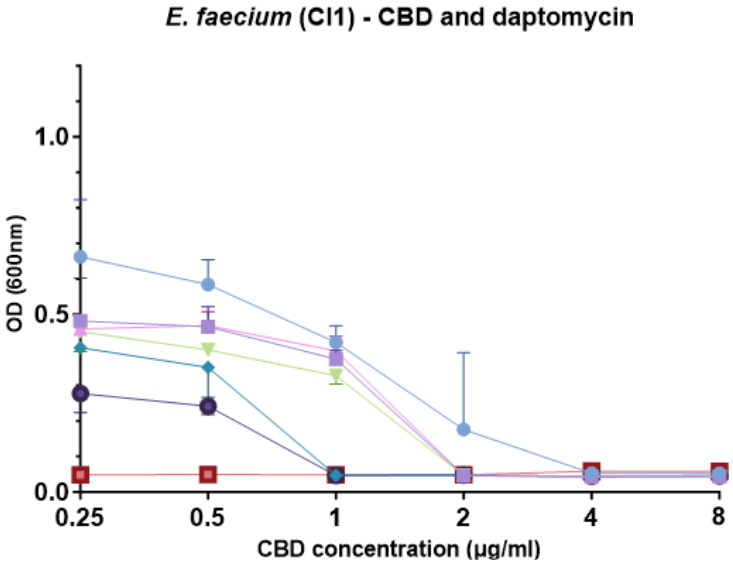
a) MRSA USA300

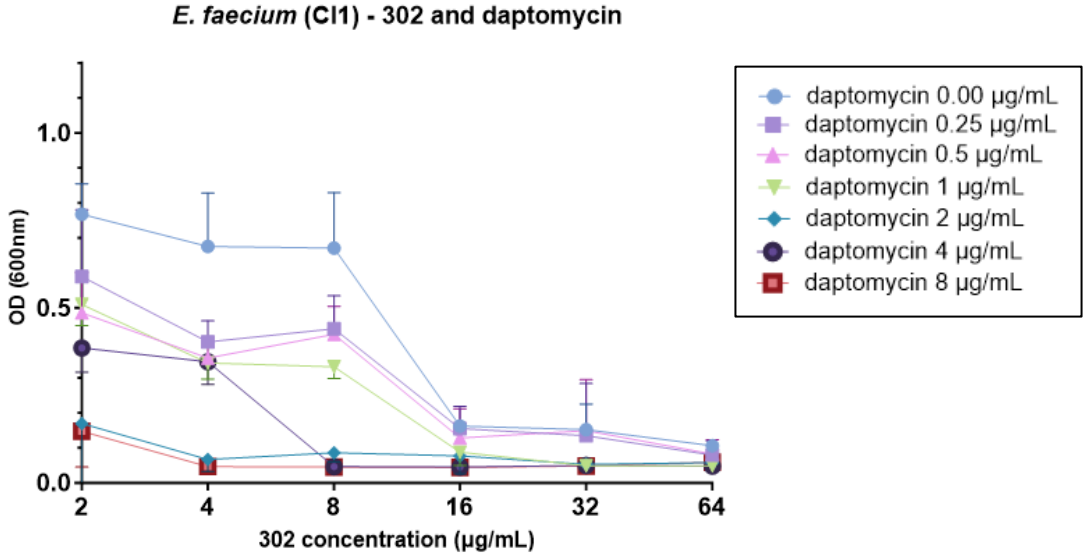


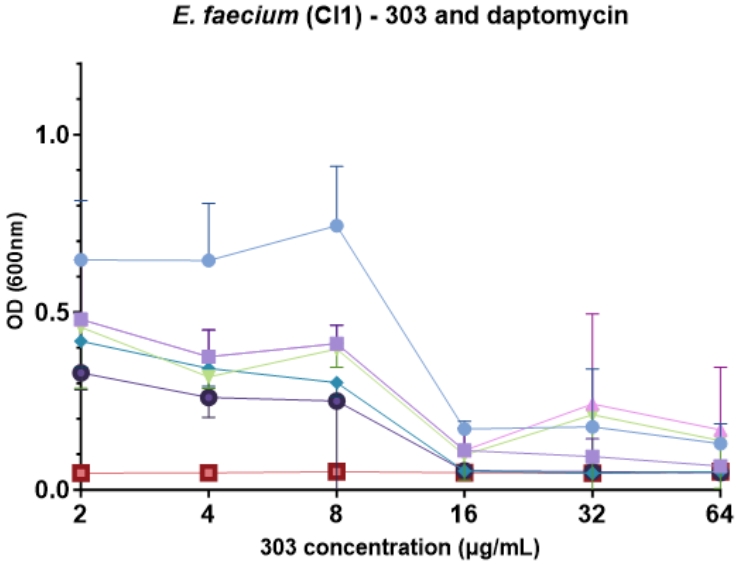




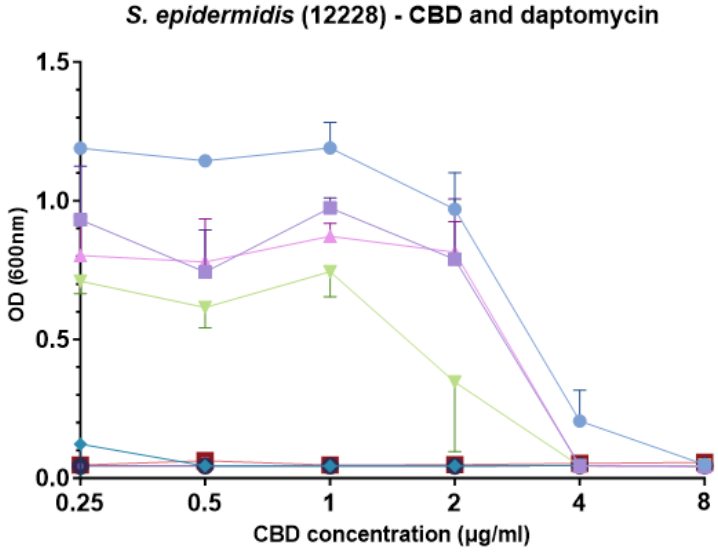
b) ***E. faecium (CI1)***

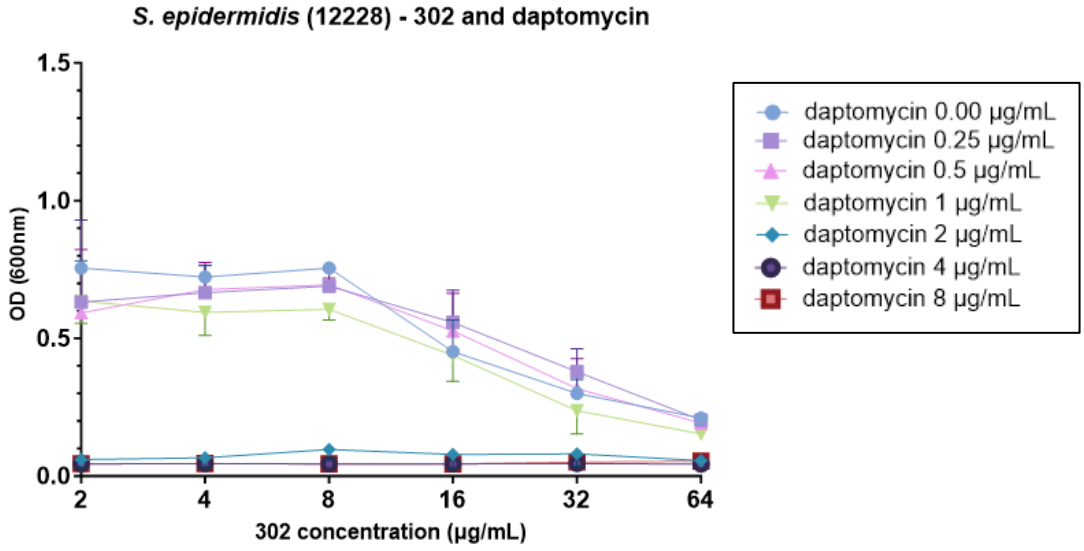






c) *S. epidermidis* (12228)





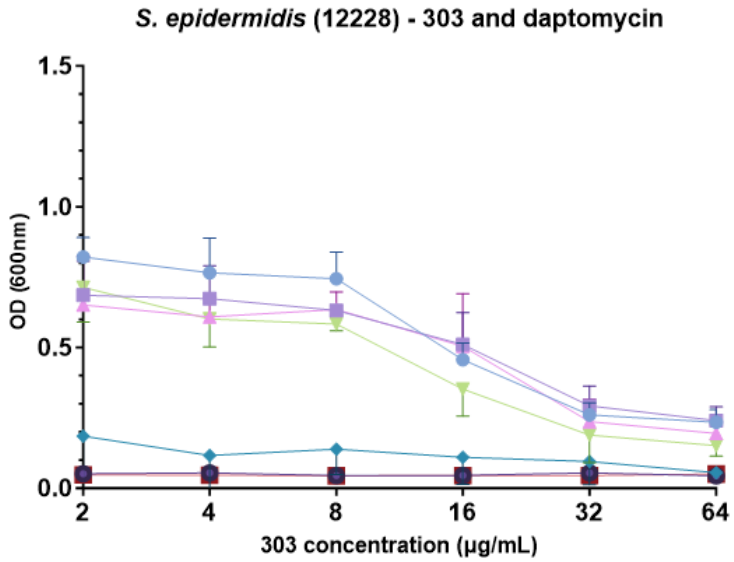


Figure 2-3. a-c) – Concentration-response curves for effects on bacterial growth in response to daptomycin (0.25 to 8 μg/mL) in combination with CBD (0.25 to 8 μg/mL) or CBD derivatives (302 or 303) (2 to 64 μg/mL) against MRSA USA300 (**a**), *E. faecium* (CI1) (**b**) and *S. epidermidis* (12228) (**c**). Bacterial growth was measured as optical density at 600 nm (OD_{600}) after 24 h incubation. Each curve represents the growth profile of bacteria under different treatment concentrations, represented by different colours. Each data point represents the mean OD_{600} ± standard error of the mean (SEM) of three independent experiments. The percentage relative growth between treated wells and the growth control was calculated using Equation 1. The MIC was defined as the lowest drug concentration reducing bacterial growth by more than 80%.

2.3.5 AlamarBlue biofilm viability assay

The AlamarBlue biofilm viability assay was used to assess the concentration-dependent impact of the five most active CBD derivatives (247, 290, 291, 302 and 303) on biofilm viability. Treatment concentrations ranged from 0.0625 – 64 μg/mL for CBD and 0.25 – 256 μg/mL for CBD derivatives. Treated plates were incubated for 24 h. MIC values for CBD against MRSA (USA300), *E. faecium* (CI1) and *S. epidermidis* (ATCC 12228) were 8 – 16 μg/mL, 8 μg/mL and 4 – 8 μg/mL respectively. MIC values for daptomycin against MRSA (USA300), *E. faecium* (CI1) and *S. epidermidis* (ATCC 12228) were 32 μg/mL, 16 to 32 μg/mL and 32 μg/mL respectively. None of the five CBD derivatives displayed activity against biofilms formed.

2.3.6 Cytotoxicity assessment

Mammalian cell cytotoxicity assessment was performed on Caco-2 human colon adenocarcinoma cells via the MTT cell viability assay. A concentration-dependent response was observed for each compound tested (CBD, 247, 290, 291, 302 and 303), including the vehicle control DMSO (Figure 2-4). It was important to understand the influence of DMSO as the vehicle because DMSO itself exerts concentration-dependent cytotoxic effects on cells (Galvao *et al.,* 2014). To help differentiate the influence of the CBD or derivatives from that of the vehicle, we included control groups treated with DMSO alone. Our results

showed that at 1% DMSO, cell viability remained high at 92%, however, at 2% and 4% DMSO, cell viability significantly decreased to 80% and 70%, respectively.

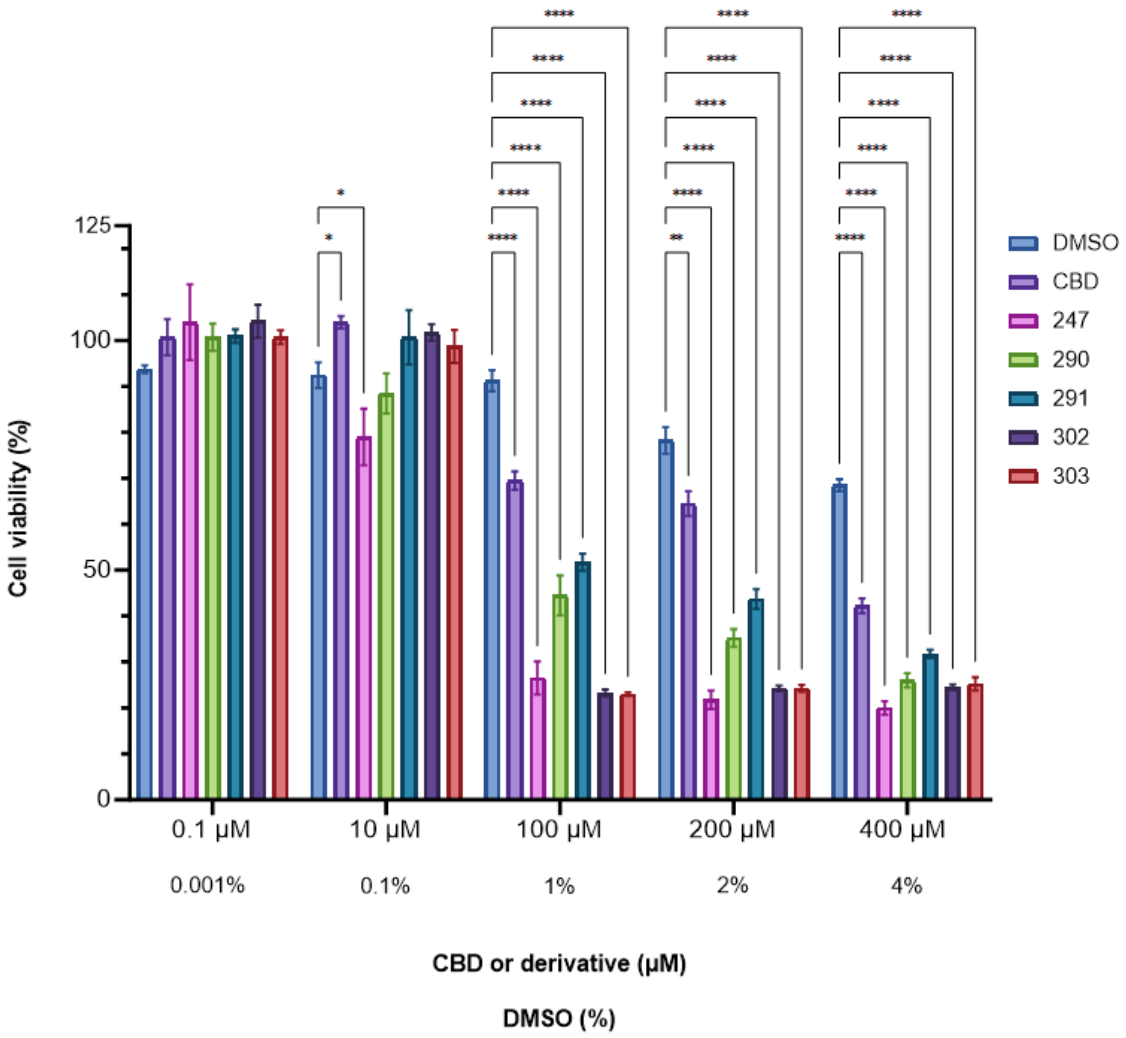


Figure 2-4. Concentration-response graph illustrating the effect of DMSO, CBD and CBD derivatives 247, 290, 291, 302 and 303 on Caco-2 cell viability by the MTT assay. Compound concentrations ranged from 0.1 to 400 µM and DMSO concentrations ranged from 0.001 to 4%. Each data point represents the mean viability percentage ± standard error of the mean (SEM) from four independent experiments. * <0.05, ** <0.01, *** <0.001, **** <0.0001 compared to vehicle control within each treatment group.

2.4 DISCUSSION

In January 2022, “The GRAM report” provided comprehensive estimates of the global burden of bacterial AMR as the contributing factor in 4.95 million deaths in 2019; of those, 1.27 million deaths were directly attributable to AMR (Murray *et al.,* 2022). The impact of AMR is more severe in lower and upper middle-income countries, not only threatening public health, but also economic growth and development (Alvarez-Uria, Gandra and Laxminarayan 2016; Sulis, Sayood and Gandra 2022). This results in prolonged hospitalisation and recovery times often requiring expensive medicines and specialised care for patients (Smith and Coast 2013; Dadgostar 2019). To address this challenge it's critical to explore innovative alternatives, harness the potential of existing agents and invest in research to develop sustainable strategies that safeguard our ability to combat infections effectively. Promising avenues, include compounds like Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), have shown potential for a range of therapeutic applications. THC is known for its analgesic, antiemetic and appetite-stimulating effects, while CBD has demonstrated anti-inflammatory, analgesic, anxiolytic and antipsychotic properties (Mechoulam, Parker and Gallily 2002; Pertwee 2008). Additionally, the minor phytocannabinoid cannabigerol (CBG) has shown promise in preclinical trials for its potential anti-inflammatory, neuroprotective and anti-cancer properties (Borrelli *et al.,* 2013). Additionally, within the cannabis plant are terpenes and flavonoids which have also demonstrated anti-inflammatory, antioxidant and analgesic activity, with cannflavin A and B inhibiting the production of certain inflammatory mediators (Russo 2011; Katsuyama *et al.,* 2013; Gertsch, Pertwee and Marzo 2010). Investigating such compounds for antimicrobial treatment may offer novel solutions for a pressing global health issue.

Previous studies have investigated the antimicrobial activity of different phytocannabinoids, including the five major cannabinoids (CBD, CBG, THC, cannabichromene [CBC] and cannabinol [CBN]), all indicating Minimum Inhibitory

Concentration (MIC) values between 0.5-2 μg/mL against six methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Farha *et al.,* 2020). Although primarily effective against Gram-positive bacteria, there is observed activity against some Gram-negative bacteria at concentrations >64 μg/mL (Blaskovich *et al.,* 2021).

In this study, we explored the antimicrobial potential of 42 CBD derivatives against Gram-positive and Gram-negative bacteria from the ESKAPE pathogen group. Preliminary investigations revealed intriguing results with five derivatives (247, 290, 291 302 and 303) demonstrating bactericidal activity against Gram-positive bacteria, including MRSA (USA300). This aligns with previous research indicating the efficacy of CBD against Gram-positive bacteria (Blaskovich *et al.,* 2021). Growth curve analysis indicated rapid concentration-dependent activity within the first six hours of exposure, while synergistic activity was observed with two derivatives (302 and 303) when used in combination with daptomycin. While the MIC and MBC values for CBD derivatives demonstrated promising bactericidal activity, it was noteworthy that CBD derivatives required higher concentrations when compared with CBD. It was also interesting to observe varying degrees of the antimicrobial activity among different strains of the same species.

Synergistic activity was observed with CBD and CBD derivatives 302 and 303 in combination with daptomycin against *E. faecium* clinical isolate 1 (CI1). This suggests that these combinations with traditional antibiotics could provide a multi-target treatment with reduced propensity for developing antimicrobial resistance (Brotz-Oesterhelt and Brunner 2008; Oldfield and Feng 2014; Gray and Wenzel 2020). However, for CBD derivatives we observed no activity against established bacterial biofilms. This lack of activity suggests that the tested CBD derivatives might encounter biofilm-specific resistance mechanisms or challenges in penetrating the biofilm matrix (Dufour, Leung and Lévesque 2012). This highlights the complexity of biofilm tolerance and resistance mechanisms and the need for

tailored approaches to address these resilient bacterial communities (Wu *et al.,* 2015; Kragh and Richter 2022).

Our growth curve analysis provided valuable insights into the kinetics of the antimicrobial activity, highlighting the concentration-dependent response and the rapid onset of activity (within 4-6 h), indicating a potential for fast-acting antimicrobial effects. However, the results of the cytotoxicity assessment of CBD derivatives in Caco-2 cells also demonstrated a concentration-dependent reduction in cell viability. Each CBD derivative exhibited varying extents of cytotoxicity against Caco-2 epithelial cells, suggesting that their chemical structures or interactions with Caco-2 cells or DMSO influenced their cytotoxic effects. These compound-specific effects should be considered when evaluating their suitability in various applications. While CBD and CBD derivatives show promise as antimicrobial agents, the observed cytotoxic effects on human intestinal epithelial cells raise important safety considerations. Further research is needed to develop drug-delivery platforms for safe and effective therapeutic use of CBD derivatives, particularly in the context of direct exposure to the human intestinal epithelium arising from oral administration.

Future mechanistic studies could explore alternative methods like membrane depolarisation, membrane permeability or bacterial cytological profiling assays, which could offer a clearer understanding of how CBD and CBD derivatives affect bacteria at the molecular level. Previous studies suggest that CBD may affect the integrity and/or permeability of bacterial membranes. Farha *et al.,* revealed that CBG acts by targeting the cytoplasmic membrane of Gram-positive bacteria (2020) and Blaskovich *et al.,* (2021) suggested membrane disruption as CBD's primary mechanism. Membrane depolarisation could lead to a disruption in ion gradients affecting essential cellular processes. Moreover, influences on membrane permeability could impact nutrient uptake, waste expulsion and overall cell homeostasis (Kapoor, Saigal and Elongavan 2017; Khodaparast *et al.,* 2021).

Differences in activity against Gram-positive and Gram-negative bacteria could be explained by the presence of the outer membrane and lipopolysaccharide (LPS). Such as in the case of bacteria like *Neisseria gonorrhoeae* and *Moraxella catarrhalis*, where membrane disrupting drugs or being LPS-deficient increases susceptibility to CBD, this was not the case for *Acinetobacter baumannii* (Blaskovich *et al.,* 2021). Alternatively, there could be specific molecular targets within bacterial cells influencing key regulatory pathways or gene expression, which could explain the differential activity observed between strains of the same species (Oldfield and Feng 2014).

When assessing synergy, the results suggest the possibility of multiple mechanisms which appeared more pronounced against *E. faecium* CI1. This could be due to complementary mechanisms of action causing simultaneous disruption of vital functions or structures, resulting in a multi-faceted cellular disruption (Oldfield and Feng 2014). This finding not only highlights the potential of combining existing antibiotics with novel antimicrobial agents but also demonstrates the importance of leveraging synergistic interactions to enhance therapeutic outcomes. Further investigations are warranted to elucidate the mechanistic basis of this observed synergy and to explore its suitability to other strains and bacterial species.

Blaskovich *et al.,* (2021) explored the structure-activity relationship (SAR) of various cannabinoid derivatives, focusing on modifications aimed at enhancing activity and bioavailability. Clear differences in SAR between Gram-positive MRSA and Gram-negative *N. gonorrhoeae* were revealed, with certain modifications showing differential effects on activity. For instance, oxidation of the cyclohexene methyl group to a hydroxymethyl (7-hydroxy-cannabidiol [7-OH-CBD]) or carboxyl group (7-nor-7-carboxy-cannabidiol [7-COOH-CBD]) was detrimental to MRSA activity but had minimal impact on *N. gonorrhoeae* activity. Notably, the placement of a carboxyl substituent on the resorcinol aromatic ring had a less of an impact on MRSA activity.

In contrast, the CBD derivatives assessed in this study had extensive modifications in the resorcinol core, including a longer bridge between the terpene and phenolic groups. Given the overall antimicrobial activity of these derivatives was reduced compared to CBD, this suggests these modifications were less favourable but nonetheless provide further insight for future SAR studies and derivative development. These findings highlight the intricate SAR of CBD derivatives and underscore the importance of considering bacterial specificity when designing antimicrobial agents based on cannabinoids. This complexity of interactions emphasises the need for further studies to fully understand the mechanisms of cannabinoids and their derivatives. Future research should focus on determining the exact mode of action of CBD or derivatives, as this remains poorly understood and may not necessarily involve easily discernible morphological changes.

Acknowledging the study's limitations, including the reliance on a single cell line for cytotoxicity assessment, we emphasise the need for further investigations. Future research should collectively focus on elucidating the specific molecular targets of CBD and derivatives within bacterial cells, drug-delivery formulation development, *in vivo* safety assessments and efficacy profiles.

2.5 CONCLUSION

In conclusion, our results highlight the antimicrobial activity of CBD and selected derivatives, particularly against Gram-positive bacteria. The concentration-dependent effects, rapid onset of action and synergy potential with traditional antibiotics make these compounds promising candidates for further research. By uncovering their potential and assessing their comparative efficacy, this research may aid the development of innovative antimicrobial therapies and strategies combating AMR.

CHAPTER 3

INVESTIGATING THE GASTROPROTECTIVE AND CYTOTOXIC PROPERTIES OF TERPENES REVEALS A SELECTIVE PROTECTIVE ROLE FOR LINALOOL ON INTESTINAL BARRIER FUNCTION IN VITRO

Statement of Authorship

Title of Paper	INVESTIGATING THE GASTROPROTECTIVE AND CYTOTOXIC PROPERTIES OF TERPENES REVEALS A SELECTIVE PROTECTIVE ROLE FOR LINALOOL ON INTESTINAL BARRIER FUNCTION IN VITRO
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Principal Author

Name of Principal Author (Candidate)	Luke Wooley		
Contribution to the Paper	Performed the experiments, performed the analysis and wrote the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	09/07/2025

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

iv. the candidate's stated contribution to the publication is accurate (as detailed above);

v. permission is granted for the candidate in include the publication in the thesis; and

vi. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr Scott Smid		
Contribution to the Paper	Conceived the project and provided critical review of the manuscript		
Signature		Date	9/07/2025

PREFACE

This manuscript was written to the specifications of the University of Adelaide. Essential formatting rules were followed in order to satisfy the thesis guidelines for the University of Adelaide's School of Biomedicine Doctor of Philosophy in Pharmacology program:

- 12pt Times New Roman font
- Double spacing
- Block justified
- Left margin 3.5cm; top, bottom and right margins 2cm
- Figures and tables are embedded in the appropriate places within the text, rather than at the end of the document or in a separate document
- Highlight section is not included.
- References are not a numbered style.

The word count for the manuscript (excluding references, acknowledgements and supplementary material) is 6067.

TITLE

Investigating the Gastroprotective and Cytotoxic Properties of Terpenes Reveals a Selective Protective role for linalool on intestinal barrier function *in vitro*

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3.0 ABSTRACT

Gastrointestinal (GI) disorders such as peptic ulcers, mucositis and inflammatory bowel disease (IBD) pose a significant health burden worldwide, often resulting from a myriad of factors including microbial imbalance, nonsteroidal anti-inflammatory drug (NSAID) overuse, oxidative stress and immune dysregulation. While current treatments effectively manage symptoms, they are frequently associated with adverse side effects, highlighting the need for safer therapeutic options. Terpenes, aromatic compounds abundant in a wide variety of plants have gained attention for their anti-inflammatory, antioxidant, and gastroprotective properties.

This study evaluated the cytotoxic and protective effects of 16 cannabis predominant terpenes on intestinal epithelial Caco-2 cells using the MTT cell viability assay, Trans-Epithelial Electrical Resistance (TEER) measurements of barrier function and analysis of tight junction protein expression. The MTT results demonstrated a variable but otherwise innocuous viability profile of Caco-2 cells in response to terpenes, with protective effects demonstrated for selected compounds such as linalool and α-bisabolol, but also with concentration-dependent cytotoxicity for several terpenes (>200 μM). Functional TEER analysis revealed that in the presence of pro-inflammatory cytokines, linalool significantly improved epithelial barrier integrity ($p < 0.01$) after 48 hours, suggesting its potential to mitigate inflammation-induced intestinal epithelial monolayer damage. However, western blot analysis revealed that in cytokine-treated cells, occludin tight junction protein expression was reduced. The ability for linalool to maintain TEER in inflammatory conditions without upregulation of claudin-1 or occludin suggests alternative mechanisms are responsible for maintaining TEER under such settings.

These findings further highlight the potential of terpenes as natural gastroprotective agents with promising applications in food products, essential oils and in clinical therapeutic

formulations such as medicinal cannabis. However, further research is required to understand the mechanisms underlying their protective effects and to identify therapeutic concentrations safe for use in managing GI disorders.

Keywords: Gastrointestinal disorders, Gastroprotection, Terpenes, Tight junctions, Caco-2 cells, linalool, Oxidative stress.

3.1 INTRODUCTION

Gastrointestinal (GI) disorders such as peptic ulcers, mucositis and inflammatory bowel disease (IBD) represent a substantial global health burden, affecting millions and costing billions annually (Peery *et al.,* 2022; Ng *et al.,* 2017). These health conditions are often attributed to damage to the mucosal lining of the stomach and intestines due to many factors including microbial imbalance, genetic predisposition, immune dysregulation, excessive use of nonsteroidal anti-inflammatory drugs (NSAIDs) and dietary habits. (Kaser, Zeissig and Blumberg 2010; Camilleri *et al.,* 2012; Melcarne, García-Iglesias and Calvet 2016). While current therapies, including glucocorticosteroids and aminosalicylates have demonstrated efficacy in the treatment of IBD, their use is frequently accompanied by adverse side effects and potential long-term complications, emphasising the need for safer and more effective alternatives. (Van Staa *et al.,* 2005; Gisbert, González-Lama and Maté 2007; Curkovic, Egbring and Kullak-Ublick 2013).

Gastroprotective agents are compounds that assist in regulating the GI mucosa by promoting protective factors like mucus and bicarbonate secretion, or by inhibiting harmful factors such as acid production, oxidative damage and/or microbial imbalance (Kaser, Zeissig and Blumberg 2010; Camilleri *et al.,* 2012). The search for more effective gastroprotective agents has increasingly focused on natural compounds including terpenes, particularly due to their favourable safety profiles and versatile bioactivity. Terpenes are

naturally occurring organic compounds found primarily in plants and some insects, responsible for the distinctive aromas and flavours of many fruits, herbs, and flowers. Structurally composed of isoprene units, terpenes are abundant in essential oils and serve various roles such as attracting pollinators and defending plants from pests and environmental stress (Gershenzon and Dudareva 2007; Lange and Ahkami 2013).

Known for their diverse pharmacological properties, terpenes exhibit anti-inflammatory, antioxidant, antimicrobial, anxiolytic, cytoprotective and gastroprotective effects. Common examples include limonene, myrcene, (-)-β-caryophyllene, linalool, and pinene, which have been widely studied for their therapeutic potential (Paduch *et al.,* 2007; Nuutinen 2018; Gertsch *et al.,* 2008; Tomko *et al.,* 2020; Kim *et al.,* 2020). Terpenes can be administered via inhalation, topically or via the GI tract, with their mechanisms of action influenced by the route of intake, chemical composition and concentration (Gershenzon and Dudareva 2007). Factors such as the rate of uptake and any synergistic interactions between constituents play a critical role in their effects (Koyama and Heinbockel 2020).

Select cannabis-predominant terpenes such as (-)-β-caryophyllene have been shown to activate peroxisome proliferator-activated receptors (PPARs) and is a selective cannabinoid receptor 2 (CB2-R) agonist, reducing oxidative stress and inflammation (Bento *et al.,* 2011; Horváth *et al.,* 2012). This activity plays a critical role in regulating gut homeostasis, inflammation, and barrier integrity and has been shown to quell colitis in experimental models (Bento *et al.,* 2011; Horváth *et al.,* 2012; Cho *et al.,* 2015). This activity aligns with studies on minor phytocannabinoids such as cannabichromene (CBC) and cannabigerol (CBG), which have demonstrated similar activity in modulating colitis, suggesting the potential for synergistic activity (Anderson *et al.,* 2024; Izzo *et al.,* 2012). Interestingly, many terpenes have also shown a modest degree of cannabinoid receptor 1 (CB1-R) activation and when combined with cannabinoids like Δ^9-tetrahydrocannabinol (THC), selectively enhanced CB1-R activation (Raz *et al.,* 2023). Through this multifaceted

regulation terpenes may help reduce intestinal permeability, alleviate inflammation-associated damage and support overall GI health, making them promising candidates for managing gut disorders such as IBD, functional dyspepsia and leaky gut syndrome (Bento *et al.,* 2011; Horváth *et al.,* 2012; Youssef, El-Favoumi and Mahmoud 2019; LaVinge *et al.,* 2021; Wardill *et al.,* 2023).

While abovementioned examples allude to a range of GI bioactivities of terpenes which may improve a broad suite of markers of gut health generally, the direct role of terpenes on intestinal epithelial barrier function has yet to be systematically profiled. With increasing use of broad-spectrum medicinal cannabis formulations for gut disorders, we aimed to investigate the effects of both cannabis-prevalent and minor terpenes on intestinal epithelial barrier integrity in an in vitro functional epithelial barrier model.

This study focused on the potential of cannabis predominant terpenes (α-pinene, (-)-β-caryophyllene, β-caryophyllene oxide, α-bisabolol, β-pinene, borneol, citronellol, friedelin, humulene, limonene, linalool, myrcene, nerolidol, ocimene, terpinolene, and zerumbone) to improve intestinal barrier function, cytotoxicity and protective properties using Caco-2 epithelial monolayers as a model of the intestinal epithelium. To implement these we employed assays for biochemical mitochondrial activity (MTT assay), epithelial barrier integrity (TEER measurements) and tight junction protein expression (Western blot).

3.2 MATERIALS AND METHODS

Corning CoStar Transwells - Polyester membrane (Polyethylene Terephthalate - PET), 6.5 mm diameter, 0.4 μm pore size, 0.33 cm2 24-well plate, 12 inserts per plate (Corning, Cat. No. CLS3402), trypan blue, dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT), Dulbecco's Modified Eagle Medium (DMEM), non-essential amino acids (NEAA), penicillin/streptomycin, 0.25% w/v trypsin with phenol red and

ethylenediaminetetraacetic acid (EDTA), 250 mg/mL amphotericin B (Cat. No. A2942), phosphate buffered saline (PBS) and α-pinene, (-)-β-caryophyllene, β-caryophyllene oxide, α-bisabolol, β-pinene, borneol, citronellol, friedelin, humulene, limonene, linalool, myrcene, nerolidol, ocimene, terpinolene, zerumbone and pro-inflammatory cytokines tumour necrosis factor-α (TNF-α) (Cat. No. SRP3177) and Interleukin 1-β (IL-1β) (Cat. No. H6291) were purchased from Sigma-Aldrich (St. Louis, MI, USA). Pierce™ BCA Protein Assay Kit, Tris-buffered saline, radioimmunoprecipitation buffer (RIPA), protease inhibitor cocktail, 0.2 μM nitrocellulose membrane, bicinchoninic acid (BCA) assay, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from ThermoFisher Scientific (Waltham, MA, USA). Foetal bovine serum (FBS) was purchased from Fischer Biotec (Wembley, WA, Australia) and bovine serum albumin (BSA) was obtained from Bovogen Biologicals (East Keilor, VIC, Australia). Rabbit monoclonal antibody to β-actin cytoskeleton marker (ab115777), rabbit monoclonal antibody to Occludin (ab216327), rabbit monoclonal antibody to Claudin 1 (ab211737), HRP-conjugated goat anti-rabbit antibody (Invitrogen #SA535571) and protease inhibitor cocktail (EDTA-free) (ab2011111) were purchased from Abcam (Cambridge, United Kingdom). All materials were autoclaved for sterility and all analysis was conducted under sterile conditions.

Standard *in vitro* assessment protocols, including the Thiazolyl blue tetrazolium bromide (MTT) assay and transepithelial electrical resistance (TEER) measurement, were employed to evaluate the effects of 16 selected terpenes on Caco-2 human colorectal adenocarcinoma cell line, including α-pinene, (-)-β-caryophyllene, β-caryophyllene oxide, α-bisabolol, β-pinene, borneol, citronellol, friedelin, humulene, limonene, linalool, myrcene, nerolidol, ocimene, terpinolene, and zerumbone. The MTT assay was used to assess mitochondrial activity as an indication of cell viability and compound toxicity, while TEER measurements evaluated mucoprotective properties in the presence of pro-inflammatory cytokines tumour necrosis factor-α (TNF-α) and interleukin 1-β (IL-1β). Western blot

analysis was subsequently performed to investigate the influence of three selected terpenes (myrcene, (-)-β-caryophyllene and linalool) on tight junction protein expression (claudin-1 and occludin). These three terpenes were selected based on TEER results and their reported anti-inflammatory and gastroprotective properties, as well as their potential to modulate intestinal barrier function. Terpenes were dissolved in 70% ethanol to a concentration of 10 mM and stored at -20 °C until required and pro-inflammatory cytokines, TNF-α and IL-1β were dissolved in PBS to a concentration of 10 μg/mL and stored at -80 °C until required.

3.2.1 Cell culture

Human colonic carcinoma (Caco-2) cells obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) were used to replicate the gut epithelium *in vitro*. Cells were cultured in complete DMEM, containing 10% v/v FBS, 1% v/v non-essential amino acids and 1% v/v penicillin/streptomycin then incubated at 37°C with 5% CO_2 as per previously established protocols (Marsh and Smid 2022).

3.2.2 Thiazolyl Blue Tetrazolium Bromide (MTT) assay

Cell viability after treatment was measured by MTT assay as per previously established protocol (Marsh and Smid 2022). Briefly, cells were seeded in 96-well plates at a density of 2×10^4 cells per well in complete DMEM, then incubated at 37 °C with 5% CO_2 for 24 h prior to treatment. Cells were treated with each terpene at ranging concentrations (0, 25, 50, 100, 200 and 400 μM) then incubated for 48 hours at 37°C with 5% CO_2, after which time cell viability was measured using the MTT assay. Culture media was aspirated and replaced with serum-free media containing 0.25 mg/mL MTT then incubated for 2 hours at 37°C, 5% CO_2. MTT solution was then aspirated and cells lysed with DMSO. Absorbance

was measured at 570 nm using a Synergy MX microplate reader (Bio-Tek, Bedfordshire, UK). Cell viability was calculated using Equation 1:

$$Cell\ Viability\ (\%) = \left(\frac{Absorbance\ of\ control}{Absorbance\ of\ treated\ sample}\right) \times 100 \quad (1)$$

3.2.3 Transepithelial Electrical Resistance (TEER)

Transepithelial electrical resistance (TEER) was measured using previously established protocols with modifications (Marsh and Smid 2022). Briefly, experiments were conducted in Corning CoStar Transwells - Polyester membrane (Polyethylene Terephthalate - PET), 6.5 mm diameter, 0.4 μm pore size, 0.33 cm^2 24-well plate, 12 inserts per plate. The basolateral compartment was filled with 600 μL of complete DMEM containing 1% v/v amphotericin B. Cells were seeded in the apical compartment at a density of 1.65×10^5 cells/mL in 100 μL of complete DMEM; one insert per plate was left blank containing media only for the purpose of normalising TEER values. Plates were then incubated for 6 hours at 37 °C with 5% CO_2, at which time media in the apical chamber was replaced with complete DMEM to remove non-adherent cells and prevent excessive clumping. Media in both the apical and basolateral chambers was changed every 2 to 3 days for up to 25 days until the cells reached confluence and differentiated; These subsequent media changes were supplemented with 1% v/v amphotericin B to avoid fungal contamination. Before TEER measurements were taken, media was replaced with complete DMEM without amphotericin B. Only inserts with TEER values over 500 Ω/cm^2 were used for analysis. Once cells reached threshold TEER values, the apical chamber was treated with each terpene at 50 μM and allowed to equilibrate for 15 minutes prior to the addition of pro-inflammatory cytokines TNFα and IL-1β to the basolateral chamber (each at 100 ng/mL).

Treatment concentrations were based on initial concentration-dependent toxicity results, where 50 μM was generally non-toxic to Caco-2 cells across all terpenes tested, and each terpene was tested without pro-inflammatory cytokines to determine their individual effects on epithelial permeability. Cytokines were diluted to desired concentrations using PBS as a vehicle. TEER measurements were taken 6, 24 and 48 hours after treatment. TEER was measured using the EVOM3 Epithelial Volt/Ohm meter (World Precision Instruments, Sarasota, FL, USA) to determine monolayer integrity. Initial resistance values for each monolayer were normalised to the blank well using Equation 2:

$$Resistance\ of\ Monolayer\ (\Omega) = Resistance\ Initial\ (\Omega) - Resistance\ Blank\ (\Omega) \quad (2)$$

Final TEER values were then calculated using Equation 3:

$$TEER\ Final\ (\Omega.cm^2) = Resistance\ Monolayer\ (\Omega) \times A\ Culture\ Area\ (cm^2) \quad (3)$$

3.2.4 Western Blot

Caco-2 cells were pre-treated with terpene linalool, myrcene or (-)-β-caryophyllene (50 μM) incubated for 2 hours before addition of both TNF-α and IL-1β (10 ng/mL each) then incubated for 24 hours alongside an untreated control containing vehicle (ethanol 0.7%).

After incubation, cells were washed in ice-cold PBS and lysed with ice-cold RIPA buffer containing protease inhibitors (aprotinin 2 mg/ml, pepstatin A 1 mg/ml, PMSF 1 mM and EDTA 5 mM). The cells were scraped into a microfuge tube and agitated on ice for 30 minutes prior to centrifugation at 12,000 rpm at 4 °C. Lysate supernatant was stored at -70

°C until needed. Lysate protein concentrations were determined via BCA assay then normalised accordingly. Samples were loaded into Mini Protein TGX precast (any kD) gel (Bio-Rad, Gladesville, NSW, Australia) with a protein loading of 30 µg/lane alongside protein standards. Electrophoresis was performed at 80V for 15 min then increased to 150V for 1 hour in 1× running buffer (1.88% glycine, 0.3% Tris, 0.2% SDS, adjusting volume with Milli-Q water). Proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane at 100V for 1.5 hours in transfer buffer (1.44% Glycine, 0.3% Tris, 25% methanol, adjusting volume with Milli-Q water). The membrane was then blocked with 5% non-fat dry milk and 0.1% Tween 20 in Tris-buffered saline. Western blot analysis was performed using primary monoclonal occludin antibody (Abcam #ab216327) with dilution 1:1000 and monoclonal claudin-1 antibody (Abcam #ab211737) with dilution 1:1000, as well as monoclonal β-actin antibody (Abcam #ab115777) with dilution 1:200 as a control used to normalise the data. The membrane was incubated with the primary antibody overnight at 4 °C with agitation, then incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Invitrogen #SA535571), diluted 1:2000. After incubation, immunoreactive bands were detected using a Li-COR Odyssey infrared imaging system at 800 nm and 700 nm (Li-Cor Biosciences, Cambridge, UK). The density of each protein was quantified using ImageJ software, normalised relative to β-actin density then expressed as a percentage relative to the untreated control.

3.2.5 Statistical analysis

All experiments were performed in 4 independent experiments. The data were expressed as mean ± SEM. GraphPad Prism 10 was used for statistical analysis and graph creation (GraphPad Software, San Diego, USA). Data from MTT and TEER assays was analysed

by two-way analysis of variance (ANOVA) with Dunnett's multiple comparisons to determine significance versus control for cell viability and versus cytokine treatment for TEER data ($p < 0.05$). Data from Western blots was analysed using one-way ANOVA with Bonferroni's multiple comparisons to determine significance versus control and cytokine treatment groups ($p < 0.05$).

3.3 RESULTS

3.3.1 Effects of terpenes on Caco-2 cell viability via MTT assay

The MTT assay, which measures mitochondrial activity as an indicator of cell metabolic function, was used to evaluate the effects of various terpenes on the viability of Caco-2 cells across a concentration range (25–400 μM), illustrated in Figure 3-1(a–d).

Results revealed that 5 of the 16 terpenes (friedelin, limonene, α-bisabolol, linalool and citronellol) either had no impact or significantly improved viability. Friedelin and limonene indicated no significant difference, while linalool and citronellol significantly increased cell viability at higher concentrations (≥ 200–400 μM) ($p < 0.001$ to $p < 0.0001$ compared to the vehicle control). Interestingly, α-bisabolol showed a significant increase in viability at 200 μM ($p < 0.0001$) however, there was no significant difference at 400 μM. The most pronounced protective effects were observed for α-bisabolol at 200 μM and for linalool and citronellol at concentrations ≥ 200 μM ($p < 0.001$ to $p < 0.0001$). These results suggest that certain terpenes may promote cellular metabolism or confer resistance to stress at these concentrations.

Moderate cytotoxic effects were observed with myrcene ($p < 0.05$) and terpinolene ($p < 0.01$) at 400 μM while, β-pinene and borneol produced significant cytotoxicity at 400 μM ($p < 0.0001$).

Several terpenes (nerolidol, ocimene, (-)-β-caryophyllene and humulene) exhibited concentration-dependent reductions in cell viability, displaying a steep decline with increasing concentrations (≥ 25-400 µM), suggesting cytotoxic potential ($p < 0.05$ to < 0.0001). Interestingly, zerumbone, α-pinene and β-caryophyllene oxide significantly increased viability at 25 µM ($p < 0.01$) and 200 µM ($p < 0.05$) respectively. However, viability significantly decreased at 50 and ≥ 200 µM ($p < 0.0001$) for zerumbone and at 400 µM ($p < 0.0001$) for α-pinene and β-caryophyllene oxide. These findings suggest potential hormetic effects of selected terpenes, with cytoprotective effects at lower concentrations and cytotoxicity at higher concentrations.

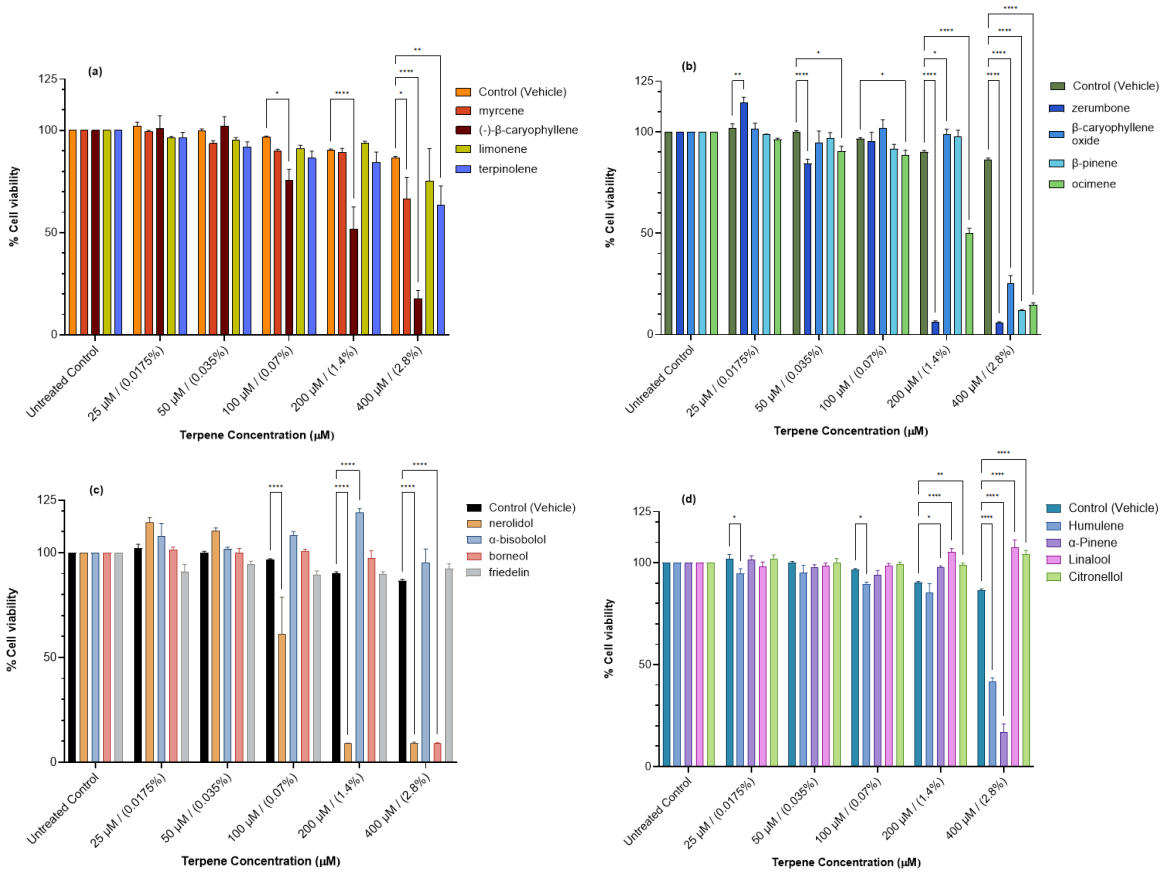
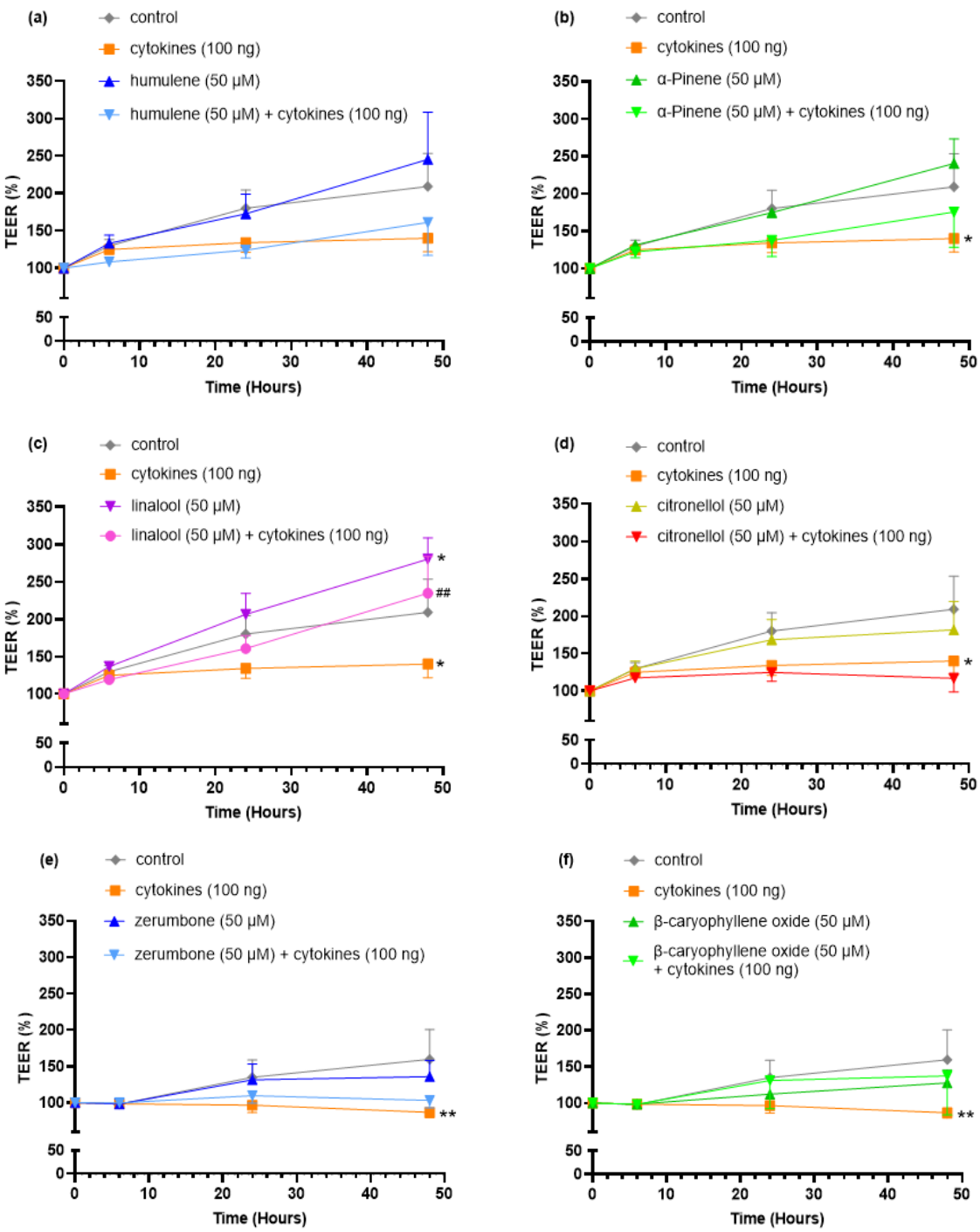
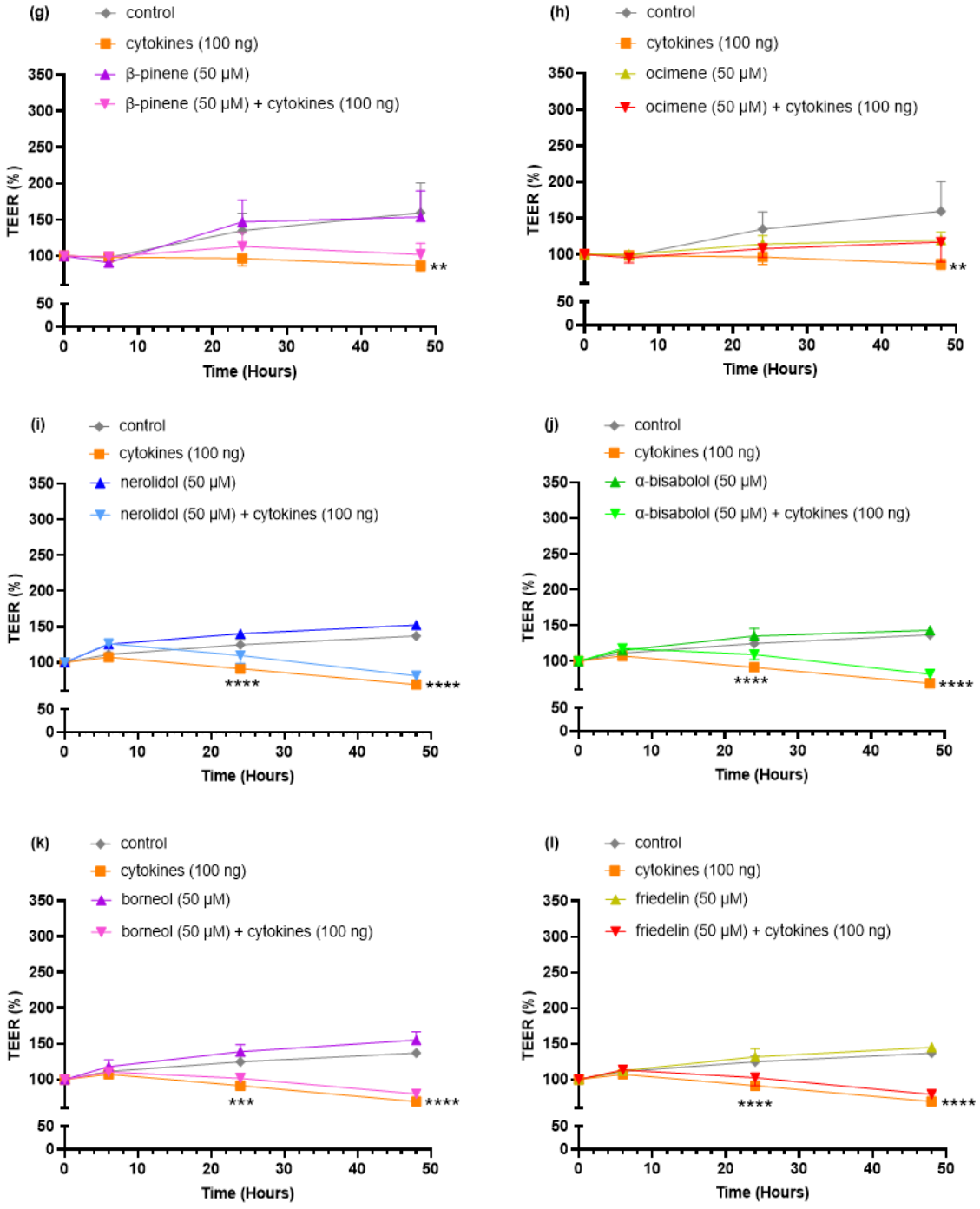


Figure 3-1. MTT assay of Caco-2 cell viability following 48 h incubation with varying concentrations of terpenes (0 – 400 µM): **(a)** myrcene, (-)-β-caryophyllene, limonene and terpinolene, **(b)** zerumbone, β-caryophyllene oxide, β-pinene and ocimene, **(c)** nerolidol, α-bisabolol, borneol and friedelin, **(d)** humulene, α-pinene, linalool and citronellol. Vehicle (ethanol) concentrations ranged from 0 – 2.8%. Each data point represents the mean viability percentage ± SEM from four independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to vehicle control within each group.

3.3.2 Effects of terpenes on Transepithelial Electrical Resistance (TEER)

The effect of terpenes on Caco-2 monolayer integrity was assessed using TEER measurements at a sub-toxic test concentration (50 μM), as shown in Figure 3-2(a-p). TEER reflects the functional status of the epithelial barrier, with higher values indicating stronger barrier integrity. When treated with inflammatory cytokines alone, there was a significant reduction in TEER compared to the control (Figure 3-2a-l), demonstrating that cytokines disrupted epithelial barrier integrity. In Figure 3.2 (m-p), there was a trend towards a reduction with cytokines vs control, but this was not significant. When treated with terpenes alone, both limonene and linalool demonstrated a significant increase in TEER at 48 h compared to the control ($p < 0.05$). When treated with both cytokines and terpenes, linalool was the only terpene to show a significant difference in TEER compared to the cytokine treatment alone. Linalool maintained a significantly higher TEER ($p < 0.01$) after 48 hours compared to inflammatory cytokines alone (Figure 3-2d). These findings suggest that linalool may maintain barrier integrity under both baseline (control) and inflammatory conditions.


(a)
control
cytokines (100 ng)
humulene (50 µM)
humulene (50 µM) + cytokines (100 ng)
TEER (%)
Time (Hours)
(b)
control
cytokines (100 ng)
α-Pinene (50 µM)
α-Pinene (50 µM) + cytokines (100 ng)
*
(c)
control
cytokines (100 ng)
linalool (50 µM)
linalool (50 µM) + cytokines (100 ng)
*
##
*
(d)
control
cytokines (100 ng)
citronellol (50 µM)
citronellol (50 µM) + cytokines (100 ng)
*
(e)
control
cytokines (100 ng)
zerumbone (50 µM)
zerumbone (50 µM) + cytokines (100 ng)
**
(f)
control
cytokines (100 ng)
β-caryophyllene oxide (50 µM)
β-caryophyllene oxide (50 µM) + cytokines (100 ng)
**


(g)
control
cytokines (100 ng)
β-pinene (50 μM)
β-pinene (50 μM) + cytokines (100 ng)
TEER (%)
Time (Hours)
**
(h)
control
cytokines (100 ng)
ocimene (50 μM)
ocimene (50 μM) + cytokines (100 ng)
TEER (%)
Time (Hours)
**
(i)
control
cytokines (100 ng)
nerolidol (50 μM)
nerolidol (50 μM) + cytokines (100 ng)
TEER (%)
Time (Hours)
****
****
(j)
control
cytokines (100 ng)
α-bisabolol (50 μM)
α-bisabolol (50 μM) + cytokines (100 ng)
TEER (%)
Time (Hours)
****
****
(k)
control
cytokines (100 ng)
borneol (50 μM)
borneol (50 μM) + cytokines (100 ng)
TEER (%)
Time (Hours)
***
****
(l)
control
cytokines (100 ng)
friedelin (50 μM)
friedelin (50 μM) + cytokines (100 ng)
TEER (%)
Time (Hours)
****
****

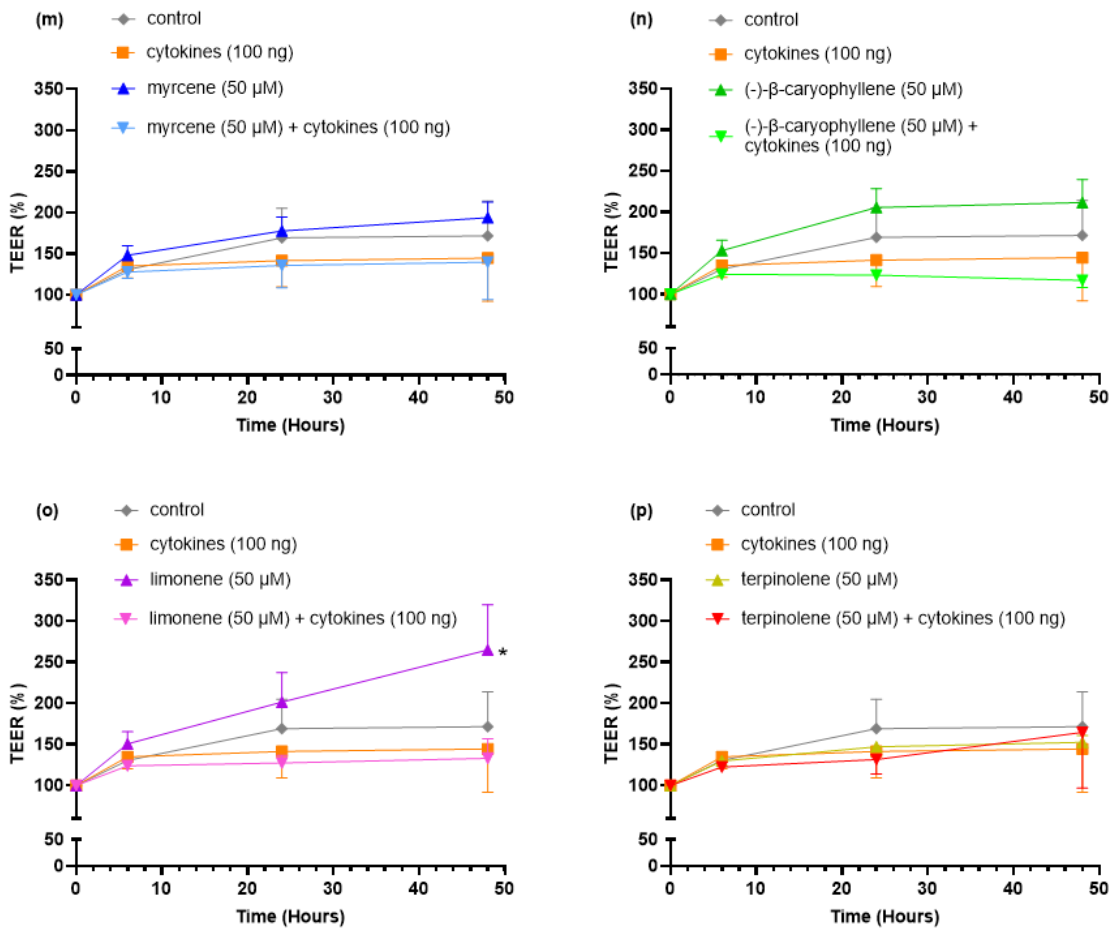


Figure 3-2(a-p). TEER measurements for four groups: control (ethanol vehicle at 0.035%), terpene (50 µM), terpene (50 µM) with IL-1β and TNF-α (100 ng each), and IL-1β and TNF-α alone (100 ng each). **(a)** humulene, **(b)** α-pinene, **(c)** linalool, **(d)** citronellol, **(e)** zerumbone, **(f)** β-caryophyllene oxide, **(g)** β-pinene, **(h)** ocimene, **(i)** nerolidol, **(j)** α-bisabolol, **(k)** borneol, **(l)** friedelin, **(m)** myrcene, **(n)** (-)-β-caryophyllene, **(o)** limonene, **(p)** terpinolene. Linalool was the only terpene to show a significant increase in TEER compared to the cytokine-only control group, suggesting protective effects on barrier integrity. No significant TEER changes were observed for other terpenes. Each data point represents the mean ± SEM from four independent experiments. (*) Compared to control, (#) compared to cytokine treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$.

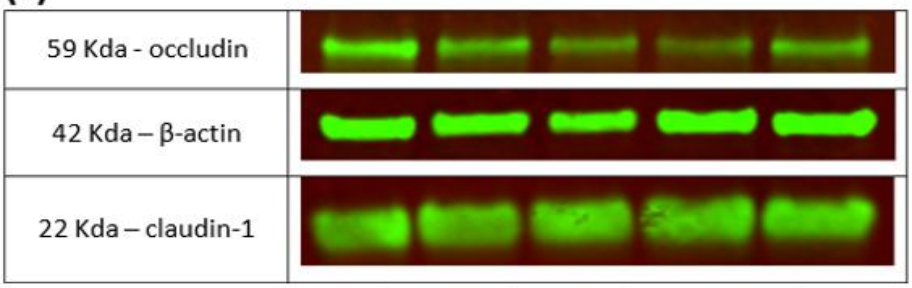
3.3.3 Western Blot analysis of tight junction protein expression

Western blot analysis was performed to evaluate the expression of claudin-1 and occludin after treatment with terpenes myrcene, (-)-β-caryophyllene or linalool (50 µM) and pro-inflammatory cytokines TNF-α (10 ng/mL) and IL-1β, (10 ng/mL) compared to a control

(Figure 3-3a-c)**.** For claudin-1 expression, as shown in Figure 3-3b, cytokine treatment alone did not induce significant changes compared to the control. However, treatment with (-)-β-caryophyllene or linalool and cytokines significantly decreased claudin-1 expression ($p < 0.05$ compared to the cytokine-treated group), while there was no significant difference between the cytokine-treated group and the group treated with myrcene and cytokines.

For occludin expression, as shown in Figure 3-3c there was no significant difference between the control and cytokine treatment groups. However, all terpene-treated groups exhibited a significant reduction in occludin levels ($p < 0.0001$ relative to the cytokine-treated group), suggesting that these compounds did not mitigate cytokine-induced occludin loss and may have contributed to further loss. These findings suggest that the protective effect of linalool observed in TEER analysis is not mediated by upregulation of claudin-1 or occludin under these conditions.

(a)



Lane conditions	Control	Cytokines (CYT)	CYT + myrcene	CYT + (-)-β-caryophyllene	CYT + linalool
TNF-α (10 ng/mL + IL-1β (10 ng/mL)	-	+	+	+	+
vehicle (ethanol) (0.7%)	+	+	+	+	+
myrcene (50 µM)	-	-	+	-	-
(-)-β-caryophyllene (50 µM)	-	-	-	+	-
linalool (50 µM)	-	-	-	-	+

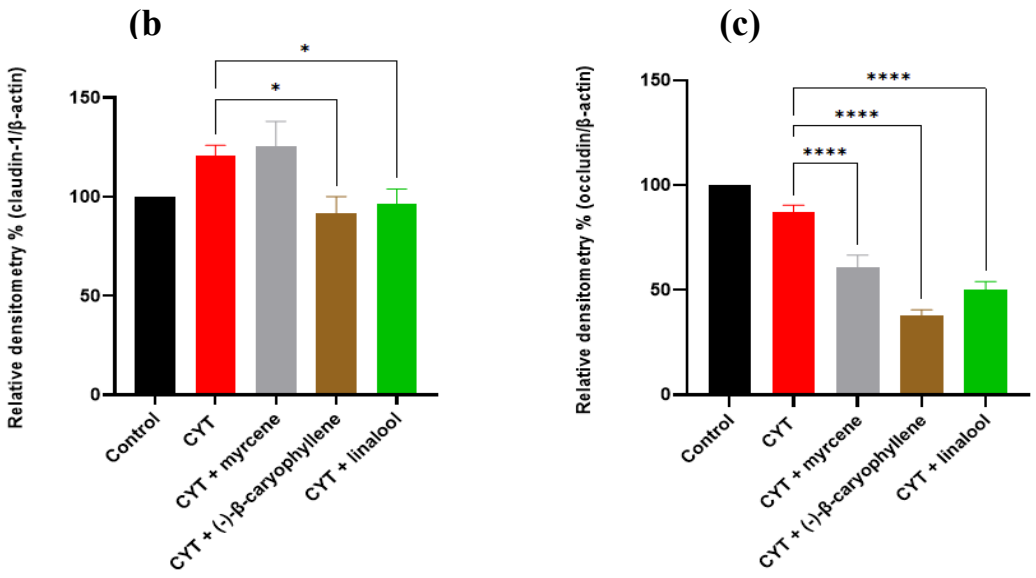


Figure 3-3. Western blot treatment conditions and blot results **(a)** and relative densitometry of claudin-1 **(b)** and occludin **(c)** expression in Caco-2 cells treated with TNF-α (10 ng/mL) and IL-1β (10 ng/mL), with or without terpenes, myrcene, (-)-β-caryophyllene or linalool (50 µM). Protein expression was quantified by densitometric analysis, normalised to β-actin, and expressed as a percentage relative to the untreated control. Bar graphs show mean ± SEM (n = 8). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.4 DISCUSSION

Terpenes are considered to have a favourable safety profile due to their natural origin, low toxicity and long history of use in traditional medicine, aromatherapy, cosmetics and food products. Generally recognised as safe, they are well-tolerated when used appropriately, though their safety depends on factors such as concentration and administration route, with key considerations being potential for allergic reactions and cytotoxicity at high concentrations (Dharmagunawardena *et al.,* 2003; Matura *et al.,* 2005; Koyama and Heinbockel 2020). Terpenes are the largest group of natural bioactive compounds, with their production influenced by both genetic factors and environmental conditions (Gershenzon and Dudareva 2007). As a result, each terpene exhibits distinct bioactivity, leading to varied effects on cellular processes and physiological responses.

This study investigated the role of terpenes as gastroprotective compounds, focusing on their effects on Caco-2 colonic epithelial cell viability and monolayer barrier function. Using a combination of MTT assays, TEER analysis and Western blot, we evaluated the concentration-dependent effects of 16 different terpenes and explored potential mechanisms underlying their cytoprotective properties.

The results from the MTT assay revealed a degree of concentration-dependent cytotoxicity for nerolidol, humulene, ocimene and (-)-β-caryophyllene, with significantly reduced cell viability at 50 to 100 μM further decreasing with higher concentrations. In contrast, myrcene, terpinolene, β-caryophyllene oxide, α-pinene, β-pinene and borneol exhibited a significant decrease only at 400 μM, suggesting lower cytotoxicity. Notably, α-bisabolol, linalool and citronellol significantly increased viability, suggesting potential as cytoprotective agents. Interestingly, zerumbone, α-pinene and β-caryophyllene oxide displayed a hormetic effect, where low concentrations increased viability but higher concentrations induced cytotoxicity, suggesting biphasic concentration-dependent activity.

Previous studies have shown that terpene-induced cytotoxicity can involve multiple pathways and be cell-type-specific. Typically, generation of reactive oxygen species (ROS), mitochondrial depolarisation, increased membrane permeability and/or depletion of adenosine triphosphate (ATP) can compromise homeostasis leading to increased cell death (De-Oliveira, Fidalgo-Neto and Paumgartten 1999; Gertsch *et al.,* 2008; Usta *et al.,* 2009; Cherneva *et al.,* 2012; Kumari *et al.,* 2019; Lu *et al.,* 2019; Agus, 2021).

The hormetic behaviour observed for zerumbone, α-pinene, and β-caryophyllene oxide suggests that mild oxidative stress may trigger cytoprotective pathways. Zerumbone's ability to induce proteotoxic stress and activate heat shock factor 1 (HSF1) could influence Caco-2 cells by altering stress adaptation mechanisms (Igarashi *et al.,* 2016).

Similarly, α-pinene and β-caryophyllene oxide could activate adaptive stress responses such as HSF1 or nuclear factor erythroid 2-related factor 2 (Nrf2) pathways, promoting cell survival and anti-inflammatory effects at low concentrations. However, excessive oxidative stress, mitochondrial dysfunction or apoptosis may occur at higher concentrations. Unlike (-)-β-caryophyllene which interacts with CB2-R receptors, β-caryophyllene oxide exerts its bioactivity through multiple pathways, including Nrf2 activation, mitochondrial apoptotic signalling and modulation of inflammatory mediator production (Chavan, Wakte and Shinde 2010; Park *et al.,* 2011; Kim *et al.,* 2014a; Kim *et al.,* 2014b; Sain *et al.,* 2014; Kim *et al.,* 2015; Fidyt *et al.,* 2016).

Notably, as discussed by Laws III and Smid (2024), zerumbone exhibits concentration-dependent cytotoxicity that is not strongly correlated with ROS generation. In PC12 neuronal cells, zerumbone demonstrated neurotoxicity at higher concentrations, while lower concentrations had minimal impact. This response suggests that zerumbone's effects may involve oxidative and/or reductive stress mechanisms. Unlike oxidative stress, which results from excess ROS, reductive stress arises from an overabundance of reducing

agents, such as glutathione (GSH) and nicotinamide adenine dinucleotide + hydrogen (Lloret *et al.,* 2016). While antioxidant responses are essential for oxidative phosphorylation, detoxification and biosynthesis, excessive reductive stress can impair redox balance, disrupting epithelial repair and immune signalling. Given the intestine's reliance on redox signalling for barrier function and immune modulation, dysregulated reductive stress may contribute to epithelial dysfunction in conditions such as IBD and colorectal cancer (Gostner *et al.,* 2015; Kornienko *et al.,* 2019).

Given that both oxidative and reductive stress contribute to GI disorders, the protective effects observed for α-bisabolol, linalool and citronellol may be therapeutically relevant. These monoterpene alcohols have demonstrated a broad range of pharmacological properties and mechanisms of action, including anti-cancer, neuroprotective, anti-inflammatory, antioxidant, analgesic, anticonvulsant, antidepressant, antimicrobial and hepatoprotective effects (Hada *et al.,* 2003; Trombetta *et al.,* 2005; Bastos *et al.,* 2009; Brito *et al.,* 2012; Nuutinen 2018; Santos *et al.,* 2019; Kim *et al.,* 2020; Tomko *et al.,* 2020; Jayaraj *et al.,* 2022).

For example, linalool's effects on the central nervous system are mediated through monoaminergic, glutamatergic, and neuropeptide systems, while its anti-tumour effects involve cell cycle arrest, induction of apoptosis and immune modulation (Usta *et al.,* 2009; Chang and Shen 2014; Cheng, Dai and Zheng 2017). Furthermore, its antioxidant properties contribute to its neuroprotective and anti-inflammatory activities (Huo *et al.,* 2013; Li *et al.,* 2015).

Additionally, previous studies indicate a cell-type-specific effect of linalool. For instance, linalool demonstrated inhibition of mitochondrial complexes I and II in HepG2 human liver cancer cells leading to a reduction in ATP, GSH and cell viability (Usta *et al.,* 2009). However, Sabogal-Guáqueta *et al.,* (2019) observed neuroprotective properties in HT-22

mouse hippocampal neuronal cells, providing protection from glutamate-induced oxidative stress by preserving mitochondrial function. They demonstrated that linalool reduced ROS, calcium and lipid accumulation while improving mitochondrial morphology, membrane potential and respiration, ultimately improving cell viability. Furthermore, Jana *et al.,* (2014) found that linalool was a pro-oxidant in tumour tissues but functioned as an antioxidant in liver tissue, further highlighting its dual functionality and cell-type-specific effect. In the context of GI health, the observed protective effects of linalool may extend to intestinal epithelial cells, where mitochondrial integrity is essential for maintaining barrier function and resisting oxidative damage.

Similarly, citronellol and α-bisabolol exhibit diverse pharmacological properties. Their anticancer effects are mediated through pro-apoptotic mechanisms that target mitochondrial pathways and apoptosis-related proteins. They have shown efficacy against pancreatic, breast, leukemia and glioma cancers by modulating key signalling pathways, including BH3-interacting domain death agonist, Fibroblast Growth Factor and AKT (Gomes-Carneiro *et al.,* 2005; Anter *et al.,* 2011; Chirumbolo and Bjørklund 2017; Nuutinen 2018; Jayaraj *et al.,* 2022). Moreover, they display anti-inflammatory properties by inhibiting nuclear factor-kappa B (NF-κB) and MAPK pathways, contributing to gastroprotection, nephroprotection and reduced lung inflammation (Kim *et al.,* 2011b; Rocha *et al.,* 2011b; Mauyra *et al.,* 2014; Sampaio *et al.,* 2016; D'Almeida *et al.,* 2017; Santos *et al.,* 2019). In the context of gastroprotection, these properties are particularly relevant given α-bisabolol has been found to decrease oxidative stress and inflammatory events associated with gastric lesions induced by ethanol and NSAIDs (Rocha *et al.,* 2010; Rocha *et al.,* 2011a). Given the role of inflammatory mediators in regulating tight junction integrity, further studies are needed to assess how terpenes influence these pathways in the context of intestinal barrier function and IBD.

In this regard, the nominal test concentration of each terpene chosen (50 μM) for TEER analysis was broadly representative of an innocuous concentration on Caco-2 cells across the broad range of terpenes used as indicated by the MTT viability assay, which is a commonly used and valid marker for Caco-2 cell viability when used in standardised, in vitro permeability assays (Kus, Ibragimow and Piotrowska-Kempisty 2023).

3.4.1 Preservation of functional barrier integrity under inflammatory conditions

TEER analysis revealed that linalool was the only terpene capable of preserving barrier integrity in cytokine-treated Caco-2 cells, highlighting its unique potential as a modulator of epithelial barrier function. This result suggests that linalool may counteract inflammation-induced damage or stabilise tight junctions under inflammatory conditions, either by directly influencing their dynamics or through anti-inflammatory effects that mitigate cytokine-induced barrier dysfunction.

It was also noted that linalool and limonene alone induced an intrinsic and significant increase in TEER at 48 h in the absence of cytokine treatment, retaining the possibility that such an improvement in barrier function may have occurred independently of any cytokine-mediated effects on permeability. However, unlike linalool, treatment with limonene did not result in any restitution in TEER in the presence of cytokines. For linalool, the improved TEER in both contexts highlights its potential as a versatile therapeutic compound. This activity could benefit gut health generally across a wide range of conditions by enhancing epithelial barrier integrity overall and simultaneously protecting against a range of stressors.

Linalool's protective effects on intestinal epithelial barrier integrity may involve both anti-inflammatory and antioxidant mechanisms to help counteract cytokine-induced barrier disruption. Despite inhibiting mitochondrial complexes I and II in HepG2 cells, linalool

may promote a metabolic shift, reduce oxidative stress and modulate redox-sensitive pathways, ultimately preserving tight junction integrity and cell survival in Caco-2 cells (Usta *et al.,* 2009; Sabogal -Guáqueta *et al.,* 2019).

Furthermore, Huo *et al.,* (2013) found that linalool decreased phosphorylation of Inhibitor of NF-κB alpha (IκBα), attenuating NF-κB activation and blocked phosphorylation of p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase and extracellular signal-regulated kinase, inhibiting the MAPK pathway in LPS-stimulated RAW 264.7 macrophages. Additionally, Lee *et al.,* (2018) demonstrated that linalool inhibits NF-κB activation in the spleen and mesenteric lymph nodes, which aligns with its ability to decrease phosphorylation of IκBα in RAW 264.7 macrophages. Since NF-κB and MAPK activation contribute to intestinal barrier dysfunction, its inhibition by linalool may explain the preserved TEER in Caco-2 cells under inflammatory conditions.

Moreover, linalool has been shown to activate the Nrf2 pathway in LPS-stimulated BV2 microglial cells, a key regulator of antioxidant defence (Li *et al.,* 2015). Given the interplay between inflammation and oxidative stress in epithelial barrier dysfunction, Nrf2 activation could further contribute to linalool's protective effects by enhancing cellular resilience against inflammatory damage. These findings highlight linalool's potential as a multi-target modulator of inflammation and oxidative stress across different cell types with a cell-type-specific effect (Nuutinen 2018; Kim *et al.,* 2020; Tomko *et al.,* 2020).

3.4.2 Tight junction protein expression

Western blot analysis revealed that cytokine treatment alone did not significantly alter occludin or claudin-1 expression compared to control, despite the observed reduction in TEER. This suggests that cytokine-induced barrier dysfunction may have occurred through mechanisms other than direct downregulation of these tight junction proteins, potentially

involving post-translational modifications, altered localisation, or increased turnover indirectly resulting in reduced detection due to increased turnover (Poritz *et al.,* 2011; Van Itallie and Anderson 2018; Feldman, Mullin, and Ryan 2005).

Interestingly, all terpene-treated groups exhibited a significant reduction in occludin levels compared to cytokine treatment alone, suggesting that these compounds did not mitigate cytokine-induced occludin loss and may have contributed to additional degradation. The mechanism underlying this effect remains unclear but may involve post-translational modifications such as phosphorylation, which regulates occludin stability and tight junction integrity. Occludin interacts with several scaffolding proteins, including ZO-1, ZO-2, and JAM, which influence its role in epithelial barrier function (Wong and Gumbiner 1997; Wong 1997; Van Itallie and Anderson 2018).

In contrast, (-)-β-caryophyllene and linalool significantly reduced claudin-1 expression relative to cytokine treatment alone, suggesting a potential exacerbation of tight junction disruption in inflammatory conditions. Since occludin has been implicated in stabilising claudin interactions within the tight junction complex, its reduction in terpene-treated groups may have indirectly contributed to claudin-1 destabilisation or internalisation, resulting in reduced detection (Feldman, Mullin, and Ryan, 2005). Interestingly, Rodríguez-Mejía *et al.,* (2022) reported that (-)-β-caryophyllene restored claudin-1 expression in an obesity-induced barrier dysfunction model but reduced claudin-1 levels in healthy controls, suggesting its expression may be disease-dependent.

Notably, Poritz *et al.,* (2011) explored the impact of TNF-α on intestinal barrier function in IEC-18 cells and IBD patient tissues, focusing on tight junction proteins claudin-1 and occludin. Consistent with our findings on TEER and tight junction protein regulation, they observed that TNF-α treatment reduced TEER and decreased occludin expression while increasing claudin-1. However, claudin-1 predominantly localised to the cytoplasm rather

than integrating into the tight junction, indicating dysregulation rather than a compensatory strengthening of barrier function. Given that claudin-1 is a key component of the multi-protein tight junction structure, its localisation to the cytoplasm likely indicates disrupted tight junction assembly and barrier dysfunction.

Moreover, in IBD tissues, they observed the claudin-1 to occludin (C:O) ratio was elevated, but with distinct patterns: in ulcerative colitis, the increase was limited to inflamed tissue, indicating barrier disruption as a consequence of inflammation, whereas in Crohn's disease, the C:O ratio was elevated regardless of disease state, suggesting an inherent tight junction abnormality. These findings highlight fundamental differences in tight junction regulation between ulcerative colitis and Crohn's disease, suggesting that barrier dysfunction in ulcerative colitis is secondary to inflammation, while in Crohn's disease, an intrinsic tight junction abnormality may contribute to disease development. These findings reinforce the complexity of epithelial barrier regulation under inflammatory conditions, highlighting that barrier integrity is not solely dictated by tight junction protein expression levels. The ability of linalool to preserve TEER suggests that it may exert protective effects through actin cytoskeletal stabilisation or post-translational modifications rather than direct regulation of occludin or claudin-1.

The actin cytoskeleton is a key regulator of barrier function, providing structural support and facilitating dynamic remodelling in response to inflammation or injury (Rodgers and Fanning 2011; Oshima *et al.,* 2016; Gao and Nakamura 2022). Since actin filaments interact directly with tight junction proteins, their dysregulation could contribute to barrier disruption. Pro-inflammatory cytokines are known to disrupt cytoskeletal structures such as lamellipodia and filopodia, which are crucial for epithelial repair and junction stability (Youakim and Ahdieh 1999; Small *et al.,* 2002; Pollard and Borisy 2003). Linalool may help preserve actin filament organisation, preventing excessive cytoskeletal remodelling that would otherwise weaken cell-cell junctions. Given its reported anti-inflammatory and

antioxidant properties, its ability to maintain TEER despite reductions in occludin and claudin-1 suggests a role in stabilising cytoskeletal components or modulating tight junction dynamics.

Given the gut barrier plays a critical role beyond local epithelial integrity, influencing systemic inflammation, immune homeostasis and metabolic health, disruptions in gut permeability have been linked to various diseases, including IBD, metabolic syndrome and neuroinflammation (Kaser, Zeissig and Blumberg 2010; Camilleri *et al.,* 2012; Melcarne, García-Iglesias and Calvet 2016). Increased gut permeability can lead to translocation of bacterial endotoxins, which may contribute to systemic inflammation observed in conditions such as metabolic syndrome and neurodegenerative diseases (Boquet *et al.,* 1998; Sears and Kaper 1998; Oshima *et al.,* 2016; Hess *et al.,* 2001; Alouf 2000). The ability of linalool to preserve TEER suggests that it may have broader implications for maintaining intestinal homeostasis, particularly in inflammatory conditions.

Since gut motility affects nutrient absorption and local inflammatory responses, understanding how terpenes influence both motility and epithelial integrity is crucial in determining their therapeutic potential (Olsson and Holmgren 2001). Some terpenes, particularly those that influence the endocannabinoid system (e.g., (-)-β-caryophyllene and α-humulene) may impact gut motility and secretion patterns, which could have different implications for acute inflammation compared to chronic inflammation (Lian, Casari and Falasca 2022; Kwon *et al.,* 2018).

Furthermore, the antimicrobial properties of terpenes are known to influence the gut microbiome, which in turn affects intestinal permeability and immune responses (Sears and Kaper 1998; Boquet *et al.,* 1998; Alouf 2000; Hess *et al.,* 2001; Zhao *et al., 2022*). Some terpenes such as D-limonene have been reported to modulate gut microbial composition by inhibiting growth of potential pathogens while promoting beneficial bacteria associated

with anti-inflammatory effects (Minnaard *et al.,* 2004; Ouwehand *et al., 2010;* Ambrosio *et al.,* 2019; Zhao *et al., 2022*). Linalool may also support a favourable microbial balance by exerting antimicrobial properties against pathogenic bacteria while preserving commensal species. Future *in vivo* studies should explore whether terpene-induced changes in gut barrier integrity are mediated by alterations of the microbiome.

3.5 CONCLUSION

This study demonstrates that terpenes exhibit dual effects on intestinal epithelial cells, with both cytotoxic and potential cytoprotective properties. Linalool uniquely preserved TEER in cytokine-treated cells, suggesting a barrier-protective effect that was not associated with occludin or claudin-1 upregulation. In contrast, (-)-β-caryophyllene reduced claudin-1 expression compared to cytokine treatment alone, potentially exacerbating barrier disruption under inflammatory conditions. These findings underscore the complex nature of GI regulation and tight junction proteins, highlighting the need for further mechanistic studies to determine select terpene-mediated effects on barrier integrity, but propose a role for acyclic monoterpenoid linalool in preserving intestinal barrier function.

CHAPTER 4

CANNABIDIOL AND CANNABIDIOL HYDROXYQUINONE EXHIBIT DIFFERENTIAL CYTOTOXICITY IN BLADDER UROTHELIAL CANCER CELLS

Statement of Authorship

Title of Paper	CANNABIDIOL AND CANNABIDIOL HYDROXYQUINONE EXHIBIT DIFFERENTIAL CYTOTOXICITY IN BLADDER UROTHELIAL CANCER CELLS
Publication Status	☐ Published ☐ Accepted for Publication ■ Unpublished and Unsubmitted work written in manuscript style ☐ Submitted for Publication
Publication Details	NA

Principal Author

Name of Principal Author (Candidate)	Luke Wooley		
Contribution to the Paper	Performed the experiments, performed the analysis and wrote the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	09/07/2025

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr Scott Smid		
Contribution to the Paper	Conceived the project and provided critical review of the manuscript		
Signature		Date	9/07/2025

PREFACE

This manuscript was written to the specifications of the University of Adelaide. Essential formatting rules were followed in order to satisfy the thesis guidelines for the University of Adelaide's School of Biomedicine Doctor of Philosophy in Pharmacology program:

- 12pt Times New Roman font
- Double spacing
- Block justified
- Left margin 3.5cm; top, bottom and right margins 2cm
- Figures and tables are embedded in the appropriate places within the text, rather than at the end of the document or in a separate document
- Highlight section is not included.
- References are not a numbered style.

The word count for the manuscript (excluding references, acknowledgements and supplementary material) is 3741.

TITLE

Cannabidiol and cannabidiol hydroxyquinone exhibit differential cytotoxicity in bladder cancer cells

AUTHOURS

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4.0 ABSTRACT

Bladder cancer is a significant health challenge, particularly in high-grade muscle-invasive disease, which is associated with poor prognosis and resistance to current therapies such as radical cystectomy, radiation therapy or neoadjuvant chemotherapy (e.g. cisplatin-based regimens). Cannabidiol (CBD) and its oxidative metabolite cannabidiol hydroxyquinone (CBDHQ) have demonstrated anticancer potential in various tumour models, yet their effects in bladder cancer remain less well known.

This study assessed the cytotoxic effects of CBD and CBDHQ in RT4 and T24 human urothelial carcinoma cells, representing low-grade and high-grade bladder cancer, respectively. Cell viability was measured using the MTT assay and intracellular reactive oxygen species (ROS) levels were evaluated using the DCFDA assay.

Both compounds reduced cell viability in a concentration-dependent manner, with greater sensitivity observed in T24 cells. Neither CBD nor CBDHQ significantly increased ROS levels under these conditions, suggesting a non-redox mechanism of action. Co-treatment with the thiol antioxidant N-acetylcysteine (NAC) reduced ROS in RT4 cells but had minimal effect in T24 cells treated with CBDHQ, indicating potential redox-sensitive interactions in high-grade cancer cells. This heightened sensitivity of T24 cells may be due to their reliance on DNA replication and increased susceptibility to thiol-reactive compounds.

These findings suggest that CBDHQ selectively impairs viability in aggressive bladder cancer cells without inducing oxidative stress. This highlights its promise as a therapeutic candidate with potential advantages over conventional cytotoxic agents. Further investigation into pharmacokinetics, *in vivo* efficacy, and safety is required.

Keywords: cannabidiol, CBDHQ, bladder cancer, urothelial carcinoma, cytotoxicity, redox signalling, topoisomerase II, reactive oxygen species, cannabinoid quinones.

4.1 INTRODUCTION

Bladder cancer is among the ten most diagnosed cancers worldwide, with urothelial carcinoma accounting for over 90% of all cases (Lenis, Lec and Chamine 2020). It presents in two major forms: low-grade, non-invasive tumours and high-grade, muscle-invasive disease. While low-grade tumours often recur, high-grade tumours are associated with poor prognosis and significant mortality (Lenis, Lec and Chamine 2020). Despite advances in surgical and chemotherapeutic approaches, recurrence and resistance remain persistent challenges, highlighting the need for novel therapeutic strategies (Lenis, Lec and Chamine 2020).

Cannabidiol (CBD), a non-psychoactive phytocannabinoid derived from *Cannabis sativa* L., has attracted considerable interest for its anticancer potential (Trac, Keck, and Deweese 2021; Tomko *et al.,* 2022). Preclinical studies across multiple cancer types have demonstrated its ability to impair cell viability, induce apoptosis, disrupt mitochondrial function, and elevate reactive oxygen species (ROS) (Ruiz, Miguel and Díaz-Laviada 1999; Carracedo *et al.,* 2006; Greenhough *et al.,* 2007; Aviello *et al.,* 2012; Hernán Pérez de la Ossa *et al.,* 2013; Massi *et al.,* 2013; De Petrocellis *et al.,* 2013; Kosgodage *et al.,* 2018; Honarmand *et al.,* 2019; Simmerman *et al.,* 2019).

Cannabidiol hydroxyquinone (CBDHQ or HU-331), an oxidative metabolite of CBD, has emerged as a promising anticancer agent due to its ability to induce cytotoxicity in various cancer cell lines. Notably, it has been shown to induce apoptosis via topoisomerase II (TOP2) inhibition, resulting in cell cycle arrest without significant ROS production (Kogan *et al.,* 2007; Regal, Mercer and Deweese 2014; Wilson *et al.,* 2018). Importantly, CBDHQ has shown selective toxicity towards cancer cells with minimal effects on healthy cells, a feature highlighted in recent reviews discussing its potential as a therapeutic cannabinoid-quinone (Massi *et al.,* 2013; Jiang, Tashiro and Ikejima 2020; Trac, Keck and Deweese

2021). Although CBDHQ does not significantly increase ROS, its redox activity may involve antioxidant effects or interfere with cellular redox-sensitive pathways, contributing to its cytotoxicity. CBDHQ behaves as a quinone/hydroquinone compound, which can donate electrons or hydrogen, potentially neutralising ROS and influencing cellular redox buffering systems (Bęben *et al.,* 2024). In colon cancer cell models, CBDHQ reduced intracellular ROS release by 20–34%, whereas CBD reduced it by only 17–26% indicating stronger antioxidant activity tied to its hydroquinone structure (Bęben *et al.,* 2024). These distinct mechanisms of action, including TOP2 inhibition and modulation of redox homeostasis, suggest that CBDHQ may provide a multifaceted approach to targeting cancer cells. Research into the effects of CBDHQ in cancer models remains limited, and to date no studies have evaluated its impact on bladder carcinoma cells.

In this study, the cytotoxic effects of CBD and CBDHQ were evaluated in RT4 and T24 human bladder carcinoma cells, which represent models of low-grade and high-grade urothelial cancer respectively. Mitochondrial metabolic activity and intracellular ROS accumulation were assessed using thiazolyl blue tetrazolium bromide (MTT) and 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA) assays to characterise cannabinoid-induced cytotoxicity and redox alterations in a concentration-dependent manner.

4.2 MATERIALS AND METHODS

75 cm^2 tissue culture flasks (Corning Life Sciences, Lowell, MA, USA), flat bottom polystyrene 96-well microtiter plates (Corning, Cat. No. 3370), trypan blue, dimethyl sulfoxide (DMSO), MTT, Dulbecco's Modified Eagle Medium (DMEM), phenol red-free media, non-essential amino acids (NEAA), penicillin/streptomycin, 0.25 % w/v trypsin with phenol red and ethylenediaminetetraacetic acid (EDTA), foetal bovine serum (FBS), phosphate buffered saline (PBS), tert-butyl hydroperoxide (t-BHP), edaravone and N-

acetylcysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MI, USA). Cellular ROS (DCFDA) assay kit was purchased from Abcam, Australia (Abcam, Cat. No. ab113851). Cannabidiol (CBD) was purchased from Australian Government National Measurement Institute (Lindfield, NSW, Australia) and cannabidiol hydroxyquinone (CBDHQ) was purchased from Mineral Innovative Technologies (Boonah, QLD, Australia).

Standard *in vitro* assessment protocols, including the Thiazolyl blue tetrazolium bromide (MTT) assay and 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA) assay, were used to evaluate the effects of CBD and CBDHQ on RT4 and T24 human bladder carcinoma cell lines. The MTT assay was used to assess mitochondrial activity as a measure of cell viability and cytotoxicity, while DCFDA was used to measure intracellular ROS levels. The treatments included in this study CBD, CBDHQ and DMSO vehicle. Compounds were dissolved in DMSO to a concentration of 10 mM and stored at -20 °C until required. All materials were autoclaved for sterility and all analysis was conducted under sterile conditions.

4.2.1 Cell culture

Human bladder carcinoma cell lines (RT4 and T24) were kindly donated by Dr Luke Grundy, Flinders University, Adelaide, South Australia. Cells were cultured in complete DMEM, containing 10 % v/v FBS, 1 % v/v NEAA and 1 % v/v penicillin/streptomycin in 75 cm^2 tissue culture flasks and incubated at 37 °C with 5 % CO_2 as per previously established protocols (Karkoulis *et al.,* 2010).

4.2.2 Thiazolyl blue tetrazolium bromide (MTT) assay

Cell viability after treatment was measured by MTT assay as per previously established protocol (Marsh and Smid 2022). Briefly, cells were seeded in 96-well plates at a density of 2×10^4 cells per well in complete DMEM, then incubated at 37 °C with 5 % CO_2 for 24 h prior to treatment. Cells were treated with either CBD, CBDHQ at (0, 0.1, 1, 10, 50 and 100 μM) or DMSO vehicle control at (0, 0.001, 0.01, 0.1, 0.5 and 1 %) then incubated for 48 h at 37 °C with 5 % CO_2, after which time cell viability was measured using the MTT assay. Culture media was aspirated and replaced with serum-free media containing 0.25 mg/mL MTT then incubated for 2 h at 37 °C, 5 % CO_2. MTT solution was then aspirated, and cells lysed with DMSO. Absorbance was measured at 570 nm using a Synergy MX microplate reader (Bio-Tek, Bedfordshire, UK). Cell viability was calculated using Equation 1:

$$Cell\ Viability\ (\%) = \left(\frac{Absorbance\ of\ control}{Absorbance\ of\ treated\ sample}\right) \times 100 \quad (1)$$

4.2.3 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA) assay

Intracellular ROS levels were measured by DCFDA assay as per previously established protocols and manufacturer's instructions (Laws III and Smid 2024). Briefly, cells were stained with DCFDA (20 μM) for 30 min in the dark at 37 °C with 5% CO_2. Cells were then washed with PBS and seeded in black walled, clear bottom 96-well plates at a density of 1×10^5 cells per well in phenol red-free media. To evaluate the generation of any reactive quinones or oxidative species from either CBD or CBDHQ incubation, plates were pretreated with either of two antioxidants, n-acetyl cysteine (NAC) (1 mM) or edaravone (1 μM), for 15 min prior to treatment with either the lipid peroxidant t-BHP (200 μM), CBD, CBDHQ (100 μM) or vehicle solvent control DMSO (1 %). Following treatment,

plates were incubated for 4 h at 37 °C with 5% CO_2 in the dark. Fluorescence intensity was then measured with excitation/emission wavelengths at 485 nm / 535 nm in a BioTek Synergy Mx microplate reader (BioTek, Vermont, USA).

Statistical analysis

All experiments were performed in 4 independent experiments (n=4). The data were expressed as mean ± SEM. GraphPad Prism 10 was used for statistical analysis and graph creation (GraphPad Software, San Diego, USA). Data from MTT was analysed by two-way analysis of variance (ANOVA) with Dunnett's multiple comparisons to determine significance versus control and between different treatment conditions within each group for cell viability ($p < 0.05$). Data from DCFDA was analysed by one-way ANOVA with Tukey's multiple comparisons to determine significance versus the control and between different treatment conditions ($p < 0.05$).

4.3 RESULTS

4.3.1 Effects of CBD and CBDHQ on RT4 and T24 cell viability via MTT assay

The MTT assay, which measures mitochondrial activity as an indicator of cell metabolic function, was used to evaluate the effects of CBD and CBDHQ on the viability of RT4 and T24 cells across a concentration range (0–100 μM), illustrated in Figure 4-1(a–b**)**.

Results revealed that both CBD and CBDHQ demonstrated significant reductions in viability at 10 μM (CBD $p < 0.05$ and CBDHQ $p < 0.001$) for RT4 cells and at 1 μM (CBD $p < 0.01$ and CBDHQ $p < 0.0001$) for T24 cells compared to the vehicle control. A further reduction in viability was observed for both compounds at concentrations $\geq$ 50 μM ($p < 0.0001$) and at $\geq$ 10 μM ($p < 0.0001$) for RT4 and T24 cells respectively. Notably, T24 cells exhibited a greater sensitivity to CBD and CBDHQ, with significant reductions in viability

observed at concentrations as low as 1 µM, indicating a lower threshold for cytotoxicity. This decrease in viability demonstrated that CBD and CBDHQ exhibited concentration-dependent cytotoxicity, with a more pronounced effect at higher concentrations. Notably, at 1 µM for T24 cells and 10 µM for RT4 cells, CBDHQ indicated a significantly greater reduction in viability ($p < 0.001$) compared to CBD.

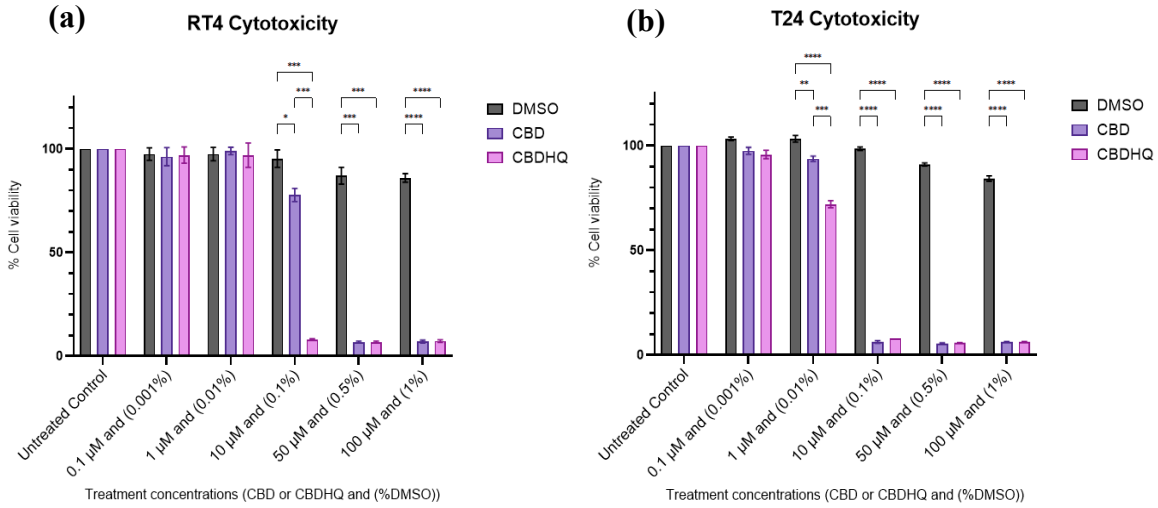


Figure 4-1. MTT assay of RT4 (**a**) and T24 (**b**) cell viability following 48 h incubation with varying concentrations of CBD or CBDHQ (0 – 100 µM). Vehicle (DMSO) concentrations ranged from 0 – 1%. Each data point represents the mean viability percentage ± standard error of the mean (SEM) from four independent experiments (n=4). Data was analysed by two-way analysis of variance (ANOVA) with Dunnett's multiple comparisons to determine significance versus control and between different treatment conditions within each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to vehicle control within each group.

4.3.2 Effects of CBD and CBDHQ on RT4 and T24 intracellular reactive oxygen species and antioxidant activity

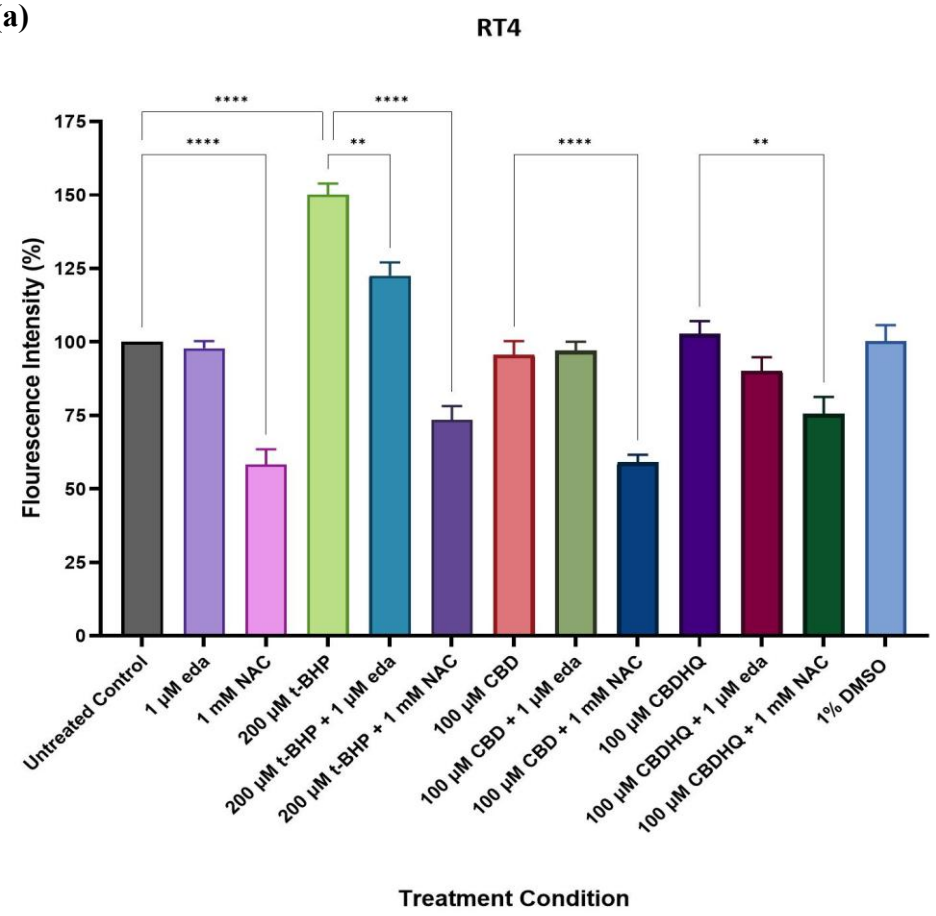
Reactive oxygen species (ROS) levels were measured by the DCFDA assay, which detects intracellular ROS through fluorescence intensity. Untreated cells represent baseline ROS levels, while the lipid peroxidant t-BHP-treated cells served as the positive control, inducing elevated ROS. Edaravone and N-acetylcysteine (NAC) were included as

reference antioxidants. CBD and CBDHQ were tested for their potential to modulate ROS levels, either alone or in combination with antioxidants. Data represent the fluorescence intensity relative to untreated control, indicating the level of ROS production across treatment groups, shown in Figure 4-2(a-b). For both RT4 and T24 cells, treatment with t-BHP (200 μM) generated a significant increase in DCFDA fluorescence, indicating elevated ROS compared to the untreated control ($p < 0.0001$), confirming t-BHP as a pro-oxidant producing corresponding elevated ROS in the assay.

Treatment with edaravone alone (1 μM) did not significantly alter ROS compared to the control. Co-treatment with edaravone (1 μM) and t-BHP (200 μM) significantly reduced ROS in both cell lines, RT4 ($p < 0.01$) and T24 ($p < 0.0001$) when compared to t-BHP alone, confirming the antioxidant capacity of edaravone. Treatment with NAC alone (1 mM) significantly reduced ROS in both RT4 and T24 cells compared to the control ($p < 0.0001$). Co-treatment with NAC (1 mM) and t-BHP (200 μM) also significantly reduced ROS in both cell lines ($p < 0.0001$) when compared to t-BHP alone, confirming the antioxidant capacity of NAC.

Neither CBD nor CBDHQ (100 μM) alone however significantly affected ROS compared to the control. Similarly, co-treatment with edaravone (1 μM) and either CBD or CBDHQ (100 μM) did not significantly reduce ROS compared to CBD or CBDHQ alone. While co-treatment with CBD (100 μM) and NAC (1 mM) significantly reduced ROS compared to CBD alone ($p < 0.0001$), the extent of the reduction was no greater to that of NAC alone. Co-treatment with CBDHQ (100 μM) and NAC (1 mM) resulted in a lessened and non-significant reduction in ROS.

(a)


RT4
Flourescence Intensity (%)
175
150
125
100
75
50
25
0
****
****
****
**
****
**
Untreated Control
1 µM eda
1 mM NAC
200 µM t-BHP
200 µM t-BHP + 1 µM eda
200 µM t-BHP + 1 mM NAC
100 µM CBD
100 µM CBD + 1 µM eda
100 µM CBD + 1 mM NAC
100 µM CBDHQ
100 µM CBDHQ + 1 µM eda
100 µM CBDHQ + 1 mM NAC
1% DMSO
Treatment Condition

(b)

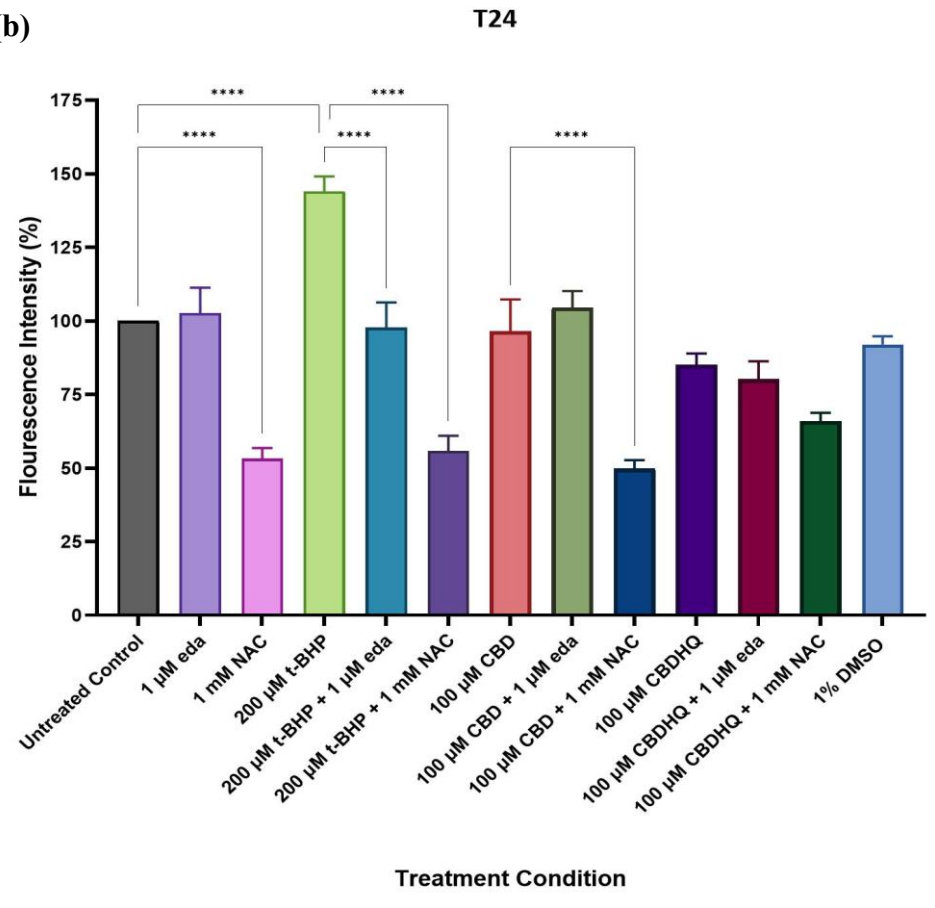

T24
Flourescence Intensity (%)
175
150
125
100
75
50
25
0
****
****
****
****
****
Untreated Control
1 µM eda
1 mM NAC
200 µM t-BHP
200 µM t-BHP + 1 µM eda
200 µM t-BHP + 1 mM NAC
100 µM CBD
100 µM CBD + 1 µM eda
100 µM CBD + 1 mM NAC
100 µM CBDHQ
100 µM CBDHQ + 1 µM eda
100 µM CBDHQ + 1 mM NAC
1% DMSO
Treatment Condition

Figure 4-2. Effects of edaravone (eda), N-acetylcysteine (NAC), t-BHP, CBD and CBDHQ on intracellular ROS formation in RT4 (**a**) and T24 cells (**b**) (as % ROS formation) via DCFDA assay after 4 h incubation. Cells were pre-treated with either edaravone or NAC, followed by exposure to t-BHP, CBD or CBDHQ then incubated for 4 hours. Each data point represents the mean ROS percentage ± SEM from four independent experiments (n=4). Data was analysed by one-way ANOVA with Tukey's multiple comparisons to determine significance versus the control for baseline measurements and against t-BHP for oxidative-stress conditions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.4 DISCUSSION

The RT4 and T24 cell lines represent distinct stages of urothelial carcinoma progression. RT4 cells are derived from a well-differentiated, non-invasive papillary tumour, characteristic of low-grade bladder cancer. In contrast, T24 cells originate from a poorly differentiated, high-grade carcinoma, indicative of muscle-invasive bladder cancer (Liu *et al.,* 2013). In our study, both CBD and CBDHQ exhibited concentration-dependent cytotoxic effects on both cell lines. Notably, T24 cells demonstrated greater sensitivity, with significant reductions in viability observed at lower concentrations (1 μM) compared to RT4 cells (10 μM). This heightened sensitivity in T24 cells may be attributed to their aggressive phenotype with higher proliferative capacity, poor differentiation, enhanced invasiveness and genomic instability (Liu *et al.,* 2013). More aggressive cancer cells like T24 cells exhibit an altered response to therapy with resistance to certain treatments, such as cisplatin (Shi *et al.,* 2022). However, given their greater reliance on expeditious and efficient DNA replication, they may be more susceptible to agents that disrupt DNA replication or cellular homeostasis.

Previous research supports the theory that cannabinoids can selectively target aggressive cancer cells. For instance, Chen *et al.,* (2021) reported that CBD induced apoptosis in bladder cancer cells, with enhanced effects observed in more malignant cell lines. Similarly, other studies have demonstrated that cannabinoids can reduce viability and invasion in a variety of high-grade cancer models (Ruiz, Miguel and Díaz-Laviada 1999;

Carracedo *et al.,* 2006; Greenhough *et al.,* 2007; Aviello *et al.,* 2012; Hernán Pérez de la Ossa *et al.,* 2013; De Petrocellis *et al.,* 2013; Kosgodage *et al.,* 2018; Honarmand *et al.,* 2019; Simmerman *et al.,* 2019; Trac, Keck, and Deweese 2021; Tomko *et al.,* 2022). These findings suggest that the differential sensitivity of RT4 and T24 cells to CBD and CBDHQ is likely reflective of their respective degree of malignancy.

CBD is metabolised by hepatic microsomes to form the quinone CBDHQ, which has been identified as a reactive metabolite due to its ability to undergo Michael addition reactions with thiol groups in proteins, glutathione and cytochrome P450 3A11 (Watanabe *et al.,* 1991; Bornheim and Grillo, 1998; Jiang *et al.,* 2013; Li *et al.,* 2016). This can impair antioxidant defences and alter redox-sensitive signalling pathways, without generating large amounts of ROS (Li *et al.,* 2016). Thiol groups play a crucial role in maintaining the structural and functional integrity of many enzymes, including TOP2. Depletion of cellular thiols can therefore impair TOP2 activity. Wu and Jan (2010) demonstrated that CBDHQ induces apoptosis in splenocytes primarily through thiol depletion, an effect associated with TOP2 inhibition. As TOP2 is essential for DNA replication, transcription and chromosome segregation, its inhibition disrupts these vital processes and can ultimately lead to cell death (Nitiss 2009a; Nitiss 2009b).

Given that T24 cells are highly proliferative, with a doubling time of approximately 24 hours, they are dependent on efficient DNA replication (Kim *et al.,* 2011a). Therefore, inhibition of TOP2 as is purported for CBDHQ may result in a more pronounced impact on their viability. In contrast, RT4 cells have a slower doubling time of approximately 48 hours and are less dependent on rapid TOP2 activity for proliferation (Kim *et al.,* 2011a). This difference in proliferative capacity may be a contributor to the differential sensitivity observed in the MTT assay between the two cell lines.

Studies have demonstrated that CBDHQ acts as a catalytic inhibitor of TOP2, stabilising the enzyme in a closed conformation without inducing DNA strand breaks, a mechanism distinct from other TOP2 poisons such as doxorubicin, which generate genotoxic stress and cardiotoxicity (Kogan *et al.,* 2007; Regal, Mercer and Deweese 2014; Wilson *et al.,* 2018; Colvin *et al.,* 2022). This unique mode of action suggests that CBDHQ may offer more selective anticancer efficacy with a reduced risk of adverse effects. While an original aim of this study was to characterise TOP2 inhibitory activity of CBD and CBDHQ, protracted delays in the provision of a commercial TOP2 assay kit by the manufacturer meant we were unable to pursue this mechanistic line of investigation.

Treatment with CBD or CBDHQ alone did not significantly alter ROS compared to the untreated control aligning with previous studies (Kogan *et al.,* 2007; Trac, Keck, and Deweese 2021; Colvin *et al.,* 2022). While co-treatment with NAC significantly reduced ROS levels compared to CBD or CBDHQ alone in RT4 cells, the level of reduction was equivalent to that of NAC alone, inferring merely that NAC continued to reduce basal ROS associated with cell metabolic activity irrespective of cannabinoid co-treatment. However, in T24 cells, this effect was only observed with CBD and NAC co-treatment; CBDHQ co-treatment did not significantly alter ROS, suggesting potential cell-line specific redox interactions or an altered antioxidant response to CBDHQ in high-grade cells. Previous findings that thiol antioxidants like NAC, but not non-thiol antioxidants such as edaravone, abrogate CBDHQ-induced apoptosis and thiol depletion, support the involvement of thiol-sensitive redox mechanisms rather than overt oxidative stress in the cellular response to CBDHQ (Wu and Jan 2010). This is consistent with findings by Wu *et al.,* (2010), who reported that CBD-induced apoptosis in freshly isolated human monocytes was strongly dependent on intracellular glutathione levels and redox status. In contrast, precultured monocytes, which exhibited elevated antioxidant capacity, showed resistance to CBD-induced cytotoxicity. Our findings indicate that neither CBD nor CBDHQ significantly

induce oxidative stress under these experimental conditions, making it likely that the observed reductions in cell viability are mediated through alternative mechanisms independent of ROS generation, which may be concordant with the purported catalytic inhibition of TOP2 by CBDHQ (Kogan *et al.,* 2007; Regal, Mercer and Dewsee 2014; Wilson *et al.,* 2018). These results highlight the importance of intracellular redox environment in modulating cannabinoid bioactivity and further support that CBDHQ may act via redox-independent cytotoxic pathways in certain cell types.

In the context of clinical applications, CBD has gained interest for its potential role as an adjunctive therapy in cancer treatment (Velasco, Sánchez and Guzmán 2015; Colvin *et al* 2022; Tomko *et al.,* 2022; Braun *et al.,* 2024). Studies suggest that CBD may enhance the efficacy of conventional chemotherapeutic agents and radiation therapy, which is particularly valuable in cancers like bladder carcinoma, where resistance to chemotherapy is common (Choi *et al.,* 2014; Rosewall, Feuz and Bayley 2020). Additionally, the anti-inflammatory properties of CBD may offer complementary benefits in mitigating the side effects of cancer treatments, such as nausea, pain, and loss of appetite (Massi *et al.,* 2012; Jiang, Tashiro and Ikejima 2020; Braun *et al.,* 2024).

However, as observed by Ewing *et al.,* (2019), high doses of CBD-rich cannabis extract led to liver injury in mice. This was indicated by elevated liver enzymes, histological changes, and altered gene expression associated with oxidative stress and metabolism. These findings indicate that high doses of CBD may induce dose-dependent hepatotoxicity, underscoring the importance of dose regulation and monitoring of liver function in clinical settings (Florian *et al*., 2025). As discussed by Millar *et al.,* (2018), CBD is metabolised primarily by hepatic enzymes (CYP3A4 and CYP2C19), with peak plasma concentrations typically occurring 1–6 hours post-dose. Factors such as food intake, formulation and dosage were shown to impact pharmacokinetic outcomes with significant variability in absorption and bioavailability, especially following oral administration. The authors

concluded that while CBD is generally well-tolerated, more standardised clinical trials are needed to better define its pharmacokinetic profile.

Despite promising preclinical data, clinical evidence on the efficacy and safety of CBDHQ remains absent, requiring additional studies to assess therapeutic relevance. The unique mechanism of action, distinct from traditional TOP2 inhibitors, highlights the need for further investigation into its therapeutic potential and safety. CBDHQ's selective toxicity toward cancer cells represents a possible therapeutic advantage over conventional chemotherapy agents, which typically exhibit high systemic toxicity. This specificity is particularly beneficial for patients suffering from cancers such as bladder carcinoma, where aggressive treatments may exacerbate symptoms and reduce quality of life.

Chemical modification of CBDHQ has demonstrated enhanced activity in both *in vitro* and *in vivo* cancer models indicating promise for the design of a new class of antineoplastic derivatives (Petronzi *et al.,* 2013; Morales *et al.,* 2013). Notably, the synthesised quinone V assessed by Petronzi *et al.,* (2013) displayed the ability to inhibit the expression of antiapoptotic protein X-linked Inhibitor of Apoptosis Protein (XIAP) in M14 cancer cells, a regulatory protein that suppresses apoptosis by binding to caspase proteins (Dandawate *et al.,* 2010; Riedl *et al.,* 2001).

4.5 CONCLUSION

In conclusion, these mechanistic insights position CBD and CBDHQ as promising potential candidates in bladder cancer therapy. Their preferential cytotoxicity toward high-grade urothelial carcinoma cells, coupled with a non-genotoxic mechanism of action may reduce adverse effects while maintaining therapeutic efficacy. The differential sensitivity between RT4 and T24 cells highlights their potential to selectively target aggressive, treatment-resistant bladder cancer.

While further studies are necessary to evaluate pharmacokinetics, *in vivo* efficacy, and safety profiles, our findings support continued investigation of CBD and CBDHQ as standalone or adjunctive agents in bladder cancer treatment. Their ability to modulate redox-sensitive targets, impair TOP2 activity and selectively impact rapidly proliferating cancer cells offers a novel therapeutic option to enhance existing chemotherapeutic treatment, or serve as an alternative for patients with treatment-resistant disease.

CHAPTER 5

GENERAL DISCUSSION

The current and increasing global burden of antimicrobial resistance, inflammatory gastrointestinal disorders, and urothelial disease underscores an urgent need for safer, more effective pharmacotherapeutic strategies. These conditions, often chronic, multifactorial and difficult to treat present limitations in current treatments that include toxicity, development of resistance and limited tissue selectivity. The therapeutic versatility of plant-derived compounds presents an opportunity to develop multi-functional agents that target disease through multiple pathways with potentially improved safety profiles.

This study aimed to provide a broad characterisation of bioactivity and therapeutic potential of cannabis-derived phytochemicals, including cannabidiol (CBD), its synthetic derivatives and oxidative metabolite cannabidiol hydroxyquinone (CBDHQ), in addition to a panel of cannabis related terpenes across three distinct yet interconnected biological systems; bacterial pathogens, the gastrointestinal tract and the urothelium.

The first study identified five CBD derivatives with potent bactericidal activity against Gram-positive bacteria and methicillin-resistant *Staphylococcus aureus*, *Enterococcus faecium*, and *Staphylococcus epidermidis*. These derivatives exhibited low micromolar minimum inhibitory concentrations and acted rapidly within six hours of exposure. Checkerboard assays revealed synergy between select derivatives and daptomycin against *E. faecium*, demonstrating a potential to improve antibiotic activity in combination. While no antibacterial activity was observed against Gram-negative bacteria or bacteria cultured to biofilm, the selective action against Gram-positive strains highlights the promise of structural modification of CBD to maintain a degree of antimicrobial activity. Importantly, cytotoxicity profiling using Caco-2 epithelial cells indicated a concentration window with acceptable safety margins, supporting further preclinical development.

The second study demonstrated that most terpenes were well tolerated at concentrations ≤ 200 μM, with linalool, α-bisabolol, and citronellol significantly enhancing cell viability.

TEER analysis identified linalool as the only terpene capable of preserving barrier integrity in the pro-inflammatory conditions tested, suggesting it provides protective effects on intestinal epithelial monolayers. Western blot analysis revealed no upregulation of claudin-1 or occludin despite preserved TEER, indicating linalool may act through alternative pathways such as cytoskeletal stabilisation or modulation of redox and/or inflammatory signalling. These results position linalool as a candidate for adjunctive gastrointestinal therapies aimed at restoring or preserving gut barrier function during inflammation.

The third study assessed the impact of CBD and its metabolite CBDHQ on low-grade (RT4) and high-grade (T24) bladder cancer cell lines. Both compounds reduced viability in a concentration-dependent manner, with greater sensitivity observed in the aggressive T24 cells. Neither CBD nor CBDHQ induced significant ROS generation at cytotoxic concentrations. The selective cytotoxicity toward high-grade cells, combined with its non-ROS-mediated action, highlights CBDHQ as a candidate for novel anticancer therapies with selective toxicity.

Together, these findings demonstrate the broad therapeutic potential of cannabis-derived phytochemicals across distinct disease contexts and how shared mechanisms of actions across organ systems could be leveraged for addressing key challenges like antibiotic-resistant microbial infection, mucosal damage, and tumour cell viability. This comprehensive approach not only advances understanding of individual phytochemical actions but also highlights their potential for adjunctive or multi-targeted therapies.

The studies are limited by a reliance on *in vitro* models, which, while highly controlled, do not replicate the complexity of multicellular tissues, immune interactions, or in vivo pharmacokinetics. The TEER and Western blot assays do not capture spatial localisation or dynamic reorganisation of tight junctions *in vivo*. Similarly, the antimicrobial assays were limited to a panel of laboratory and clinical strains, and further testing against Gram-

negative pathogens remains necessary. The anticancer findings for CBDHQ require confirmation in animal models to validate pharmacodynamic and toxicity profiles. We were also unable to test the non-malignant UROtsa urothelial cell line as originally planned, because these cells were not viable from cryogenic thaw. This would have provided a characterisation of any cytotoxicity from CBD and CBDHQ on cells with a more normal urothelial phenotype, while a prolonged delay in the acquisition of a TOP2 isomerase assay kit meant we were unable to proceed with experiments that may have confirmed a target site of action of CBD and CBDHQ.

Future research should focus on expanding the structure–activity relationship (SAR) profiling of CBD derivatives to optimise their antimicrobial potential, particularly against resistant Gram-positive pathogens. *In vivo* efficacy should be validated using clinically relevant infection models, alongside the development of formulation strategies to enhance penetration into Gram-negative bacteria. For gastrointestinal applications, TEER findings warrant validation in animal models of IBD or NSAID-induced intestinal injury, with additional studies needed to assess impacts on microbiome composition and gut motility. Oral delivery strategies, such as enteric capsules or dietary formulations, may further support therapeutic translation. In oncology, *in vivo* evaluation of CBDHQ in bladder cancer models is essential to determine therapeutic relevance. Detailed pharmacokinetic profiling and metabolite stability studies are also required, with future development of synthetic analogues aimed at improving tumour selectivity and enabling targeted cannabinoid delivery.

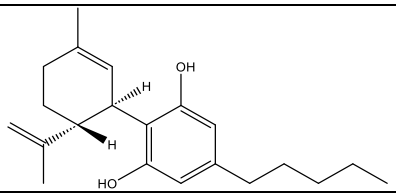
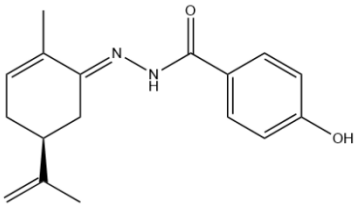
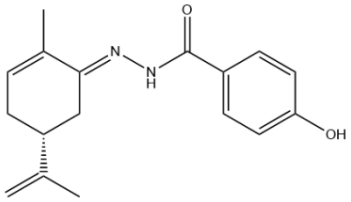
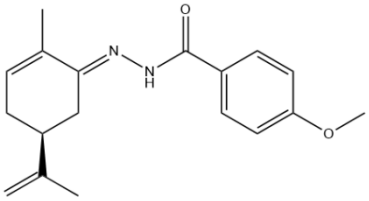
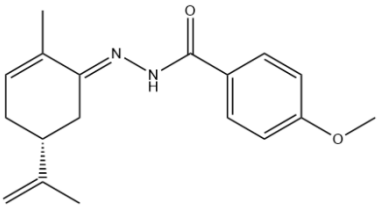
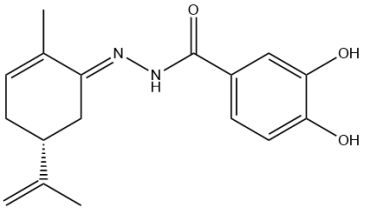
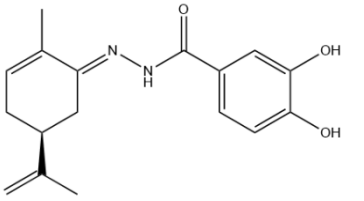
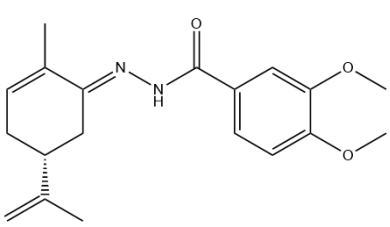
Furthermore, bioprospecting cannabis as a source of novel phytochemicals underpins the research presented in this thesis, highlighting the immense potential of this understudied plant. Cannabis and the *Cannabaceae* family produce hundreds of unique compounds, many with distinct chemical structures and biological activities. As medical cannabis use expands globally there is an increasing requirement to thoroughly investigate these

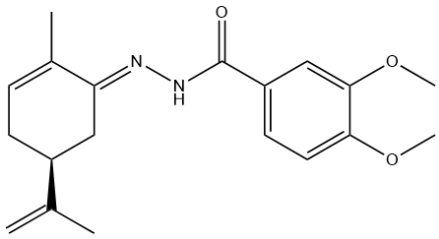
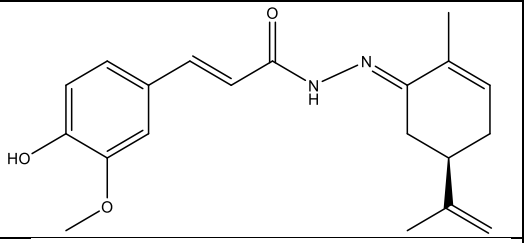
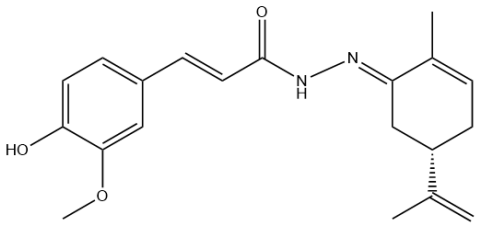
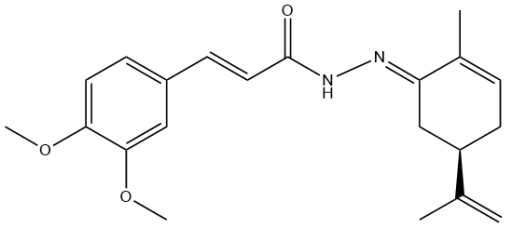
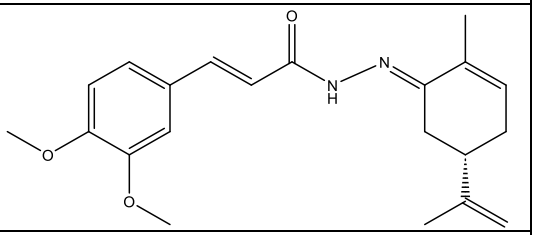
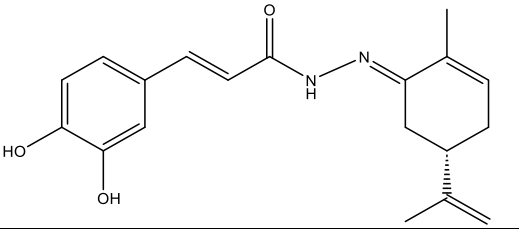
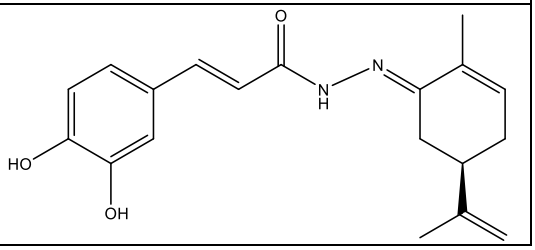
phytochemicals to understand their therapeutic potential, mechanisms of action, and safety profiles. This work contributes foundational knowledge that develops understanding in cannabinoid and terpene activity, supporting future development of therapies and formulations. Beyond advancing pharmacological insight, such research may impact drug discovery, personalised medicine and healthcare approaches ultimately facilitating the translation of cannabis-derived compounds.

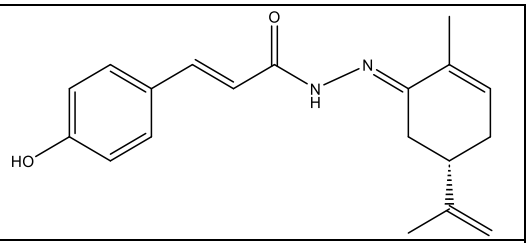
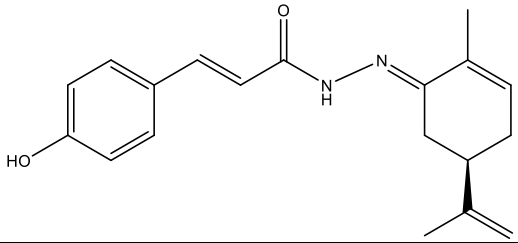
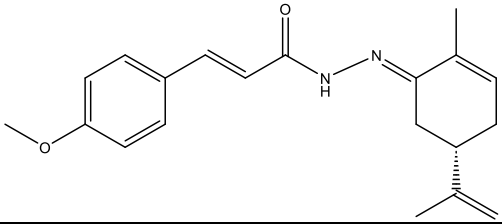
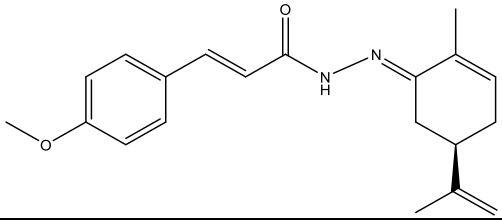
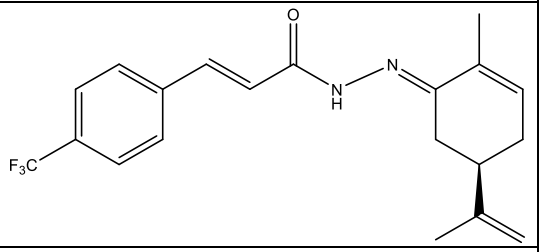
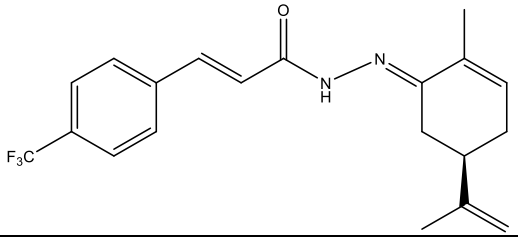
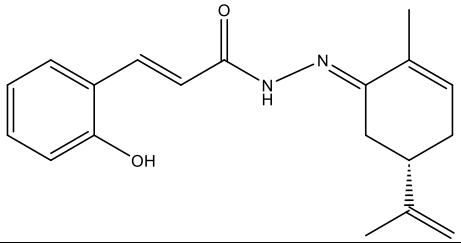
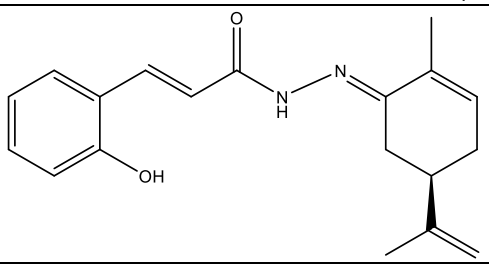
Collectively, these studies support the potential of cannabis-derived phytochemicals as safe and effective modulators of antimicrobial resistance, epithelial barrier function and cancer cell viability. By exploring their activity across distinct pathophysiological contexts, this research supports a multi-target model for phytochemical-derived therapeutics, one that aligns with the complexity of chronic diseases and their underlying dysregulation. The findings highlight the need for continued research to fully understand the therapeutic value of cannabis constituents and contribute to the development of mechanism-informed treatments for persistent global health challenges.

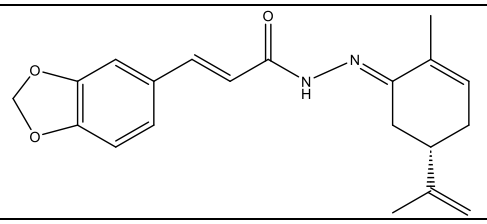
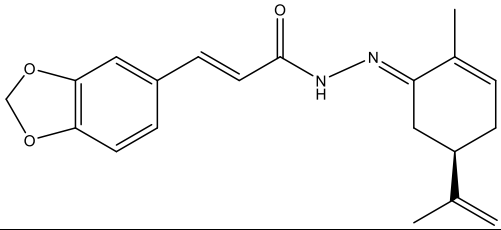
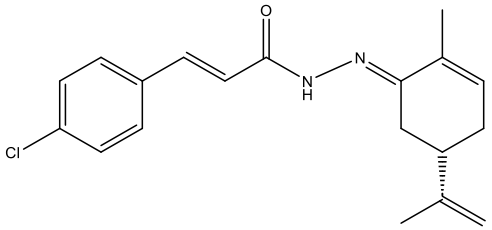
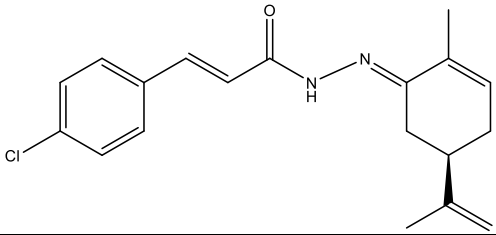
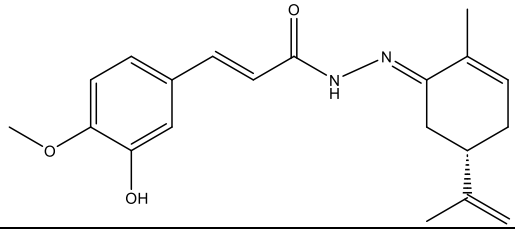
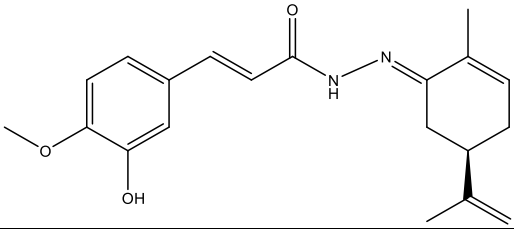
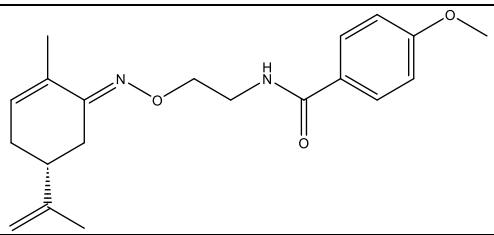
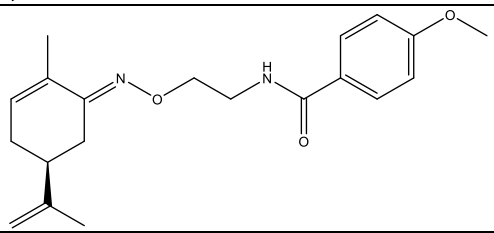
CHAPTER 6

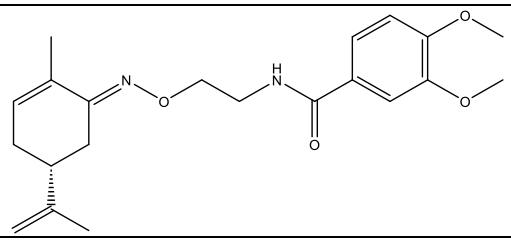
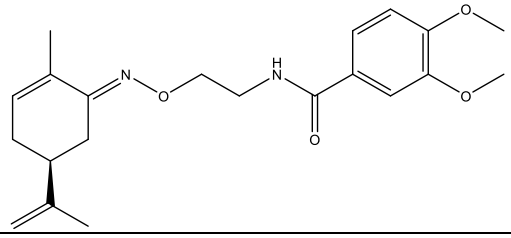
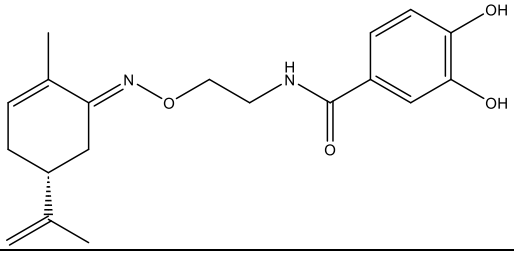
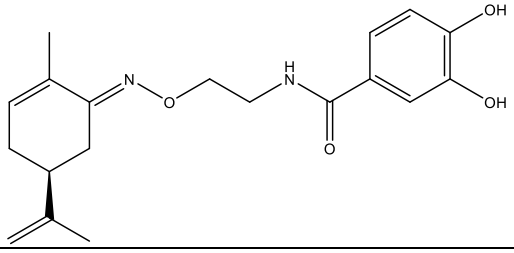
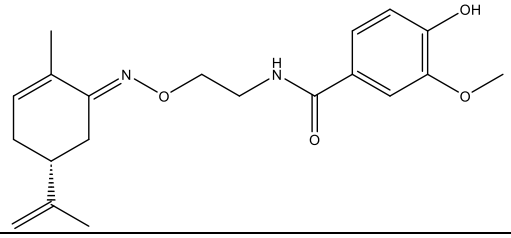
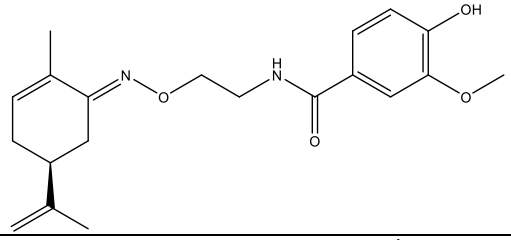
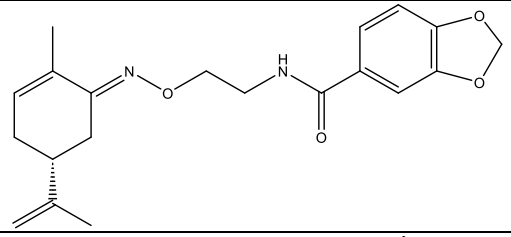
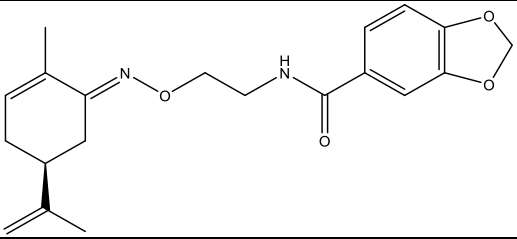
SUPPLEMENTARY INFORMATION

Compound ID	Nomenclature	Compound Structure
Cannabidiol (CBD)	2-[(1R,6R)-6-Isopropenyl-3-methyl-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol	
242	(S,E)-4-hydroxy-N'-(2-mehtyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)benzohydrazide	
243	(R,E)-4-hydroxy-N'-(2-mehtyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)benzohydrazide	
244	(S,E)-4-methoxy-N'-(2-mehtyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)benzohydrazide	
245	(S,E)-4-methoxy-N'-(2-mehtyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)benzohydrazide	
246	(R,E)-3,4-dihydroxy-N'-(2-mehtyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)benzohydrazide	
247	(S,E)-3,4-dihydroxy-N'-(2-mehtyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)benzohydrazide	
248	(R,E)-3,4-dimethoxy-N'-(2-methyl-5-prop-1-en-2-yl)cyclohex-2-en-1-ylidene)benzohydrazide	

249	(S,E)-3,4-dimethoxy-N'-(2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)benzohydrazide	
273	(E)-3-(4-hydroxy-3-metoxyphenyl)-N'-(R,E)-2-methyl-5-(prop-1-en-2-il)cyclohex-2-en-1-ilidene)acrilohidrazide	
274	(E)-3-(4-hydroxy-3-methoxyphenyl)-N'-((R,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
275	(E)-3-(3,4-dimethoxyphenyl)-N'-((R,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
276	(E)-3-(3,4-dimethoxyphenyl)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
290	(E)-3-(3,4-dihydroxyphenyl)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
291	(E)-3-(3,4-dihydroxyphenyl)-N'-((R,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	

292	(E)-3-(4-hydroxyphenyl)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
293	(E)-3-(4-hydroxyphenyl)-N'-((R,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
294	(E)-3-(4-methoxyphenyl)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
295	(E)-3-(4-methoxyphenyl)-N'-((R,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
300	(E)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)-3-(4-(trifluoromethyl)phenyl)acrylohydrazide	
301	(E)-N'-((R,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)-3-(4-(trifluoromethyl)phenyl)acrylohydrazide	
302	(E)-3(2-hydroxyphenyl)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
303	(E)-3(2-hydroxyphenyl)-N'-((r,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	

304	(E)-3-(benzo[d][1,3]dioxol-5-yl)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
305	(E)-3-(benzo[d][1,3]dioxol-5-yl)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
306	((E)-3-(4-chlorophenyl)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
307	((E)-3-(4-chlorophenyl)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
308	((E)-3-(3-hydroxy-4-methoxyphenyl)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
309	((E)-3-(3-hydroxy-4-methoxyphenyl)-N'-((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)acrylohydrazide	
337	(R,E)-4-methoxy-N-(2-(((2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)benzamide	
338	(S,E)-4-methoxy-N-(2-(((2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)benzamide	

339	(R,E)-3,4-dimethoxy-N-(2-(((2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)benzamide	
340	(S,E)-3,4-dimethoxy-N-(2-(((2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)benzamide	
341	(R,E)-3,4-dihydroxy-N-(2-(((2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)benzamide	
342	(S,E)-3,4-dihydroxy-N-(2-(((2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)benzamide	
343	(R,E)-4-hydroxy-3-methoxy-N-(2-(((2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)benzamide	
344	(S,E)-4-hydroxy-3-methoxy-N-(2-(((2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)benzamide	
345	(R,E)-N-(2-(((2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)benz0[d][1.3]dioxole-5-carboxamide	
346	(S,E)-N-(2-(((2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)benz0[d][1.3]dioxole-5-carboxamide	

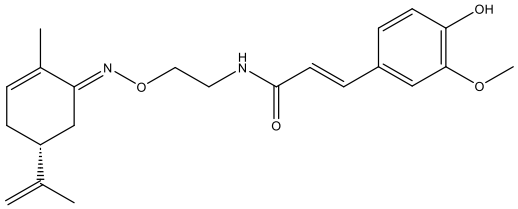
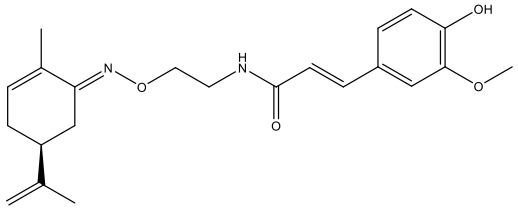
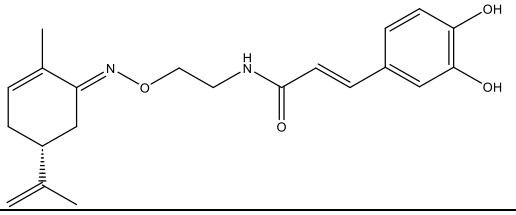
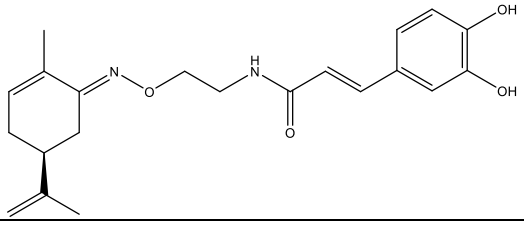
347	(E)-3-(4-hydroxy-3-methoxyphenyl)-N-(2-((((R,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)acrylamide	
348	(E)-3-(4-hydroxy-3-methoxyphenyl)-N-(2-((((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)acrylamide	
349	(E)-3-(3,4-dihydroxy)-N-(2-((((R,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)acrylamide	
350	(E)-3-(3,4-dihydroxy)-N-(2-((((S,E)-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-en-1-ylidene)amino)oxy)ethyl)acrylamide	

Figure S2-1. Chemical structure of cannabidiol (CBD) and CBD derivatives. Images of the chemical structures were generated using ChemDraw Prime Version 22.2.0.

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