The Antinociceptive Effects of Cannabinoids and Terpenoids on a Zebrafish Model of Nociception

by

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Abstract

With many communities affected by the opioid crisis, it has become of paramount importance to explore methods of pain management outside of treatments with opioids. One alternative treatment that has seen a recent surge in interest is cannabis and its related phytochemical-based treatments. Indeed, some cannabinoids and terpenoids have been demonstrated to show antinociceptive effects in various model systems. However, for the vast majority of terpenoids in cannabis, these effects remain unexplored. In this study I used a zebrafish model of nociception to test the antinociceptive effects of cannabinoids, terpenoids and a combination of the two compounds. Zebrafish provides a robust and high-throughput model system for such inquiry. Using behavioural assays, I confirmed the ability of acetic acid to elicit a change in locomotion in larval zebrafish. Exposure to low concentrations of acetic acid (0.001% - 0.01%) led to a four-fold increase in mean activity levels of 5-day old larvae. When zebrafish larvae were exposed to cannabidiol (CBD) at 2.5 mg/L and 5 mg/L and the terpenoids, trans-nerolidol and caryophyllene oxide at 10mg/L, the acetic acid-induced increase in activity was prevented. However, tetrahydrocannabinol (THC) and other terpenoids failed to prevent nociception from acetic acid. Combinations of the terpenoids with CBD did not lead to a greater antinociceptive effects compared with each individual compound. Using specific blockers, I determined that the process through which CBD, trans-nerolidol, and caryophyllene oxide display antinociceptive effects involves the transient receptor potential (TRP) cation channels, specifically TRPA1 and TRPV1 channels. On the other hand, intervention with specific blockers of μ-opioid receptor, δ-opioid receptor, and cannabinoid receptor types 1 and 2 was insufficient in preventing the acetic acid-induced activity. These findings lay the foundation for a more thorough investigation of the

therapeutic effects of cannabinoids, terpenoids and combinations of the two, as well as a mechanistic understanding of how such effects arise at a cellular level.

Preface

This thesis is an original work by Andrew M. Kim. The research project, which includes this thesis, was granted its research ethics approval from the University of Alberta Research Ethics Board: Project Name: “The Antinociceptive Effects of Cannabinoids and Terpenoids on a Zebrafish Model of Nociception,” AUP #00000816

Research done for this thesis is part of a collaboration between Ali lab and Allison lab at the University of Alberta. The equipment used for the behavioural assays are graciously provided by the Allison lab, and parts of this thesis will be published as part of a research paper alongside other experiments performed by myself and researchers in the collaborating lab. All research and analysis presented in the thesis are my original work.

No part of this thesis has been previously published.

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Abbreviations

AA: acetic acid

AITC: allyl isothiocyanate

CBD: cannabidiol

CNS: central nervous system

CO: (-)-caryophyllene oxide

DMSO: dimethylsulfoxide

DOR: δ-opioid receptor

IQR: Inter-quartile range

KOR: κ-opioid receptor

LM: limonene

LN: linalool

MOR: μ-opioid receptor

ORL-1: opioid receptor-like 1

PAG: periaqueductal grey

PNS: peripheral nervous system

PT: phytol

RVM: rostral ventromedial medulla

THC: (-)-Δ^9-tetrahydrocannabinol

TN: *trans*-nerolidol

TRP: transient receptor potential

- TRPA1: ankyrin type-1
- TRPV1: vanilloid type-1

αP: α-pinene

βM: β-myrcene

Chapter 1. Introduction

1.1 Issues with the Conventional Methods of Pain Management

In the medical field, options pertaining to the management of pain can be quite limited. Many of the commonly used methods for managing pain entail the use of opiates and synthetic opioids as anti-nociceptive (pain medication) agents. Many advantages, such as the ease of use from their multiple modalities of administration (Hagemeier, 2018) and their non-diminishing ability to alleviate pain (Holden et al., 2005), make opioids a more desirable option than some alternatives such as nonsteroidal anti-inflammatory drugs (NSAIDs), with their gastrointestinal and renal side effects (Tai & McAlindon, 2021; Grosser, Ricciotti, FitzGerald, 2017), and acetaminophen with its liver toxicity (Ghanem et al., 2016). The near unrestricted medical use of opioids from the 1990's has led to various issues throughout the world. The prescription of opioids in the United States nearly tripled in twenty years from 76 million in 1991 to 219 million in 2011 (Hagemeier, 2018), resulting in an epidemic of opioid drug abuse, overdose, and related deaths aptly named the Opioid Crisis. In Alberta, the use of fentanyl, a synthetic opioid, for pain management has also led to a province-wide medical emergency. During the covid-19 pandemic, there was an increase in the number of overdose cases and deaths related to fentanyl and other opioid use (Government of Alberta, 2022). These epidemics have led to various social, economic, and health issues (Nebhinani et al., 2013; Guy, Pasalic, & Zhang, 2018; Walwyn, Miotto, & Evans, 2010). In order to resolve these issues and prevent future instances of misuse and abuse, it is of paramount importance to explore methods of pain management outside of treatments with opioids.

1.1.1 Pain Pathway

In order to develop an alternate method of pain management, it is important to understand the mechanism underlying the perception of pain. There are three major classifications of pain: nociceptive, neuropathic, and inflammatory pain (Yam et al., 2018). Of the three, opioids are used to treat nociceptive, and acute and chronic inflammatory pain (Przewlocki & Przewlocki, 2001). Nociceptive pain refers to the pain felt in response to an external noxious stimulus detected by nociceptors of the sensory neurons (Vardeh, Mannion, & Woolf, 2017). Noxious stimuli have various forms, including mechanical, chemical, and thermal stimuli. When any of these stimuli exceeds a certain threshold and becomes potentially damaging or harmful, they are classified as a noxious stimulus. The signal from the nociceptor is propagated via the sensory afferent neurons to the central nervous system (CNS). Throughout this process, the signal is further processed and regulated, and in combination with the emotional response, it forms what we perceive as pain. On the other hand, nociception, unlike pain, lacks an emotional element; it is the neuronal signal that occurs in response to the noxious stimulus (Lee and Neumeister, 2020).

Noxious stimuli at the cutaneous region are detected at the free nerve endings of the peripheral neurons (Zylka, Rice, & Anderson, 2005). The various types of stimuli (mechanical, chemical, heat, or cold) activate different types of receptors (Raja, Meyer, & Campbell, 1988; Dubin & Patapoutian, 2010) and are converted into electrical signals in a process called transduction (Yam et al., 2018; Dubin & Patapoutian, 2010). The detection of noxious heat has been closely linked with the opening of the transient receptor potential vanilloid type 1 (TRPV1) channel, along with activation of the vanilloid receptor types 2, 3, and 4 (Caterina et al., 1999; Moqrich et al., 2005; Lee et al., 2005) and the inhibition of the potassium channel, TREK-1

(Alloui et al., 2006). On the other hand, noxious cold has shown to require the transient receptor potential ankyrin 1 (Kwan et al., 2006) and TRPM8 (Patapoutian, Tate, & Woolf, 2009). The TRPA1 channel is involved in chemo-nociception of various non-capsaicin chemicals (Patapoutian, Tate, & Woolf, 2009), and several TRP channels are thought to be involved in mechano-nociceptive processes (Dubin & Patapoutian, 2010).

The resulting signals, propagated by myelinated Aδ and unmyelinated C fibres to the CNS, form the initial transmission of the pain pathway (Yam et al., 2018). The primary afferents synapse on to various pathways at the dorsal horn of the spinal cord, including the spinothalamic tract and the spinoparabrachial tract (Lee & Neumeister, 2020; Yam et al., 2018).

1.1.2 Descending Modulation of the Pain Pathway

The descending pain pathway is highly regulated and can be modulated at several levels. According to the "gate theory" (Melzack and Wall, 1965), the non-nociceptive and myelinated Aβ fibres synapse onto inhibitory neurons in the dorsal horn of the spinal cord. These inhibitory neurons subsequently inhibit synaptic transmission from the unmyelinated and slower C fibres, thus reducing overall nociception (Lee & Neumeister, 2020) (Fig 1). An opioid-dependent inhibitory top-down regulation of the pain pathway further reduces this activity; it increases the activity of the inhibitory dorsal horn cells and decreases the activity of the excitatory circuitry that perpetuates the pain signals at the dorsal horn. (Basbaum & Fields, 1984).

A three-tier model of the regulatory system involves the periaqueductal grey (PAG), rostral ventromedial medulla (RVM), and the spinal dorsal horn. The connections between each region of this pathway contain the endogenous opioids (β-endorphin, met- and leu-enkephalins, and dynorphins) and their receptors (μ-, δ-, and κ-opioid receptors (MOR, DOR, and KOR,

respectively) and opioid receptor like-1 (ORL1)). PAG receives modulation from the limbic system, especially the central nucleus of the amygdala, which plays a significant role in a μ-opioid receptor dependent manner (Finnegan, Chen, & Pan, 2005). The hypothalamus also affects the PAG signaling through the opioid system (Bach & Yaksh, 1995; Finley, Lindström, & Petrusz, 1981). The projections from PAG to RVM also express enkephalin (Williams & Beitz, 1990), as well as MOR and DOR (Wang & Wessendorf, 2002). Finally, the neurons of the RVM also express MOR, DOR, and KOR (Kalyuzhny et al., 1996; Gutstein et al., 1998), and they contain enkephalin expressing efferents to lamina I, II, and V of the spinal dorsal horn (Zhang et al., 2015). It is hypothesised that the projections to RVM from PAG are inhibited in a normal state by a GABA-mediated modulation. The opioid signal inhibits the release of GABA, thereby disinhibiting the descending pathway of pain modulation (Moreau & Fields, 1986; Bobeck et al., 2014). The projections from central nuclei of amygdala to periaqueductal grey to rostral ventromedial medulla are represented in Figure 2.

In addition to the opioid system, many other neurotransmitter systems, including but not limited to the serotonergic, norepinephrinergic (Basbaum & Fields, 1984; Gu & Wessendorf, 2007), and GABAergic (Osborne et al., 1996; Lau, Winters, & Vaughan, 2020) systems, are also involved at the various tiers of the descending modulation of the pain pathway. The direct way in which the opioid system is linked to the pain pathway made it an easy target for pharmacological interventions of unwanted pain, contributing to the popularity of opiates as a tool for pain management.

1.1.3 Opiates and Their Limitations

Opiates and synthetic opioids work by interacting with the receptors of the endogenous opioids. These opioid receptors are G-protein coupled receptors (GPCR) with seven transmembrane domains (Waldhoer, Bartlett, & Whistler, 2004). When bound by the endogenous ligands or opiates, the receptors become activated, and this leads to an inhibition of the adenylyl cyclase (AC)/cyclic AMP (cAMP) messenger system (Yam et al., 2018; Connor & Christie, 1999). This subsequently leads to changes in cellular function. For example, inhibition of the cAMP messenger system may decrease neuronal excitability through inhibiting voltage gated calcium and activating inwardly rectifying potassium channels (Al-Hasani & Bruchas, 2011). It is through this process that the neurons of the pain pathway are modulated, leading to analgesia.

However, such interactions are multifaceted in nature and may result in various side effects. One such side effect is the hedonic effects that opioids exert, which can lead to addiction and dependence on the opiates if abused or improperly used (Malwyn, Miotto, & Evans, 2010). MOR, the most common of the opioid receptors to be targeted for pain management due to its effectiveness, was shown to be expressed in the nucleus accumbens and basolateral amygdala (Wassum et al., 2011). It is linked to the reward pathway and its activation has been shown to lead to drug-seeking behaviour and dependence (Contet, Kieffer, & Befort, 2004; Mitchell et al., 2012). The activation of KOR is also linked to dysphoria, which has multiple mental health implications in addicted individuals (Wang, 2019) and may be involved in relapses in drug taking behaviours (Bruchas & Chavkin, 2013; Koob & Volkow, 2016). The dependence on opiates has become a serious issue due to the health impact on the affected individuals and the social and economic impact it exerts. As the rate of misuse, abuse, and addiction to these

medications have been directly linked to their over-prescription, it is important to explore safer alternatives that can help counter the massive issue of pain management without significant drawbacks.

1.2 Cannabis as an Alternative Pain Management Methods

In recent years, the legalisation of cannabis in Canada, as well as its relatively low risk of addiction and complications in comparison to opiates and synthetic opioids, led to a heightened interest in the use of cannabis and related phytochemical-based treatments as an alternative to combat the rise in opioid dependence and related deaths. This interest extends beyond the confines of the research community, with the proportion of pregnant individuals and patients suffering from chronic pain and anxiety using cannabis as a treatment for their symptoms increasing drastically in the recent years (Avila et al., 2020).

Cannabis sativa is a species of plant with over 1000 constituents (Hanuš and Hod, 2020) that has been utilised for therapeutic, medical, and ritualistic purposes in various cultures throughout history (Ren et al., 2019; Simiyu, Jang, and Lee, 2022; Sommano et al., 2020). It also provides some additional benefits, such as its ability to alleviate migraine headaches and certain inflammatory pains that opioids fail to provide relief from (Baron, 2018). Despite the centuries of traditional and modern medical applications, however, there is a general lack of knowledge on the mechanism of actions for the pharmaceutical effects the plant displays, including its ability to subdue pain and nociception.

Cannabinoids and terpenoids serve as important targets of research due to their wide and varied physiological functionality and applications. Studies have shown that some cannabinoids and terpenoids have anti-nociceptive and anxiolytic effects in various model systems and many

have suggested the existence of a combinatorial effect of these phytochemicals (Altun et al., 2015; Avila et al., 2020; Crivelaro et al., 2020; Foss et al., 2021). With the sheer number of its substituents and the variety of effects that each phytochemical exerts on different endogenous systems (Alves et al., 2020), it is critical that the individual and combinatorial effects of the phytochemicals are determined and understood for an effective combination and dosing in treatments.

1.2.1 Pharmacological Targets of Cannabinoids

There are various types of cannabinoids found in the *C. sativa* plant. This study focuses on cannabidiol (CBD) and tetrahydrocannabinol (THC) as the main targets of interest for the antinociceptive effects of cannabinoids. THC is the major psychoactive compound found in cannabis, whereas CBD is the most prevalent non-psychoactive component of cannabis. Both CBD and THC have numerous physiological targets. They act as modulators of the receptors of the endocannabinoid system, such as CB_1R and CB_2R, and act agonistically at TRP channels. In addition to these cannabinoid receptors and TRP channels, the phytocannabinoids were also shown to modulate serotonergic receptors (Martínez-Aguirre et al., 2020), the opioid system (Kathmann et al., 2006), and other G-protein coupled receptor systems, such as GPR55 (Ryberg et al., 2007). The interactions with these receptors could individually or collectively be responsible for the wide array of functions of cannabinoids and I will explore the various interactions in order to identify the mechanism behind the antinociceptive effects of some of the compounds found within cannabis.

1.2.1.1 Endocannabinoid System

The endocannabinoid system is comprised of endocannabinoids, such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the cannabinoid receptors type 1 and type 2 (CB_1R and CB_2R) (Howlett and Abood, 2017). CB_1R and CB_2R are G-protein coupled receptors. CB_1R is mostly concentrated in the CNS. Even though CB_2R can also be found in the CNS to a small extent, they are generally thought to be more peripheral, located in the peripheral nervous system (PNS), the immune system, and the spleen (Jordan and Xi, 2019). The endocannabinoid receptors are $G_{i/o}$ coupled, and their activation leads to the inhibition of cyclic AMP signals and calcium channel activity while activating inwardly rectifying potassium ion channels (Ryberg et al., 2007; Demuth & Molleman, 2006; Zou and Kumar, 2018). Through these mechanisms, the pre-synaptically located endocannabinoid receptors suppress transmitter release from both excitatory and inhibitory terminals (Castillo et al., 2012). Both the activation (Haller, Stevens, & Welch, 2008; Cajanus et al., 2016) and the inhibition (Altun et al., 2015) of the endocannabinoid system has been shown to lead to antinociception, depending on the target site. One example is a Parkinsonian model where CBD administration led to a decrease in allodynia and hyperalgesia due to its actions on CB_1R (Crivelaro do Nascimento et al., 2020).

It is important to note, however, that CBD and THC, while both classified as cannabinoids, have vastly different interactions with the endocannabinoid system. THC has a much higher affinity for binding to both CB_1 and CB_2 receptors when compared to CBD: against human CB_1R in Sf9 cells, THC showed a K_i of 35.64 ± 12.4 nM as opposed to CBD with K_i of 1458.5 ± 158.5 nM (Rosenthaler et al., 2014). Similarly, THC had a K_i of 8.45 ± 6 nM against human CB_2R in Sf9 cells, whereas CBD had a K_i of 372.37 ± 57.5 nM (Rosenthaler et al., 2014). Additionally, the two phytocannabinoids function differently at each receptor as well. CBD acts

as an inverse agonist at CB_1 and CB_2 receptors (Thomas et al., 2007) while THC acts as a partial agonist at CB_1 and CB_2 receptors (Pertwee, 2008).

1.2.1.2 Transient Receptor Potential Channels

Endocannabinoids and phytocannabinoids do not only bind to the cannabinoid receptors, but rather to a variety of targets, including the transient receptor potential (TRP) channels (Kowalski et al., 2020). TRP channels are nonspecific cation channels that serve numerous functions and can be found in a number of sensory pathways, acting as general sensors for mechanical, chemical, thermal and other environmental stimuli (Tominaga et al., 1998; Gupta et al., 2016; Cao et al., 2013). Cannabinoids act as agonists at many of these, leading to TRP channels being considered as the ionotropic receptors of the cannabinoids. Two of these channels are of particular interest in this study: TRPV1 and TRPA1. TRPV1 (vanilloid type 1) channels are cation channels responsible for nociception and also respond to capsaicin and heat (Frias and Mirighi, 2016). TRPA1 (ankyrin) channels are activated by painful chemical stimuli and are involved in noxious cold detection (Souza Monteiro de Araujo et al., 2020). With the direct involvement of TRP channels in pain pathways and a substantial amount of evidence suggesting an interaction between cannabinoids and TRP channels, these channels have become ideal targets for investigating the analgesic and antinociceptive effects of cannabinoids. Antagonism of TRP channels is linked to antinociception, and agonist activation of TRP channels leads to desensitisation, which results in antinociception (Novakova-Tousova et al., 2007). Evidence suggests that CBD acts as an agonist at both TRPA1 and TRPV1 in rats and binding of CBD to these receptors desensitises them (De Petrocellis et al., 2011).

1.2.1.3 Opioid System

The interaction between cannabinoids and the opioid system is interesting. For instance, block of CB_1R and CB_2R has been shown to extend the duration of analgesia following opioid administration (Altun et al., 2015). Additionally, the inhibition of fatty acid amide hydrolase (FAAH), an enzyme responsible for the hydrolysis of anandamide, enhances the analgesic functions of KOR in a CB_1R dependent manner (Haller, Stevens, & Welch, 2008). Furthermore, studies have shown that THC and CBD both decreased MOR and DOR, but not KOR, binding to their ligands *in vitro* by interacting with the receptors directly to inhibit their function in a non-competitive manner (Vaysse, Gardner, & Zukin, 1987; Kathmann et al., 2006). The two ways in which cannabinoids can interact with the opioid system – indirect modulation through the endocannabinoid system or the direct modulation by the allosteric regulation of the opioid receptors – need to be further explored and tested for a more comprehensive understanding of the anti-nociceptive effects of cannabinoids.

1.2.2 The Pharmacological Targets of Terpenoids

Terpenoids are polymers of isoprene where a combination of two isoprene compounds forms one terpenoid unit. They are often found in plants and essential oils, giving them their signature scents. Within the *C. sativa* plant, there are over 200 different types of terpenoids, composed of monoterpenes, sesquiterpenes, diterpenes, and triterpenes (Sommano et al., 2020). Due to the difference in their chemical compositions and physiological targets, each terpenoid exerts highly altered effects, ranging from analgesic properties (Pellati et al., 2018; Tsuchiya, 2017) to anti-bacterial and anti-fungal effects (Iseppi et al., 2019; Djilani et al., 2012). Of the different types of terpenoids, monoterpenes and sesquiterpenes are most prevalent in *C. sativa*

(Pieracci et al., 2021). Monoterpenes are ten-carbon polymers composed of two isoprene units, of which the most abundant are α- and β-pinene, α-terpinolene, trans-ocimene, limonene, and β-myrcene (Pieracci et al., 2021; Iseppi et al., 2019). Of the sesquiterpenes – the three isoprene, 15-carbon polymers - β-caryophyllene, α-humulene, and caryophyllene oxide are the most prevalent (Iseppi et al., 2019).

The diverse array of terpenoids in cannabis suggests that there are potentially many functional and pharmacological targets for these phytochemicals and many studies have attempted to characterise these functions. For instance, (-)-linalool was shown to display antinociceptive and antihyperalgesic effects through its interactions with NMDA receptors (Peana et al., 2004). Beta-Myrcene has been demonstrated to have analgesic effects through mechanisms involving the α2-adrenergic receptor and the release of endogenous opioids (Sommano et al., 2020). Beta-caryophyllene acts as an agonist at the CB_2R and displays antinociceptive and anxiolytic effects (Gertsch et al., 2008), the effects of which has also been demonstrated on adult zebrafish (Johnson et al., 2023). Finally myrcene and nerolidol have been shown to act as agonists at TRPV1 channels (Jansen et al., 2019).

It has been speculated that cannabinoids and terpenoids have synergistic effects (Ternelli et al., 2020; Russo, 2019) and evidence to support this interaction can be found in the literature (Lorenzetti et al., 1991; Gertsch et al., 2008; Maayah et al., 2020). However, there is also evidence to the contrary (Heblinski et al., 2020), and further exploration into this topic is necessary to ascertain the interaction between cannabinoids and terpenoids and to understand the mechanisms behind their interactions.

1.3 Zebrafish as a Model System

In this project, I used a zebrafish model of nociception to test the antinociceptive effects of cannabis-based phytochemicals. Zebrafish has been used in various studies in the field of neuroscience, especially in the context of developmental neuroscience because this organism offers several important properties for developmental studies. For instance, 1) zebrafish development is similar in many ways to early human development (Demin et al., 2018), 2) the external nature of their development means that there is no maternal-placental interaction, 3) high throughput drug treatments can occur and the environment in which the embryos are kept can be much more accurately controlled while the dosage or concentration of any administered drugs can be much more precisely determined. Importantly, the high-throughput nature of the model proved especially relevant to this study due to high variability in the behavioural assays and the sheer number of compounds and combinations that need to be tested throughout this study. With hundreds of eggs being produced each day, this model system allows for a high sample size and multiple replicates for each treatment that is much more cost-effective than some alternatives.

Additionally, with relatively well conserved gene expression, many of the pharmaceutical targets within the organism are similar to their higher vertebrate counterparts, including the endocannabinoid system (Malafoglia et al., 2013). Zebrafish is more frequently used in genetic studies, with new tools becoming available for genetic manipulations on the model system (Acevedo-Canabal et al., 2019; Chiarlone et al., 2016). The dual-purpose of the model – its applicability to adult human nervous systems and its utilities as a developmental model – make it very versatile. Such versatility and an easily expandable and widely applicable nature, suggests that the preparation can serve to answer many of the questions and uncertainties surrounding the

use of cannabis-based phytochemicals in pharmaceutics and help expand its use locally and globally in a safe and controlled manner.

1.3.1 Zebrafish as a Model of Nociception

Zebrafish (*Danio rerio*) provides a robust and high-throughput model system for this study that may serve as a more ethical alternative compared to other conventional models of pain in more complex model systems. With the use of zebrafish embryos, it is also possible to test for the potential adverse effects of cannabis phytochemicals on development. Therefore, this animal model may be used to examine the viability of the increasingly popular use of cannabis-based pharmaceutical treatment on pregnant humans for managing pain and discomfort associated with pregnancy.

The different ways in which zebrafish have been used in nociceptive studies have been highlighted in several studies such as the following: acetic acid-based models of nociception in zebrafish larvae demonstrated that the activity of 5-day old larvae significantly decreased when exposed to high concentrations of acetic acid (Lopez-Luna et al., 2017; Taylor et al., 2017) and increased when exposed to low concentrations of acetic acid (Steenbergen & Bardine, 2013). An adult model of nociception based on acetic acid also exists, where it was demonstrated that an injection of acetic acid led to a drastic change in behaviour, such as reduced activity, increased freezing duration, and changes in the curvature index of the body (Costa et al., 2019). Noxious cold and heat, as well as other chemical irritants, such as allyl isothiocyanate (AITC, or mustard oil), were also used as noxious stimuli in zebrafish (Prober et al., 2008). In addition to the existence of the various models of nociception in zebrafish, the data showing that they express

conserved nociceptors and pain pathways (Sneddon, 2019; Costa et al., 2019; Ko et al., 2019) also make the zebrafish an ideal model system for studies on nociception and pain.

1.3.2 Endogenous Systems of Zebrafish

Zebrafish possess many endogenous systems that are analogous to those found in humans (Howe et al., 2013). The systems of interest for this thesis, such as the endogenous opioid system, endocannabinoids, and transient receptor potential have all been demonstrated to be well conserved throughout evolution. According to the HomoloGene database (https://www.ncbi.nlm.nih.gov/homologene), the *Danio rerio* gene for the μ-opioid receptor, *oprm1*, has 73% identity (80% from positives and 5% from gaps) with its *Homo sapiens* homologue, *ORPM1.* Similarly, the zebrafish genes for the δ-opioid receptor (*oprd1a* and *oprd1b*) have 68% (80% of positives and 3% of gaps) and 67% identities (79% positives and 2% gaps), respectively, with its human homologue, *ORPD1*. Opioid receptors are especially well conserved, with at least one zebrafish homolog for each of major opioid-related genes in humans (Bao et al., 2019).

The genetic similarities and applicability of zebrafish as a model of pain and nociception is not limited to the opioid receptors. Transient receptor potential ankyrin type 1 (TRPA1), subtypes a and b, are coded by t*rpa1a* and *trpa1b* in zebrafish respectively. *Trpa1a* shares 49% identity (67% of positives and 2% of gaps) with *TRPA1* gene in humans, whereas *trpa1b* shares 47% identity (66% of positives and 3% of gaps) with *TRPA2* gene in humans. Transient receptor potential vanilloid type 1 (TRPV1) has been tied to the genes *trpv1* in zebrafish and *TRPV1* in humans, which share 49% identity (65% of positives and 8% of gaps).

Additionally, zebrafish serve as an appropriate model for this study due to the similarities in the endocannabinoid receptors. Cannabinoid receptor type 1 (CB1) is coded by *cnr1* in

zebrafish and *CNR1* in humans. These genes share 70% identity (81% of positives and 4% of gaps). Cannabinoid receptor type 2 (CB2), coded by *cnr2* in zebrafish and *CNR2* in humans, also share 44% identities (61% of positives and 4% of gaps).

Many of these genetic similarities also translate to the receptors' functionalities as well, as explored by various pharmacological and molecular investigations (Milan et al., 2003; Schaaf et al., 2008). For example, known analgesics for humans, such as some agonists of μ-opioid receptors (MOR), demonstrate antinociceptive effects in zebrafish (Lopez-Luna et al., 2017). Agonists of human TRP channels such as AITC have also been shown to have an impact on zebrafish TRP channels (Ko et al., 2019). Cannabinoids, such as CBD and THC also interact with both the zebrafish and human endocannabinoid receptors (Amin et al., 2020).

Lastly, anatomical locations of receptors of interests, such as endocannabinoid receptors, also reflect that of mammalian systems. Much like the localisation of CB_1R in humans, CB_1R in zebrafish is also localised in brain regions, such as hypothalamus, telencephalon, and tegmentum, and also in digestive systems (Bailone et al., 2022; Son & Ali, 2022). CB_2R also shared similar localisation between zebrafish and humans, with much of the expression seen in peripheral systems including the immune system, but also found in central nervous system as well (Rodriguez-Martin et al., 2007; Klee et al., 2011). Thus, the conserved nature of these systems suggest that zebrafish may be an appropriate model for the purposes of this study.

However, when compared to higher order vertebrates, zebrafish do exhibit some differences in their morphology and cellular function. For example, TRPV3 and TRPM8, on which both CBD and THC act as an agonist and an antagonist respectively (De Petrocellis et al., 2012; Muller et al., 2019), have not been identified on zebrafish genome (Malafoglia et al.,

2013). Such differences should be taken into account when interpreting the data, deducing a mechanism of action, and applying said mechanism to human or other mammal systems.

1.4 Research Objectives and Hypothesis

This study aims to investigate the antinociceptive effects of cannabinoids, terpenoids, and the combination of these phytochemicals found in the *Cannabis sativa* plant. Many studies have demonstrated the antinociceptive effects of individual cannabinoids and terpenoids, but the mechanism of action behind the analgesic effects of cannabinoids and terpenoids remains mostly unexplored (Baron, 2018; Heblinski et al., 2020). In addition, while the synergistic effects of these phytochemicals are quite often speculated, very little evidence exists to support this hypothesis.

I will explore these effects by treating zebrafish larvae with various concentrations and combinations of CBD, THC, and several terpenoids. Using behavioural changes in zebrafish larvae as the marker of nociception, this study aims to verify the previously described pro- and antinociceptive effects of acetic acid, cannabinoids, and some terpenoids. Furthermore, it aims to further explore the antinociceptive effects of a combination of the cannabis-based phytochemicals. Specifically, this study aims to:

1. Identify which cannabinoids and/or terpenoids display antinociceptive effects, and identify a combination of phytochemicals that maximises the antinociceptive effect in the zebrafish model of nociception through behavioural assays,
2. Identify the cellular mechanisms, such as ligands and receptors involved in the antinociceptive effects of cannabinoids and terpenoids through pharmacological interventions.

For this purpose, I will be testing the antinociceptive effects of CBD, THC, and the following terpenoids found in *C. sativa* plant: α-pinene, β-myrcene, linalool, limonene, *trans*-nerolidol, (-)-caryophyllene oxide, and phytol. Once the effective concentrations of these phytochemicals have been determined, pharmacological interventions in the form of the following blockers will be used to identify the mechanism behind these effects: CTAP and naloxone for μ-opioid receptors, naltrindole for δ-opioid receptors, AMG-9090 for general TRP channels, A-784168 for TRPV1, and HC-030031 and A-967079 for TRPA1 channels.
With the information from some preliminary data and the past literature on the antinociceptive effects of cannabinoids (Baron, 2018; Ellis et al., 2018), I hypothesise that:

1. The concentrations and combinations of a cocktail of cannabinoids and terpenoids can be adjusted in such a way to empirically maximize the effectiveness of pain management while minimizing the adverse effects,
2. The endocannabinoid system is involved in the antinociceptive effects of the phytochemicals, and these effects can be isolated to specific receptor systems (CB_1R, CB_2R, TRP channels) in select neurons.

Chapter 2: Materials & Methods

2.1 Animal Care and Use

Zebrafish (Tubingen Longfin strain; *Danio rerio*) larvae were obtained through in-house breeding of adult zebrafish kept and maintained in the aquatic facility at the University of Alberta. The eggs were obtained by breeding one or two females with one male, and they were collected the following morning. Once collected, the fertilized eggs were kept in petri dishes in egg medium (60 µg/mL Instant Ocean Sea Salt) at 28.5˚C, with a 12 h-12 h dark and light cycle. The medium was replaced every day and larvae were used for experiments at 5 dpf. The animal housing and experimental procedures were approved by the Animal Care and Use Committee at the University of Alberta (AUP #00000816), adhered to the Canadian Council on Animal Care guidelines for human and animal use.

2.2 Compounds

The compounds used throughout this study are as follows: cannabidiol (CBD), (-)-Δ^9-tetrahydrocannabinol (THC), acetic acid (glacial 99.7%), AMG-9090, A-784168, HC-030031, A-967079, buprenorphine, allyl isothiocyanate, CTAP, naloxone, naltrindole, α-pinene, β-myrcene, *trans*-nerolidol, (-)-caryophyllene oxide, linalool, limonene, phytol, and lidocaine (Sigma Aldrich, St. Louis, MO, USA). The CBD, THC, and buprenorphine were purchased in solution form, dissolved in methanol, then diluted with ddH_2O to the desired concentrations of compound. Acetic acid and AITC were dissolved directly in ddH_2O. All other compounds were dissolved in dimethyl sulfoxide (DMSO) and further diluted to the desired concentrations of compound. When administered by immersion into the wells of the 96-well plates, the solutions

were further diluted by a factor of 20 to get the final solvent concentrations of 0.5% v/v methanol and 0.25% v/v DMSO. This was done to minimize the effects of the solvent on the zebrafish larvae while ensuring the compounds remained dissolved throughout the experiments. Each solution was prepared from aliquots stored at -20°C immediately prior to the behavioural experiments.

2.3 Behavioural Assay

Zebrafish larvae at 5 days post-fertilisation (dpf) were placed into the middle 48 wells of a 96-well plate, with one larva in each well. After a 30-minute period of acclimatisation in the behavioural room, the activity of the larvae was measured using the Noldus Ethovision XT software, using the Basler GenlCaM (Basler acA1300-60) scanning camera with a 75 mm f2.8 C-mount lens (Noldus, Wageningen, The Netherlands) (Fig 3A, B). The untreated activity was measured, then the treatment of choice was administered to each larva through an environmental exposure paradigm: the compound of choice was placed in each well using a micropipette. The following concentrations were administered to the larvae for each compound: 0.0001% - 0.25% acetic acid, 50 – 200 µM AITC, 0.01 – 0.1 mg/L buprenorphine, 5.0 – 50 mg/L lidocaine, 0.1 – 10 mg/L THC, 1.0 – 10 mg/L CBD, 1.0 – 50 mg/L α-pinene, 1.0 – 50 mg/L β-myrcene, 1.0 – 50 mg/L limonene, 1.0 – 25 mg/L linalool, 1.0 – 50 mg/L phytol, 1.0 – 10 mg/L *trans*-nerolidol, 1.0 – 10 mg/L (-)-caryophyllene oxide, 0.1 mg/L naloxone, 20 µM CTAP, 20 µM naltrindole, 10 – 100 µM AMG-9090, 1 – 10 µM A-784168, 1 – 50 µM HC-030031, 0.1 – 1.0 µM AM251, and 0.5 – 5.0 µM AM630.

After administration of the compound, the movement of the larvae was recorded for 10 to 30 minutes, depending on the time required for the exposure. In cases where a second compound

needed to be administered, such as with the noxious stimuli in the form of acetic acid or AITC, the movement was recorded again after the administration, also for 10 to 30 minutes. After the completion of the recording, the video was examined for any faulty detection of larval movement, at which point the incorrectly detected samples were removed from analysis.

The mean percent activity of each larva was determined by the Ethovision XT software. The parameter on the Ethovision software is defined as the mean of the changes in the ratio between active pixels (occupied by the detected sample) and the inactive pixels between each frame of the recording.

$$\%\ Activity = \frac{\Delta\ active\ pixels\ between\ each\ frame}{total\ pixels\ in\ arena} \times 100\%$$

The mean percent activity between each treatment was measured over a 10-minute interval, therefore a 30-minute recording was divided into three 10-minute measurements, thus providing 3 data points for mean activity. Figure 3C shows three the representative traces for the levels of activity obtained from zebrafish larvae under three different conditions at 5 dpf; the left trace depicts zebrafish larval activity prior to exposure to any compounds, the middle trace depicts zebrafish larval activity following exposure to acetic acid, and the right trace depicts zebrafish larval activity exposed to a combination of a known analgesic and acetic acid.

2.4 Statistical Analysis

The analysis of the behavioural data was performed using the GraphPad Prism software (Version 7.0, San Diago, CA, USA). Once collected, the outliers for each dataset were identified using the ROUT method with Q = 1%. The data was then tested for normality via the Shapiro-Wilk normality test, rejecting the normality when the p value was less than 0.05. Many of the

data sets were not normally distributed, and the data were represented using the median values with the inter-quartile range (IQR) in parentheses.

Data in figures were represented in box-and-whisker plots with the error bars representing the range between the minimum and maximum values of each group. The statistical analysis performed was the Kruskal-Willis test ($p<0.05$), followed by the Dunn's multiple comparisons test, if significant, to evaluate the significance between the medians of every treatment with one another. In order to control for large discrepancies in the data for the behavioural assays, experiments with a mean percent activity of 0.01% or less for the baseline control were discarded and redone.

Chapter 3: Results

3.1 Verification of Zebrafish as a model of nociception and pain.

The aim of this thesis was to explore the antinociceptive effects of cannabinoids and terpenoids found in the *Cannabis sativa* plant and then to identify the mechanisms underlying their effects. Larval and adult zebrafish have been used in several studies of nociception induced by different modalities of noxious stimuli (Malafoglia et al., 2013; Lopez-Luna et al., 2017; Costa et al., 2019; Demin et al., 2018), and I modelled my experimental design after some of these experiments. In this study, I used acetic acid as the primary noxious stimulus, but I also used AITC (mustard oil) as an additional noxious agent for comparison.

Because zebrafish and acetic acid have been used in several studies in the context of nociception and in the exploration of antinociceptive agents, my first goal was to confirm that acetic acid has a nociceptive effect in zebrafish larvae. Therefore, I measured the activity of 5 dpf zebrafish larvae after exposure to acetic acid. The activity of untreated zebrafish larvae was recorded for fifteen minutes from which the last ten minutes were analysed (Fig 3A). The larvae were then exposed to various concentrations of acetic acid (0.0001%, 0.001%, 0.005%, 0.01%, 0.1%, and 0.25% v/v), and the swimming activity was measured for a further ten-minutes. The ten-minute duration was selected for consistency with the literature (Lopez-Luna et al., 2017).

In my experiments, I found that administration of acetic acid at lower concentrations led to robust and reliable increases in locomotion of zebrafish larvae in a manner consistent with the literature (Steenbergen & Bardine, 2013; Lopez-Luna et al., 2017; Ellis et al., 2018). For instance, exposure to 0.001%, 0.005%, and 0.01% v/v acetic acid resulted in an increase in activity (Fig 4A). Vehicle control (ddH_2O) larvae had mean activity levels of 0.0017% (IQR =

0.019%, n = 48), whereas the mean percent activity of the larvae exposed to 0.001%, 0.005%, and 0.01% acetic acid was significantly greater at levels of 0.13% (IQR = 0.090%, n = 58; $p < 0.0001$), 0.13% (IQR = 0.093%, n = 56; $p < 0.0001$), and 0.12% (IQR = 0.060%, n = 117; $p < 0.0001$).

Exposure to higher concentrations of acetic acid (0.1% and 0.25%) did not result in a significant change in activity from controls (Fig 4A). Interestingly, previous studies have demonstrated that zebrafish larvae exposed to 0.1 - 0.5% v/v acetic acid for 60 seconds experienced cell death on the surface of their skin (Ellis et al, 2018) and exposure to solutions with pH levels of 4.0 or lower led to significant distress while pH levels of 3.0 or lower led to fatality in adult zebrafish after 24 to 48 hours (Zahanger et al, 2015). These findings suggest that higher concentrations of acetic acid are harmful and should not be used for nociceptive experiments. Taking these findings into consideration, I used acetic acid concentrations of 0.001% for all of my treatments to minimise larval distress.

A forty-minute behavioural assay following administration of 0.001% acetic acid revealed that there was a small decrease in the overall level of activity over time (Fig 4B). Even though there was a small reduction in activity after 20 minutes, the activity level was significantly greater than time-matched controls (Fig 4C). Mean activity levels during the 20-to-30-minute and 30-to-40-minute periods were about 0.20% (IQR = 0.11%, n = 48; $p = 0.0134$ for 20-30 min, and IQR = 0.12%, n = 48; $p = 0.0147$ for 30-40 min) (Fig 4B). This was consistently higher than the vehicle control (ddH_2O). Vehicle controls exhibited a low level of activity from the start of the exposure period, (0.016% activity; IQR = 0.033%) which remained unchanged throughout forty minutes of recording (n = 43-46; $p = 0.3688$) (Fig 4C). The stable activity levels

meant that changes in activity could be reliably detected within the time frame of my experiments.

To confirm that the acetic acid-induced activity was nociceptive in nature, I pre-treated zebrafish larvae with the analgesics, buprenorphine (Fig 5A, B) or lidocaine (Fig 5C, D), immediately prior to 0.001% acetic acid treatment. Buprenorphine is a semi-synthetic MOR partial agonist with a low efficacy but high affinity and potency (Coe, Lofwall, and Walsh, 2019), and its ability to act as an antinociceptive agent has been previously demonstrated in zebrafish (Steenbergen and Bardine, 2014). On the other hand, lidocaine is a local anaesthetic that acts on voltage-gated sodium channels (Lopez-Luna et al., 2017; Thomson et al., 2019).

I pre-treated larval zebrafish with either of these compounds for 30 minutes prior to the administration of 0.001% acetic acid. Both buprenorphine at 0.1 mg/L and lidocaine at 50 mg/L, prevented the acetic acid-induced increase in activity (Fig 5B, D): Compared to acetic acid treatment (0.13% activity, IQR = 0.11%, n = 45), buprenorphine pre-treated animals experienced activity of only 0.044% (IQR = 0.073%, n = 46; $p < 0.0001$). Lidocaine also decreased the acetic acid-induced activity from 0.14% (IQR = 0.10%, n = 47) to 0.090% with 50 mg/L lidocaine (IQR = 0.092%, n = 46; $p = 0.0014$).

Importantly, the lidocaine pre-treatment alone significantly decreased larval activity when used at 10 mg/L and 50 mg/L (Fig 5C). Therefore, the lidocaine-induced decrease of the acetic acid effect (Fig 5D), could also be attributed to a decrease in locomotive abilities, rather than antinociception. In adult zebrafish, exposure to lidocaine via immersion has been shown to decrease overall locomotion via a sedation mechanism that was suggested to be both peripheral and central (de Aberu et al., 2019). Similar results in rats (Weichman et al., 1981) suggest that lidocaine may indeed have a sedative effect in zebrafish, but this remains to be confirmed.

My findings from the buprenorphine exposure suggests a more direct link between acetic acid-induced activity and nociception because buprenorphine treatment alone did not alter locomotion (Fig 5A, $n = 43$; $p = 0.3019$), but it reduced the acetic-acid induced activity. This suggests that the effects of acetic acid are likely to be nociceptive in nature.

In order to verify the efficacy of acetic acid as a noxious stimulus in my experiments, I tested the actions of another noxious agent (AITC) to compare with the actions of acetic acid. Allyl isothiocyanate (AITC), or mustard oil, is a TRPA1 agonist that has been used to induce pain and nociception (Kwan et al., 2006), in animals including zebrafish (Ko et al., 2019; Taylor et al., 2017; Prober et al., 2008). I tested AITC in a similar fashion to acetic acid to determine if this different noxious stimulus could induce similar locomotor effects. Zebrafish larvae were exposed to AITC for thirty minutes. Their response to AITC was the most robust after 20 minutes of the initial exposure and the last ten minutes of the thirty-minute exposure was compared to baseline activity. I performed behavioural assays with 50 µM, 100 µM, and 200 µM AITC, because previous studies on zebrafish larvae used 100 µM AITC (Ko et al., 2019; Prober et al., 2008). Results of the behavioural assay were consistent with the literature and showed an increase in locomotor activity (Fig 6). Baseline activity levels prior to AITC treatment were 0.0094% (IQR = 0.022%, $n = 114$), whereas the mean % activity increased to 0.046% (IQR = 0.051%, $n = 42$; $p < 0.0001$), 0.051% (IQR = 0.079%, $n = 45$; $p < 0.0001$), and 0.039% (IQR = 0.032%, $n = 41$; $p < 0.0001$) when zebrafish larvae were exposed to 50 µM, 100 µM, and 200 µM of AITC, respectively. These findings support the notion that the effects of acetic acid are indeed nociceptive in nature as both it and AITC led to similar changes in locomotion.

To determine if the effects of AITC were indeed due to nociception, I exposed larval zebrafish to the MOR agonist buprenorphine prior to exposing them to 50 µM of AITC. As

previously found, buprenorphine on its own at 0.01 mg/L (n = 42; p = 0.6570) or 0.1 mg/L (n = 51; p = 0.5714) did not alter the activity levels compared with controls (Fig 7A). However, buprenorphine prevented the AITC-induced increase in activity (Fig 7B). Taken together, these findings strongly suggest that both acetic acid (0.001% v/v) and AITC (50 μM) act as nociceptive agents rather than directly altering swimming. Therefore, I used acetic acid as the noxious stimulus of choice in the remaining experiments.

3.2 The Efficacy of Cannabinoids and Terpenoids as Antinociceptive Agents

With the noxious stimulus identified and verified, I then proceeded to test if cannabinoids and terpenoids display antinociceptive effects. The compounds to test were the cannabinoids (CBD and THC) and seven terpenoids – five monoterpenes (α-pinene, β-myrcene, linalool, limonene, and phytol) and two sesquiterpenes (*trans*-nerolidol and (-)-caryophyllene oxide). These were chosen based on their relative abundance in the *Cannabis sativa* plant and their use in other studies (Pieracci et al., 2021; Iseppi et al., 2019).

3.2.1 Cannabidiol displays antinociceptive effects on the zebrafish model of nociception and pain

I first tested the effects of the cannabinoids, THC and CBD. Unlike many terpenoids, the biological effects of cannabinoids are relatively well-documented, with topics ranging from locomotion (Acevedo-Canabal et al., 2019), anxiety and psychosis (Dahlén et al., 2021; Rodriguez-Arias et al., 2013), and development (Amin et al., 2020; Sufian et al., 2019; Akhtar et al., 2013). With respect to pain and nociception, several studies have suggested that CBD and

THC exert antinociceptive effects (Haller et al., 2008; Cajanus et al., 2016; Kathman et al., 2006; Silva-Cardoso et al., 2021).

In these experiments, I pre-treated larvae with THC (0.1 mg/L, 0.5 mg/L, 1 mg/L, 5 mg/L, and 10 mg/L) or CBD (1 mg/L, 2.5 mg/L, 5 mg/L, and 10 mg/L) prior to acetic acid. Both cannabinoids were purchased as solutions dissolved in methanol. Therefore, the maximum concentration of THC and CBD that could be tested was based on the concentration of methanol used as the solvent. Initially, to minimise the toxicity of methanol in larval zebrafish, the maximum concentration of methanol was limited to 1% v/v. The effects of methanol on the locomotion of larval zebrafish were tested to validate this (Fig 8A). However, the exposure to 1% methanol led to a decrease in activity, from 0.012% in untreated control (IQR = 0.016%, n = 66) to 0.0070% (IQR = 0.011%, n = 57; p = 0.0248). On the other hand, 0.5%, methanol did not alter locomotion of the zebrafish larvae (Fig 8A, n = 102-105; p = 0.1630). Therefore, for all subsequent experiments using methanol as the vehicle, the concentration was limited to 0.5% v/v. This, meant that the highest concentration possible for either THC or CBD was 5 mg/L. The lowest concentrations were selected based on concentrations previously used at the lab (0.1 mg/L to 10 mg/L THC and 1 mg/L to 4 mg/L CBD).

CBD and THC were first tested for their effects on locomotion of 5 dpf zebrafish larvae (Fig 9). These results were then compared with their ability to prevent the locomotion induced by acetic acid or AITC, as shown in figures (Fig 10-12). Compared to the methanol control (0.016% activity, IQR = 0.045%, n = 32), THC (5 mg/L and 10 mg/L) increased activity of zebrafish larvae, to 0.069% (IQR = 0.097%, n = 31; p = 0.0007) and 0.086% (IQR = 0.086%, n = 32; p < 0.0001) (Fig 9A). Similarly, CBD increased activity in zebrafish larvae, from 0.023% in methanol control (IQR = 0.039, n = 58) to 0.072% at 1 mg/L (IQR = 0.072%, n = 48; p <

0.0001), 0.073% at 2.5 mg/L (IQR = 0.047%, n = 57; $p < 0.0001$), and 0.067% at 5 mg/L (IQR = 0.041%, n = 62; $p < 0.0001$) (Fig 9B). These results indicates that both THC and CBD increase locomotion in zebrafish larvae at 5 dpf. However, this increase in activity (Fig 9; around 0.07% to 0.08% for both CBD and THC) is not as large as acetic acid (Fig 4A; 0.13% activity for 0.001% acetic acid).

THC treatment did not alter the acetic acid-induced activity (Fig 10A, B) indicating that THC does not have antinociceptive effects on acetic-acid induced nociception.

On the other hand, administration of CBD prevented the acetic acid-induced activity at 2.5 mg/L, 5 mg/L, and 10 mg/L (Fig 10C, D). For treatment groups with 0.5% methanol as the vehicle, (2.5 mg/L and 5 mg/L) CBD reduced the activity from 0.12% in controls (IQR = 0.090%, n = 58) to 0.054% (IQR = 0.032%, n = 56; $p < 0.0001$) and 0.061% (IQR = 0.053%, n = 61; $p < 0.0001$) respectively (Fig 10C). In addition to the concentrations dissolved in 0.5% methanol, two concentrations dissolved in 1.0% methanol were also tested despite the higher methanol concentration to test for the effects of CBD at a wider range of concentrations. For groups with 1.0% methanol as the vehicle, CBD reduced the mean activity from 0.084% in controls (IQR = 0.087%, n = 38) to 0.056% (IQR = 0.038%, n = 40; $p = 0.0472$) and 0.047% (IQR = 0.032%, n = 40; $p = 0.0002$) when tested at 5 mg/L and 10 mg/L (Fig 10D). Between both sets of experiments, all tested concentrations of CBD at or above 2.5 mg/L decreased the acetic acid induced activity. This is in spite of the effects of CBD alone where it increased activity in the absence of any other treatment (Fig 9), making it less likely that this reduction in activity was an impact of CBD on zebrafish larval locomotion directly. Altogether, this suggests that the decrease in acetic acid-induced activity is potentially from antinociceptive effects of CBD.

A similar antinociceptive effect was observed when zebrafish larvae were pre-treated with CBD (1.0, 2.5, and 5.0 µg/mL) before exposure to AITC (Fig 11A). AITC was used at 50 µM, as this concentration was the lowest tested concentration but was robust enough to show a significant change in activity compared to baseline (Fig 6). 2.5 mg/L and 5.0 mg/L CBD prevented AITC-induced activity, decreasing the activity levels to 0.061% activity (IQR = 0.028%, n = 46; $p < 0.0001$) and 0.044% (IQR = 0.028%, n = 47; $p < 0.0001$) from the methanol + AITC control (0.26% activity, IQR = 0.21%, n = 47). These findings support the notion that CBD may have antinociceptive effects at concentrations at or above 2.5 mg/L.

Interestingly, unlike with acetic acid, THC was able to partially prevent the activity-increasing effects of AITC (Fig 11B). THC (at 1 mg/L) decreased the activity to 0.018% (IQR = 0.029%, n = 27; $p = 0.0205$) compared to control (0.065% activity, IQR = 0.13%, n = 35). However, the other concentrations of THC, 0.1 mg/L THC (n = 27; $p = 0.2700$) and 5 mg/L (n = 38; $p > 0.9999$), did not alter the AITC-induced activity. The lack of effect at the higher concentration of THC, in tandem with the increase in activity seen when THC is administered alone (Fig 9A), could indicate that at higher concentrations, the off-target effects of THC on locomotion override the reduction in activity from its antinociceptive effects. However, this is likely not the complete explanation, and it is also possible that the individual variance in experiments led to a false positive when there was no real antinociceptive effect. Careful consideration must be given to why acetic acid-induced activity fails to be prevented by THC administration and why only one concentration of THC reduced AITC-induced activity.

3.2.2 Combining CBD and THC led to a further decrease in the acetic acid-induced activity.

In the previous section, I noted the antinociceptive effects of CBD against both acetic acid and AITC, and the potential antinociceptive effects of THC against AITC. However, CBD and THC are rarely used alone in a practical setting. Therefore, it is important to study the effects when the two cannabinoids are used together.

When administered alone, 2.5 mg/L of CBD increased activity from 0.023% in methanol controls (IQR = 0.025%, n = 47) to 0.073% (IQR = 0.047%, n = 57; $p < 0.0001$) (Fig 9B). The administration of THC (0.5 mg/L, 2.5 mg/L and 5 mg/L) combined with 2.5 mg/L CBD resulted in activity levels that were similar to that of CBD alone ($p > 0.9999$) (Fig 9C), demonstrating that THC and CBD did not have an additive effect on the locomotive behaviour of zebrafish larvae.

Surprisingly, when zebrafish were pre-treated with a combination of THC (0.5, 1.0, and 2.5 mg/L) and 2.5 mg/L CBD and then exposed to acetic acid, their activity levels were significantly lower than with CBD pre-treatment and acetic acid exposure (Fig 12A). Specifically, compared to the acetic acid control (0.16% activity, IQR = 0.082%, n = 47), the CBD treatment decreased the activity to 0.054% (IQR = 0.032%, n = 56; $p < 0.0001$), but when co-treated with THC (0.5 mg/L, 1.0 mg/L and 2.5 mg/L) and CBD (2.5 mg/L), the activity level decreased further to 0.038% (IQR = 0.024%, n = 57; $p = 0.0158$), 0.037% (IQR = 0.029%, n = 58; $p = 0.0048$) and 0.037% (IQR = 0.027%, n = 57; $p = 0.0019$). For the tested combinations, no dose-dependent effect was observed, since the amount by which the activity decreased did not differ regardless of the concentration of THC added with 2.5 mg/L CBD. This result demonstrated that the antinociceptive effect of CBD was enhanced by the addition of THC, suggesting a potential synergistic effect between the two cannabinoids.

This enhancing effect of THC was not, however, observed when the same concentrations of THC were added to a lower dose of CBD such as 1 mg/L CBD (Fig 12B). Compared to the methanol + acetic acid control (0.079% activity, IQR = 0.099%, n = 57), 1 mg/L CBD did not change the activity level (0.083% activity, IQR = 0.072%, n = 47), as was previously observed in Figure 10C. The addition of THC did not alter this activity either (n = 55-58; $p > 0.9999$) (Fig 12B).

Figure 12 did not include any treatment groups with THC alone. Three different concentrations of THC were tested and adding each concentration as a control to compare would have drastically increased the number of experiments. Therefore, due to the fact that treatment with THC alone did not alter acetic acid-induced activity (Fig 10A, B) and because of limitations in time and resources, these experiments did not include the treatments of THC alone and instead compared the effects of a combination of CBD and THC to the effects of CBD alone.

Overall, these results indicate a possible synergistic effect between CBD at 2.5 mg/L and THC at 0.5, 1.0, and 2.5 mg/L, but not when CBD concentration was 1.0 mg/L.

3.2.3 Terpenoids show potential antinociceptive effects in acetic acid model of nociception in zebrafish

Next, I tested the antinociceptive effects of terpenoids. Terpenoids are polymers of isoprene units; two isoprene units make one terpene. Of the terpenoids, the most common ones are monoterpenes with two isoprene units and sesquiterpenes with three isoprene units (Iseppi et al., 2019). I tested seven different terpenoids found in *C. sativa* – α-pinene, β-myrcene, linalool, limonene, phytol, *trans*-nerolidol, and (-)-caryophyllene oxide.

As very little research has been done on terpenoids, it was difficult to determine the most appropriate range of concentrations to test. Based on several cell culture studies (Heblinski et al., 2020; Rahbar et al., 2019; Jansen et al., 2019), I tested concentrations ranging from 1.0 mg/L to 50 mg/L, dissolved in 0.25% v/v DMSO. DMSO was tested to ensure it did not have an impact

on the activity of zebrafish larvae (Fig 8C; $p = 0.8403$). The upper limit of the concentrations was initially calculated based on the solubility of the compounds in DMSO. However, in some cases (linalool, trans-nerolidol, and (-)-caryophyllene oxide), the higher concentrations led to the zebrafish larvae becoming completely immobile and unresponsive to mechanical stimulus and were thus removed from the experiments.

First, I tested the individual effects of these terpenoids on larval zebrafish locomotion (Fig 13). Compared to the DMSO control, 10 mg/L of α-pinene resulted in a lower level of activity ($p = 0.0066$) (Fig 13A). Application of β-myrcene, limonene, and phytol did not result in activity levels that were different from controls (Fig 13B-C, E). However, 10 mg/L and 25 mg/L of linalool decreased larval zebrafish activity from 0.036% in controls (IQR = 0.052%, n = 53) to 0.0049% activity (IQR = 0.010%, n = 41; $p < 0.0001$) and 0.00044% (IQR = 0.0060%, n = 50; $p < 0.0001$) respectively (Fig 13D).

Unlike the other terpenes, application of *trans*-nerolidol and (-)-caryophyllene oxide led to an increase in activity (Fig 13F, G). Compared to the DMSO control (0.030% activity, IQR = 0.056%, n = 77), *trans*-nerolidol increased activity levels to 0.083% at 5 mg/L (IQR = 0.070%, n = 80; $p < 0.0001$) and 0.080% at 10 mg/L (IQR = 0.057%, n = 46; $p < 0.0001$). For (-)-caryophyllene oxide, the activity increased from 0.024% in controls (IQR = 0.059%, n = 72) to 0.070% at 5 mg/L (IQR = 0.057, n = 77; $p < 0.0001$) and 0.048% at 10 mg/L (IQR = 0.052%, n = 75; $p= 0.0394$). Of the seven tested terpenoids, two – α-pinene and linalool – decreased locomotor activity and two – *trans*-nerolidol and (-)-caryophyllene oxide increased locomotor activity of zebrafish larvae.

Pre-treatment of larvae with limonene, β-myrcene, or phytol had no effect on the acetic acid-induced increase in activity (Fig 14B, C, E). Interestingly, α-pinene and linalool, both of

which decreased the activity of zebrafish by themselves, did not alter the acetic acid-induced activity either (Fig 14A, D). On the other hand, pre-treatment with *trans*-nerolidol or (-)-caryophyllene oxide prevented the effects of acetic acid (Fig 14F, G). At 5 mg/L and 10 mg/L, trans-nerolidol decreased the acetic acid-induced activity in a dose dependent manner, reducing it from 0.13% in acetic acid control (IQR = 0.098%, n = 83) to 0.10% (IQR = 0.092%, n = 80; $p = 0.0075$) and to 0.00027% (IQR = 0.0039%, n = 37; $p < 0.0001$) respectively. Caryophyllene oxide also displayed a dose dependent effect in reducing the acetic acid-induced activity: It decreased the activity from 0.19% in acetic acid control (IQR = 0.13, n = 80) to 0.075% (IQR = 0.070%, n = 78; $p < 0.0001$) and 0.018% (IQR = 0.027%, n = 65; $p < 0.0001$) when tested at 5 mg/L and 10 mg/L. These reductions in activity occurred despite the increase in activity both these terpenoids displayed when administered alone (Fig 13F, G). This suggests that the reduction in acetic acid-induced activity is not a result of the terpenoids' effect on locomotion. Therefore, these changes in acetic acid-induced activity may be attributed to antinociceptive effects.

3.2.4 Combining CBD and terpenoids improved the antinociceptive effects of (-)-caryophyllene oxide.

Some studies have suggested that synergistic effects between terpenoids and cannabinoids may exist with respect to the alleviation of pain (Heblinski et al., 2020; Sommano et al., 2020; Russo, 2019), despite a lack of empirical support. Since I had observed synergistic effects with THC and CBD, I decided to investigate the combined effects of terpenoids and cannabinoids (CBD) as well.

Since the terpenoids were dissolved in DMSO and CBD was dissolved in methanol and both solvents are potentially toxic at high concentrations, I performed an additional assay to ensure that the interaction between the two solvents did not cause changes in behaviour. When compared to methanol (0.5%) alone (n = 23), and DMSO (0.25%) alone (n = 22; p = 0.8533), the combination of 0.25% methanol and 0.125% DMSO (n = 48; p = 0.0994), or the combination of 0.5% methanol and 0.25% DMSO (n = 48; p > 0.9999) did not result in significant changes in activity levels (Fig 8D). Additionally, the combination did not alter the activity level of acetic acid when compared to either methanol or DMSO with acetic acid (Fig 8E) (n = 23-48; p = 0.8346). Based on these results, I used a combination of 0.5% methanol and 0.25% DMSO, which allowed for a higher solubility and therefore higher maximum concentrations of terpenoids and cannabinoids.

When co-administered with 2.5 mg/L CBD, five of the seven terpenoids (α-pinene, β-myrcene, linalool, limonene, and *trans*-nerolidol), did not show a significantly different response to acetic acid compared to 2.5 mg/L CBD alone + acetic acid (Fig 15A-D, F), indicating a lack of synergistic effects between these terpenoids and CBD. This also included *trans*-nerolidol, which demonstrates a similar level of reduction of acetic acid-induced activity as CBD when administered by itself (Fig 15F). The fact that either CBD or *trans*-nerolidol individually led to the same amount of reduction in activity as both combined is interesting. It could imply that they are working through the same or similar systems in which both are equally as effective. It could also indicate that the activity level cannot decrease any further from either the CBD or the *trans*-nerolidol levels. In either case, the results suggest that trans-nerolidol and CBD do not interact to alter the nociception of the zebrafish larvae.

On the other hand, phytol appears to counter the antinociceptive effects of CBD. Treatment with both phytol and CBD simultaneously eliminated the reduction of acetic acid-induced activity (Fig 15E). With 2.5 mg/L CBD alone, acetic acid-induced activity was decreased from 0.14% in vehicle + acetic acid control (IQR = 0.078%, n = 38) to 0.087% (IQR = 0.043%, n = 36; $p < 0.0001$). On the other hand, acetic acid-induced activity with both phytol and CBD was not significantly different from the acetic acid control, with activity levels of 0.13% (IQR = 0.056%, n = 40; $p = 0.1915$). A better understanding of the potential physiological targets of phytol and CBD is necessary to interpret this anti-synergy between these two phytochemicals.

(-)-Caryophyllene oxide alone decreased the acetic acid-induced activity to the same extent as CBD alone ($p > 0.9999$), to 0.058% activity (IQR = 0.092%, n = 47) from control (0.16% activity, IQR = 0.065%, n = 45) ($p = 0.0170$) (Fig 15 G). When administered simultaneously with CBD, the activity decreased even further to 0.031% (IQR = 0.045%, n = 48; $p = 0.0001$ from CBD and $p = 0.0170$ from (-)-caryophyllene oxide). This result could indicate an additive relationship between CBD and (-)-caryophyllene oxide, which could be explained if the pathway through which each phytochemical reduces the acetic acid-induced activity is different from each other and can run parallel for an additive effect. Further inquiry into the target of each compound, the dose ratio of the two, and the mechanism of action for the antinociception against acetic acid is required for a more complete understanding of the interaction between these two constituents of *C. sativa.*

3.3 Mechanism Behind the Antinociceptive Effects of Cannabinoids and Terpenoids

Now that I had tested the antinociceptive effects of cannabinoids and terpenoids, I wanted to identify the mechanism of action underlying the effects of the active compounds (CBD, *trans*-nerolidol, and (-)-caryophyllene oxide).

3.3.1 The antinociceptive effects of CBD, trans-nerolidol, and (-)-caryophyllene oxide likely do not work through opioid receptors.

I started an investigation into the mechanism by targeting the most common target of pain management: the opioid receptors. Specific blockers of opioid receptors were used to determine if the reduction of acetic acid-induced activity by the phytochemicals required the function of opioid receptors. Zebrafish larvae were independently treated with two MOR blockers, CTAP and naloxone, and one DOR blocker, naltrindole, to block mu opioid and delta opioid receptor activity. Larvae were then exposed to CBD, *trans*-nerolidol, or (-)-caryophyllene oxide, followed by acetic acid. If the opioid receptors were involved in the antinociceptive effects of CBD and the terpenoids then the opioid receptor blockers should prevent the effects of the terpenoids.

Before treating with acetic acid, I first tested the effects of the opioid blockers on locomotion. When administered alone, naloxone (0.1 mg/L) had no effect on locomotion (Fig 16A, n = 110; p = 0.0559). However, application of either CTAP (20 μM) or naltrindole (20 μM) resulted in a significant increase in activity (Fig 16A), from 0.0062% in controls (IQR = 0.012%, n = 145) to 0.013% in CTAP (IQR = 0.022%, n = 122; p = 0.0004) and 0.016% in naltrindole (IQR = 0.025%, n = 123; p < 0.0001). When administered with CBD, only naltrindole increased swimming activity (0.16% (IQR = 0.086, n = 48; p = 0.0356) from 0.13% with CBD alone (IQR = 0.045%, n = 41)) (Fig 16B). Neither of the MOR blockers altered CBD-induced activity (p =

0.9831 for CTAP and p = 0.0646 for naloxone) suggesting that the locomotor effects of CBD do not involve MOR. Similarly, the opioid receptor blockers did not alter the activity levels of *trans*-nerolidol or (-)-caryophyllene oxide exposed larvae (Fig 16C, D).

Next I tested the opioid receptor blockers with administration of acetic acid after CBD, *trans*-nerolidol, or (-)-caryophyllene oxide. My findings indicate that the opioid blockers did not alter the antinociceptive effects of CBD against acetic acid. As previously demonstrated, CBD reduced the acetic acid-induced activity from 0.18% activity (IQR = 0.12%, n = 45) to 0.091% (IQR = 0.048%, n = 44; $p < 0.0001$) (Fig 17A). However, the addition of opioid blockers did not affect this reduction at all ($p > 0.9999$) (Fig 17A), suggesting that the antinociceptive effects of CBD does not work through either MOR or DOR. Similar results were seen with both *trans*-nerolidol and (-)-caryophyllene oxide (Fig 17B, C). Compared to the DMSO + acetic acid control group (0.12% activity, IQR = 0.054%, n = 44), *trans*-nerolidol + acetic acid group showed a lower level of activity, at 0.043% (IQR = 0.055%, n = 44; $p < 0.0001$), and this level remained the same even when pre-treated with the opioid blockers (p = 0.0735 for CTAP, p = 0.2135 for naloxone, and $p > 0.9999$ for naltrindole). A similar reduction of acetic acid-induced activity is also seen with (-)-caryophyllene oxide (from 0.088% (IQR = 0.041%) to 0.020% (IQR = 0.065%), n = 37-38; $p < 0.0001$), which was also unaffected by the blocking of opioid receptors ($p > 0.9999$).

The results of these experiments suggest that neither MOR nor DOR were individually sufficient to facilitate the antinociceptive effects of CBD, *trans*-nerolidol, and (-)-caryophyllene oxide because blocking either MOR or DOR was unable to prevent the reduction of acetic acid-induced activity of CBD.

3.3.2 Acetic acid acts as a noxious stimulus via its interactions with TRP channels

Another target of cannabinoids and terpenoids that is intrinsically linked to pain and nociception is the transient receptor potential (TRP) channels. There are numerous variations of these channels, but they primarily function as non-specific cation channels that initiate signals in response to their own specialised stimuli, such as mechanical, thermal, or chemical stimuli (Caterina et al., 1999; Moqrich et al., 2005; Patapoutian, Tate, & Woolf, 2009; Dubin & Patapoutian, 2010). Many of these channels also respond to the extremes of these stimuli in a painful manner and initiate the propagation of signals along the pain pathway (Yam et al., 2018).

For a holistic understanding of the interaction between various TRP channels and the nociceptive response to acetic acid, a general TRP channel blocker, AMG-9090, was used to pharmacologically prevent responses through the channels. AMG-9090 is a broad-spectrum blocker that acts on TRPA1, TRPV1, TRPV3, TRPV4, and TRPM8 (Klionsky et al., 2007). AMG-9090 at 10, 50, and 100 μM over a 30-minute duration prevented the increase in activity that was induced by acetic acid (Fig 18A). Pre-treatment with AMG-9090 decreased the acetic acid-induced activity from 0.22% in DMSO + acetic acid control (IQR = 0.15%, n = 48) to 0.0070% at 10 μM (IQR = 0.0098%, n = 41; $p < 0.0001$), to 0.015% at 50 μM (IQR = 0.039%, n = 42; $p < 0.0001$), and to 0.033% at 100 μM (IQR = 0.047%, n = 47; $p < 0.0001$). These findings suggested that TRP channels might be involved in acetic acid-induced activity. However, treatment with AMG-9090 alone led to drastic changes in activity compared with controls (Fig 18B). For example, the mean % activity in the first ten minutes of exposure to AMG-9090 was 0.17% (IQR = 0.090%, n = 47; $p < 0.0001$), which is nearly five times higher than the DMSO control in the first ten minutes (0.035%, IQR = 0.05%, n = 47). This increased activity level was followed by a gradual decrease in activity over the thirty-minute duration of the exposure,

decreasing to 0.11% activity (IQR = 0.12%, n = 47; p = 0.0184) between 10 to 20 minutes and to 0.032% activity (IQR = 0.058%, n = 47; p < 0.0001) between 20 to 30 minutes after exposure. This data suggested that AMG-9090 may have multiple effects on locomotion. Therefore, the decrease in acetic acid-induced activity with AMG-9090 could also be explained by an inhibition of locomotion rather than a blocking of nociception.

To further investigate the effects of TRP channels, I used specific blockers of TRPV1 (A-784168) and TRPA1 (HC-030031) rather than the broad-spectrum inhibitor, AMG-9090. Treatment with 1 μM of either A-784168 or HC-030031 alone did not cause any changes in activity compared to controls (p = 0.0670 and p = 0.6187, respectively). However, higher concentrations of these blockers led to an increase in movement (Fig 19 A, B) which persisted throughout the thirty-minute exposure period (Fig 19C-D, n = 42-47; p = 0.4129 for A-784168 and p = 0.5049 for HC-030031). This meant that any decreases in acetic acid-induced activity observed with pre-treatment of either A-784168 or HC-030031 are likely not due to their direct effects on locomotion and is likely to be due to the prevention of nociception.

My data shows that the TRPV1 blocker A-784168 at 10 μM (Fig 20A) and the TRPA1 blocker HC-030031 at 10 μM and 50 μM (Fig 21A) both prevented the effects of acetic acid. For example, when compared to the DMSO controls, treatment with 10 μM A-784168 reduced larval activity from 0.11% (IQR = 0.13%, n = 46) to 0.023% (IQR = 0.027%, n = 44; p < 0.0001). HC-030031 reduced activity from 0.18% (IQR = 0.13%, n = 47) to 0.12% at 10 μM (IQR = 0.073%, n = 47; p = 0.0329) and to 0.046% at 50 μM (IQR = 0.055%, n = 45; p < 0.0001). These results show that blocking either TRPV1 or TRPA1 channels was sufficient in reducing the effects of acetic acid, suggesting that nociception from acetic acid treatment involves both TRPV1 and TRPA1 channels.

3.3.3 The mechanism of antinociceptive effect of cannabidiol involves the TRPV1 and TRPA1 channels in the zebrafish model of nociception.

Next, I explored the connection between TRP channels and CBD. CBD is capable of acting as a partial agonist at certain TRP channels (Crivelaro do Nascimento et al., 2020; De Petrocellis et al., 2011; Maggi et al., 2022), and such interactions could be involved in the mechanism underlying the antinociceptive effects of CBD.

With the current experimental design, however, it is difficult to separate the TRP channel involvement in antinociceptive effects of CBD, from its involvement in the nociceptive effect of acetic acid. Because blocking TRP channels is itself antinociceptive and prevents the acetic acid-induced activity, it would be difficult to distinguish the effects of the blockers from those of CBD. Therefore, I decided to focus on CBD-induced activity.

CBD, when administered alone, led to a robust and consistent increase in activity (Fig 9B). Application of both TRPV1 and TRPA1 blockers were able to prevent the CBD-induced activity (Fig 20B and 21B). A-784168, the TRPV1 blocker, at 10 μM decreased the CBD-induced activity to 0.041% (IQR= 0.026%, n = 39; $p = 0.0003$) compared to the DMSO + CBD control group (0.094% activity, IQR = 0.084%, n = 43). HC-030031, the TRPA1 blocker, decreased activity levels to 0.059% (IQR = 0.026%, n = 43; $p < 0.0001$) and 0.031% when tested at 10 μM and 50 μM (IQR = 0.020%, n = 46; $p < 0.0001$). This suggests that CBD may increase activity in zebrafish larvae by activating TRPV1 and TRPA1 channels. Such involvement may also indicate an involvement of these TRP channels in the antinociceptive effects of CBD, but further research and a different methodology must be used to draw a more complete conclusion.

3.3.4 TRPV1 and TRPA1 channels are also involved in the antinociceptive effects of the terpenoids.

Next, I investigated whether the effects of *trans*-nerolidol and (-)-caryophyllene oxide occurred via TRP channels. My results show that 10 μM A-784168 (TRPV1 blocker) prevented the *trans*-nerolidol-induced activity (Fig 20C) and the (-)-caryophyllene oxide-induced activity (Fig 20D). The *trans*-nerolidol-induced activity decreased from 0.072% activity in DMSO + TN control (IQR = 0.049%, n = 45) to 0.039% with 1 μM of the TRPV1 blocker (IQR = 0.041%, n = 45; $p = 0.0124$) and 0.0084% with 10 μM of the blocker (IQR = 0.0062%, n = 41; $p < 0.0001$). The TRPV1 blocker also decreased the (-)-caryophyllene oxide-induced activity. Compared to DMSO + CO control (0.040% activity, IQR = 0.063%, n = 44), (-)-caryophyllene oxide-induced activity was decreased to 0.010% with 10μM of the TRPV1 blocker (IQR = 0.019%, n = 41; $p < 0.0001$). These decreases in activity were especially convincing as A-784168, when administered alone at 10 μM, showed a higher level of activity than the DMSO control (n = 43; $p < 0.0001$) (Fig 19A). This suggests that TRPV1 channels are involved in the ability of *trans*-nerolidol and (-)-caryophyllene oxide to alter zebrafish larval locomotion and could potentially be involved in their antinociceptive effects.

When zebrafish larvae were exposed to 10 μM HC-030031, a TRPA1 blocker, with either *trans*-nerolidol or (-)-caryophyllene oxide, the activity level increased compared to the control (Fig 21D, E). DMSO + *trans*-nerolidol treatment showed an activity level of 0.046% (IQR = 0.080%, n = 42), and with 10 μM of HC-030031, this activity increased to 0.085% (IQR = 0.052%, n = 46; $p = 0.0123$). Similarly, (-)-caryophyllene oxide + DMSO treatment group showed an activity level of 0.055% (IQR = 0.081%, n = 46), which was increased to 0.091% with 10 μM of the TRPA1 blocker (IQR = 0.054%, n = 46; $p = 0.0111$). This increase was

unexpected: if the TRPA1 blocker was able to prevent the *trans*-nerolidol- and (-)-caryophyllene oxide-induced activities, the activity level should have decreased, instead of increased. This anomaly is most likely due to the effects of 10 μM HC-030031 itself on zebrafish larval locomotion, as the blocker was shown to increase the activity of zebrafish larvae at that concentration (Fig 19B, $n = 47$; $p < 0.0001$). Therefore, at a concentration of 10 μM, the TRPA1 blocker was unable to prevent the effects of *trans*-nerolidol and (-)-caryophyllene oxide on zebrafish larval locomotion.

When TRPA1 channels were blocked using a higher concentration of HC-030031 such as 50 μM, however, the application of *trans*-nerolidol and (-)-caryophyllene oxide led to a decrease in activity (Fig 21F, G). Compared to the DMSO + *trans*-nerolidol group (0.028% activity, IQR = 0.046%, $n = 48$), 50 μM HC-030031 decreased *trans*-nerolidol-induced activity to 0.0072% (Fig 21F, IQR = 0.0082%, $n = 46$; $p < 0.0001$). (-)-Caryophyllene oxide also showed a similar pattern at 50 μM HC-030031, with the activity decreasing from 0.017% in DMSO + (-)-caryophyllene oxide treatment (IQR = 0.026, $n = 47$) to 0.011% with the blocker (Fig 19G, IQR = 0.016%, $n = 47$; $p = 0.0177$). This was in spite of the increase in activity observed with administration of TRPA1 blocker at 50 μM (Fig 19B, $n = 47$; $p = 0.0314$), eliminating the possibility that the decrease in activity seen with the terpenoids + HC-030031 was a locomotor effect rather than a nociceptive effect.

Taken together, the results show that HC-030031 increases locomotion in zebrafish larvae at both 10 μM and 50 μM, but at 50 μM, HC-030031 blocks the interaction between the terpenoids and TRPA1 channels enough to reduce activity to levels that are comparable to DMSO control (0.0069% in DMSO control, as shown in Fig 19B and 0.0072% in 50 μM HC-030031 + *trans*-nerolidol or 0.011% in 50 μM HC-030031 + (-)-caryophyllene oxide).

These results suggest that both of these terpenoids influence signalling pathways that include TRPA1 and TRPV1 channels, suggesting that both TRPA1 and TRPV1 channels may be involved in the antinociceptive effects of *trans*-nerolidol and (-)-caryophyllene oxide. However, as a direct interaction between the antinociceptive effects of these terpenoids and the TRP channel blockers could not be tested due to the TRP channel blockers themselves being antinociceptive in nature, further investigation into the mechanism of action behind the antinociceptive effects of *trans*-nerolidol and (-)-caryophyllene oxide is needed to establish a proper connection between these terpenoids and TRP channels.

3.3.5 CBD-induced activity in zebrafish larvae is nociceptive in nature

Through the use of specific TRP channel blockers, I determined that CBD-induced activity involves TRPV1 and TRPA1 channels. However, these channels are also responsible for the acetic acid- and AITC-induced activities. As the increase in activity observed with acetic acid (Fig 4) and AITC (Fig 6) was used to measure the nociceptive nature of these noxious stimuli, it was important to understand if the increase in activity seen with the administration of CBD alone was also nociceptive in nature. To test this, buprenorphine, a synthetic MOR agonist, was placed in the wells of zebrafish larvae 30 minutes before the administration of CBD. Buprenorphine, as previously discussed, does not affect the locomotion of zebrafish larvae at the tested concentrations of 0.01 mg/L and 0.1 mg/L (Fig 5A). However, at 0.1 mg/L, buprenorphine + CBD treatment group showed a much lower level of activity (0.00016% activity, IQR = 0.00064%, n = 36; $p < 0.0001$) than the methanol + CBD control group (0.039% activity, IQR = 0.083%, n = 48) (Fig 22). Buprenorphine completely eliminated CBD-induced activity at 0.1 mg/L despite not affecting locomotion of zebrafish larvae directly, indicating that the increase in

activity seen with CBD may be a nociceptive response from zebrafish larvae. This means that CBD, at 2.5 mg/L, could also be noxious to the larvae.

3.3.6. Antinociceptive effect of CBD does not involve the endocannabinoid receptors CB_2 and CB_1

The final receptor system to be tested was the endocannabinoid receptors type 1 and type 2 (CB_1R and CB_2R). CB_1 and CB_2 are G-protein coupled receptors that function to regulate synaptic transmission through the inhibition of cyclic AMP signaling (Ryberg et al., 2007; Demuth & Molleman, 2006; Zou and Kumar, 2018). Their regulatory effects can be highly important in multiple systems, including the pain pathway (Anand et al., 2009; Johnson and Bradshaw, 2021).

To determine the role of these receptors in the antinociceptive effects of CBD, I used specific CB_1 and CB_2 blockers (AM251 and AM630, respectively). AM251 was used at 0.1 or 1 μM and AM630 was used at 0.5 or 5 μM. These concentrations were selected based on previous studies (in adult zebrafish; 0.1 mg/L AM251 and 1 mg/L AM630, which are equivalent to 0.18 μM and 1.8 μM) (Tran et al., 2016) and similar concentrations have been shown to be effective in embryonic and larval zebrafish (Sufian et al., 2019). After exposing the zebrafish larvae to either of these blockers for 30 minutes, CBD was then administered for 10 minutes. This was followed by acetic acid for another 10 minutes.

Neither AM251 nor AM630 affected zebrafish larval locomotion and neither altered the effects of CBD on zebrafish locomotion (Fig 23). For instance, administration of AM251 at 0.1 μM (0.0061% activity, IQR = 0.015%, n = 25; $p > 0.9999$) or 1 μM (0.00048% activity, IQR = 0.0046%, n = 22; $p > 0.9999$) did not alter the activity level from control (0.00089% activity,

IQR = 0.011%, n = 23) (Fig 23A). In comparison, the addition of CBD increased the activity to 0.034% activity (IQR = 0.035%, n = 29; p = 0.0001). This increased activity level, however, also did not change with the pre-treatment of either 0.1 μM or 1 μM AM251 (p > 0.9999). A similar pattern was observed with AM630 treatments, wherein the control activity of 0.00089% (IQR = 0.011%, n = 23) did not change with the administration of either 0.5 μM or 5 μM AM630 (p > 0.9999) (Fig 23B). The CBD-induced activity (0.034%, IQR = 0.035%, n = 29) was similarly unaffected by the pre-treatment of AM630 (p > 0.9999).

Additionally, neither AM251 nor AM630 had an impact on the acetic acid-induced activity (Fig 24). Compared to the acetic acid + vehicle control group (0.071% activity, IQR = 0.045%, n = 26), both AM251 + acetic acid and AM630 + acetic acid groups showed the same level of activity (p > 0.9999), regardless of the concentration of the blocker. This meant that the nociceptive effects of acetic acid did not involve the endocannabinoid receptors. It also meant that it was possible to directly test whether blocking the endocannabinoid receptors prevented the antinociceptive effects of CBD because the blockers themselves were not antinociceptive which made it possible to see the changes to the acetic acid-induced activity observed due to the antinociceptive effects of CBD.

When compared to CBD + acetic acid treatment (0.030% activity, IQR = 0.015%, n = 24), pre-treating with AM251 (n = 27 for 0.1 μM and n = 28 for 1 μM; p > 0.9999 for both) or AM630 (n = 28 for both; p = 0.1353 for 0.5 μM and p = 0.4603 for 5 μM) did not have a significant effect on larval activity (Fig 24). The activity levels remained significantly lower than the acetic acid treatment at either of the tested concentrations for both AM251 (p < 0.0001 for both concentration) and AM630 (p = 0.0114 for 5 μM).

The only exception was 0.5 μM AM630. At this concentration, the acetic acid-induced activity was not lower than the acetic acid control: 0.5 μM AM630 + 2.5 mg/L CBD + 0.001% acetic acid treatment did not have a significantly different level of activity from the vehicle + acetic acid group ($p = 0.0548$). However, the activity was still significantly lower than the 0.5 μM AM630 + 0.5% methanol + 0.001% acetic acid treatment (without CBD) ($p = 0.0318$) and not significantly different from the 0.25% DMSO + 2.5 mg/L CBD + 0.001% acetic acid group (without AM630) ($p = 0.1353$). This indicates that the CBD still displayed its antinociceptive effects, but not to the same extent as without AM630.

The fact that blocking CB_2 receptors with 0.5 μM of AM630 decreased the potency of the effect could indicate that the partial antagonism at CB_2 receptor by CBD is important in the antinociceptive effect of CBD, but the lack of consistency between the tested concentrations makes it difficult to draw conclusions based on these results. Additionally, the slight degree in which the activity level was impacted makes the interpretation of the results difficult as this supposed difference may not be biologically significant. There is also the possibility that the statistical insignificance in this case ($p = 0.0548$) being so close to the threshold of $p = 0.05$ was an issue of variance in the animals rather than a true difference. Further exploration is required to better understand the interaction between CB_2 receptors and the antinociceptive effects of CBD.

Chapter 4: Discussion

In this thesis, my aim was to identify potential antinociceptive effects of different cannabinoids and terpenoids found in the *C. sativa* plant. To achieve this, I used a larval zebrafish model of nociception induced by exposure to acetic acid. My key findings are as follows:

1. Acetic acid leads to nociception in zebrafish larvae that is robust enough for the study of antinociceptive agents.
2. CBD has an antinociceptive effect against acetic acid and AITC, and THC has a potential antinociceptive effect against AITC.
3. THC, when administered with CBD, enhances the antinociceptive effect of CBD.
4. *Trans*-nerolidol and (-)-caryophyllene oxide have antinociceptive effects against acetic acid.
5. CBD and (-)-caryophyllene oxide have synergistic effects on their antinociception.
6. The antinociceptive effects of CBD, *trans*-nerolidol, and (-)-caryophyllene oxide are not solely dependent on either MOR or DOR individually.
7. Nociception through acetic acid administration involves the activity of TRPV1 and TRPA1 channels.
8. The antinociceptive effect of CBD involves TRPV1 and TRPA1 channels.
9. The antinociceptive effects of *trans*-nerolidol and (-)-caryophyllene oxide involve TRPV1 and TRPA1 channels.
10. The antinociceptive effects of CBD does not involve the endocannabinoid receptors in my assays.

4.1 Robustness of the Zebrafish Model of Nociception and Pain

Zebrafish have been used as a model system for nociceptive studies using acetic acid as a noxious stimulus. For instance, Costa and colleagues (2019) demonstrated that injection of 5.0% v/v acetic acid into adult zebrafish led to abdominal constriction, and the effects were exacerbated with the simultaneous injection of the opioid antagonist naloxone (Costa et al, 2019), suggesting a nociceptive component in the observed behaviour. In a similar fashion, injection of 2.5 to 10% acetic acid into the lips of adult zebrafish led to a reduction in swimming distance and speed that was prevented by morphine injection but returned with the injection of naloxone (Taylor et al., 2017).

Larval zebrafish have also been used as a model for nociception in several studies (Malafoglia et al., 2013). For instance, Lopez-Luna and colleagues (2017) tested the nociceptive effects of acetic acid and citric acid on zebrafish larvae at 5 dpf. They found that a 10-minute exposure to 0.1% and 0.25% v/v acetic acid resulted in a significant reduction in the time spent being active whereas exposure to 0.01% v/v acetic acid increased the activity time. The reduction in activity time following exposure to 0.1% acetic acid was prevented with pre-exposure to analgesics, such as morphine, aspirin, and lidocaine. Steenbergen and Bardine (2014) demonstrated that a three-minute exposure to 0.0025% to 0.025% v/v acetic acid led to a dose-dependent increase in zebrafish larval activity. This change in activity was prevented with pre-treatment of the analgesic buprenorphine (0.1 mg/L). These studies show a bimodal effect of acetic acid where lower concentrations (0.0025% - 0.025%) resulted in an increase in activity and higher concentrations (0.1% - 0.25%) resulted in a decrease in activity, and they suggest that these changes in activity are nociceptive in nature.

Findings from my own experiments were largely in agreement with the literature. For instance, treatment of zebrafish larva with 0.001% to 0.01% acetic acid resulted in robust and reliable increases in activity (Fig 4A). This increase in activity was determined to be nociceptive in nature through the use of a known analgesic (buprenorphine, a synthetic MOR agonist), and through the comparison of behaviour with another commonly used nociceptive agent (AITC, a TRPA1 agonist). Pre-treatment with buprenorphine eliminated the increase in activity from 0.001% acetic acid (Fig 5B) whereas buprenorphine by itself did not significantly change the activity level in zebrafish larvae (Fig 5A). The nociceptive effect of acetic acid was further validated by comparison with another noxious stimulus, AITC. AITC is a TRPA1 agonist that has also been used as noxious agents in some studies of nociception (Ko et al., 2019; Taylor et al., 2017; Prober et al., 2008). The administration of AITC at 50 – 200 μM also led to a robust increase in activity in zebrafish larvae (Fig 6) that was prevented by buprenorphine (Fig 7B). Taken together, the similar effects of a known nociceptive agent (acetic acid) and a different noxious stimulus (AITC) supports the idea that acetic acid-induced activity is a nociceptive response by zebrafish larvae. Thus, my results show that acetic acid is a highly robust nociceptive agent. Furthermore, the increase in activity induced by acetic acid provides a useful whole-animal readout for observing effects of antinociceptive compounds found within the cannabis plant. Lastly, zebrafish larvae as a model of nociception provides an additional benefit of ethical favourability over more complex species, such as rodents or other mammals, or even adult fish of the same species for assays involving nociception and pain.

4.2 Cannabidiol, Tetrahydrocannabinol, trans-Nerolidol, and (-)-Caryophyllene Oxide Demonstrated Antinociceptive Effects in Zebrafish Larval Model of Nociception.

4.2.1 Cannabinoids demonstrate antinociceptive effects against acetic acid and AITC.

In this thesis, I tested for the antinociceptive effects of CBD and THC and identified CBD and THC as potential antinociceptive agents. Only CBD (2.5 – 10 mg/L) was effective in preventing the nociceptive effects of acetic acid (Fig 10C, D). In contrast, pre-treatment with 0.1 – 10 mg/L THC did not alter the acetic acid-induced activity (Fig 10A, B). With regard to AITC, both CBD and THC had some antinociceptive effect against it. CBD was the more robust of the two cannabinoids, with significant decreases in activity at both 2.5 and 5.0 mg/L (Fig 11A). THC was also able to decrease the AITC-induced activity, but only at 1.0 mg/L (Fig 11B). Thus, my results suggest that CBD is an effective antinociceptive agent, while THC, when applied alone, is not. Interestingly, however, THC does act synergistically with CBD to improve the antinociceptive effects of CBD at 2.5 mg/L (Fig 12A).

Much research has been done on the antinociceptive and analgesic effects of cannabinoids in both animal and human models: CBD has been shown to reduce allodynia and hyperalgesia in rat models of neuropathic pain (Silva-Cardoso et al., 2021), increase the threshold of nociceptive response in mouse models of Parkinson's (Crivelaro do Nascimento et al., 2020), and protect mice from chemotherapy-induced mechanical sensitivities (Foss et al., 2021), as well as improve the quality of life in adult chronic pain patients (Capano et al., 2020). The effects of CBD on both acetic acid and AITC observed in this thesis are consistent with other studies on zebrafish (Ellis et al., 2018), as well as on mice (Crivelaro do Nascimento et al., 2020; Foss et al., 2021; Kowalski et al., 2020; Silva et al., 2017) and rats (Kowalski et al., 2020).

However, the actions of CBD are likely to be complex and multi-faceted, and further investigation into its function in the context of pain management is needed. The increase in locomotor activity induced by CBD and THC add to the complexity when interpreting the results as well (Fig 9). These effects could be due to a direct action of CBD on TRP channels (Avila et al., 2020). THC also induced a significant increase in activity when administered alone (Fig 9A) but it did not prevent the activity induced by acetic acid (Fig 10A, B). If THC and CBD both increased activity of zebrafish larvae through the same TRP channels, one would expect the antinociceptive effects to also be similar. However, because the antinociceptive effects between the two compounds were different, it is probable that THC and CBD act through different pathways or their effects on TRP channels in zebrafish are different. Therefore, understanding the difference in how these cannabinoids interact with TRP channels or other biological targets, as well as how the two noxious stimuli mechanistically differ from one another, will be important in deciphering what causes the difference in the effects I observed.

4.2.2 Trans-nerolidol and (-)-caryophyllene oxide exhibit antinociceptive effects against acetic acid.

Terpenoids are phytochemicals found in the *C. sativa* plant that show various potential therapeutic applications (Pellati et al., 2018; Tsuchiya, 2017; Iseppi et al., 2019; Djilani et al., 2012). Compared to cannabinoids, however, the research on these compounds is more limited. I tested seven of the most abundant terpenoids found in the *C. sativa* plant: α-pinene, β-myrcene, linalool, limonene, phytol, *trans*-nerolidol, and (-)-caryophyllene oxide. Of these, *trans*-nerolidol and (-)-caryophyllene oxide were able to prevent acetic acid-induced nociception in zebrafish larvae (Fig 14F, G). The other terpenoids – α-pinene, β-myrcene, linalool, limonene, and phytol

– had no antinociceptive effects on my preparation (Fig 14A-E). However, evidence exists to suggest that these terpenoids display antinociceptive effects in other systems: β-myrcene reduced nociception in mice against thermal and chemical nociception as tested via hot plate latency test and acetic acid writhing test (Rao et al., 1990). Thermal nociception tested via thermal tail-flick tests on mice was reduced with intraperitoneal injections of α-pinene (Him et al., 2008). Linalool, when injected subcutaneously in rats, increased the withdrawal latency in a thermal paw withdrawal test (Peana et al., 2004). Limonene was tested in spinal nerve injury model of chronic pain in rats and showed improvements in both cold hyperalgesia and von Frey mechanical nociception (Piccinelli et al., 2015). Phytol showed antinociception against both thermal nociception, tested via hot plate method, and chemical nociception, tested via formalin and acetic acid tests, in mice (Santos et al., 2013). Lastly, THC increased the force threshold of the paw pressure test in both arthritic and nonarthritic mice (Cox and Welch, 2004).

Antinociceptive effects of *trans*-nerolidol and (-)-caryophyllene oxide have also been documented in various studies, mostly also in mammalian models. In mice, nerolidol was shown to reduce acetic acid-induced abdominal constrictions as well as reduce the licking time for formalin test (Fonsêca et al., 2016). However, this antinociceptive effect was not observed in a hot plate test (Fonsêca et al., 2016), indicating that nerolidol can reduce chemical nociception while not being as effective against thermal nociception in rodents. Caryophyllene oxide was also shown to increase latency in hot plate tests and decrease acetic acid-induced writhing in mice (Chavan, Wakte, & Shinde, 2010). Evidence for antinociceptive effects of terpenoids is often provided using mammalian models. While these studies show the antinociceptive effects of all seven terpenoids that I have tested, the fact that only two of these terpenoids were shown to display antinociceptive effects in my assays provide an intriguing insight into potential

differences between fish and rodent models of nociception and the differences in the noxious stimulus against which these compounds were tested. This inconsistency may also suggest my assays provide a model that is capable of a stricter discrimination in determining an antinociceptive effect of a given compound. This distinction, in turn, provides an interesting starting point for a future inquiry into what property makes these two terpenes different or perhaps even more effective than the others. It is also noteworthy that some studies involving terpenoids have been done using essential oils containing mixtures of various compounds that included the terpenoids in question (Baron, 2018), making it difficult to understand the role of individual terpenoids.

4.3 Mechanism of Action Underlying the Antinociceptive Effects of Cannabinoids and Terpenoids

4.3.1 Mechanism of the nociceptive effects of acetic acid

To better understand the antinociceptive effects of cannabinoids and terpenoids, it is important to understand the mechanism through which acetic acid causes nociception in zebrafish larvae. Previous studies have established a link between acetic acid-induced nociception and various receptor systems within the nervous system. For example, the ability of acetic acid to activate TRPV1 channels was demonstrated using transgenic Trpv1-Cre knock-out mice (Spencer et al., 2018) and through pharmacological interventions - TRPV1 specific antagonist, capsazepine - on wildtype mice (Yoshiyama et al., 2010). Acetic acid was also demonstrated to activate TRPA1 channels in mice (Freitas et al., 2021), and in HEK cells with either rat TRPA1 (Wang et al., 2011) or human TRPA1 (Zhao et al., 2020). Additionally, TRPM8 was shown to be modulated by acetic acid in both rats (Aizawa et al., 2018) and mice

(Liu et al., 2013). Receptor systems beyond TRP channels were also shown to be involved. Acetic acid-induced nociception can be modulated by $GABA_A$ receptor in HEK cells (Lewter et al., 2017) and in mice (Brewer et al., 2018). Agonism at the opioid receptors also reduce the nociceptive effects of acetic acid in zebrafish (Costa et al., 2023).

Consistent with these findings, I determined that nociception induced by application of acetic acid involves both TRPA1 and TRPV1 channels in zebrafish larvae using pharmacological tools. Furthermore, my data suggest that acetic acid causes nociception through mechanisms that do not rely solely on individual endocannabinoid receptors, CB_1 and CB_2, nor individually on the μ- and δ-opioid receptors (MOR and DOR), because blocking any of these receptors did not lead to changes in acetic acid-induced activity. I have also shown that opioid receptor agonists and general sodium channel blockers like buprenorphine and lidocaine can also modulate the acetic acid-induced nociception. While this thesis mainly focused on its capacity to activate TRP channels, a more thorough exploration of the other targets and interactions could help isolate the mechanism of analgesics and antinociceptive agents that work against acetic acid-induced nociception.

4.3.2 Mechanism of the antinociceptive effects of CBD

Several studies have examined the antinociceptive effects of CBD (Silva-Cardoso et al., 2021; Crivelaro do Nascimento et al., 2020; Foss et al., 2021; Cox and Welch, 2004) and terpenoids (Burcu et al., 2016; Rao et al., 1990; Him et al., 2008; Peana et al., 2004; Piccinelli et al., 2015; Santos et al., 2013; Cox and Welch, 2004), but their mechanism(s) of action remain largely unknown.

I began my investigation into the mechanism underlying the antinociceptive effects of CBD by testing if opioid receptors were involved. Application of MOR and DOR blockers – CTAP, naloxone, and naltrindole – provide no evidence that MOR and DOR were involved in the CBD-induced antinociceptive effects. Additionally, blocking CB1 and CB2 receptors did not prevent the effects of CBD, thereby failing to support that MOR, DOR, and the canonical cannabinoid receptors are involved in the effects of CBD. A study on tissues from rat cerebral cortex investigated the interaction between cannabinoids and opioid receptors MOR and DOR through radioligand binding assays and concluded that CBD and THC acted as negative allosteric modulators at the site, leading to earlier dissociation of agonists from the receptors (Kathmann et al., 2006). Another study in mice that focused on opioid receptors and cannabinoids concluded that cannabinoids enhanced the antinociceptive effects of opioids (Welch and Stevens, 1992), but little is reported on the independent effect that CBD may have on opioid receptors. My findings are consistent with these previous studies and suggest a lack of direct connection between the antinociceptive effects of CBD and the opioid receptors.

Another well-documented target of CBD is the endocannabinoid receptors. CBD is a known antagonist of the CB_1 and CB_2 receptors – for example, CBD acts against cannabinoid receptor agonists on Chinese hamster ovary cells with both mouse CB_1 and human CB_2 receptors (Thomas et al., 2007). A study by Silva and colleagues (2017) demonstrated antinociceptive effects of CBD against an acetic acid-induced writhing behaviour in rats that was independent of endocannabinoid receptors. The results of this thesis also support this idea, as the blocking of CB_1 receptor with AM251 did not prevent the antinociceptive effects of CBD. However, blocking CB_2 with 0.5 μM AM630 somewhat prevented the antinociceptive effect of CBD. A higher concentration of AM630 (5 μM) did not elicit the same result, and the activity level of the

AM630 + CBD + acetic acid treatment did not differ from either acetic acid + control treatment or CBD + acetic acid treatment groups. Due to these abnormalities, a more detailed understanding of the mechanism or site of action for CBD and AM630 at CB_2 receptor may be helpful in determining the mechanism by which the interaction between CBD and CB_2 may lead to a potential antinociceptive effect.

These findings suggest the mechanism behind the antinociceptive effects of CBD likely lie outside of endocannabinoid system. This aligns well with how CBD displays a significantly lower affinity for CB_1 and CB_2 receptors than THC (Thomas et al., 2007), and could explain why THC, which has been documented to be the more effective analgesic in mammals (Casey et al., 2017), falls short of CBD in its antinociceptive effects in this model system.

Taken as a whole, my results indicate that TRPV1 and TRPA1 channels are involved in the effects of acetic acid and the antinociceptive effects of CBD. Exposure to CBD increases zebrafish larval activity and block of TRPV1 and TRPA1 channels prevents this increase in activity (Fig 20B, 21C). When the CBD-exposed larvae were subsequently exposed to acetic acid or AITC, their activity level decreased. This reduction in activity was interpreted as antinociception, as the activity level, when compared to activity levels of larvae exposed to acetic acid or AITC, was significantly lower. Taking into consideration the fact that CBD-induced activity was lower than acetic acid- or AITC-induced activity, it was possible to hypothesize a possible mechanism of action that could explain the odd increase and decrease in activity: CBD only partially activates TRP channels, compared with a greater degree of activation by acetic acid or AITC, and this partial activation of the channels by CBD and a subsequent desensitisation of the channels could explain the antinociceptive effects of these phytochemicals.

This finding is consistent with other studies that show an exposure to various cannabinoids including CBD, led to a desensitisation of human TRPV1 channels and rat TRPA1 channels expressed in HEK293 cells (De Petrocellis et al., 2011). Another potential explanation could be derived from the unusually high efficacy of CBD to TRPV1, which rivals that of even the highest concentrations of capsaicin (Bisogno et al., 2001). This could give rise to the possibility of competitive inhibition against acetic acid, preventing activation of the channels. This, together with the desensitisation theory, could explain why the activity level of CBD and acetic acid together is sometimes lower than the activity level of CBD alone despite the presence of CBD in both cases.

Additionally, because an increase in activity was used as a measure of nociception, it is unfortunate that exposure to CBD also led to an increase in activity. To gain a more complete understanding of the mechanism, buprenorphine, the synthetic MOR partial agonist, was used to test if CBD-induced activity is also nociceptive in nature. With a pre-treatment of 0.1 mg/L buprenorphine, CBD-induced activity was completely eliminated. The activity was reduced to 0.41% of CBD when pre-treated with buprenorphine (Fig 23, IQR = 1.7%, $n = 36$; $p < 0.0001$). This suggests that the actions of CBD may also be nociceptive. Since TRP channels are responsible for initiating most nociceptive signals and CBD acts as a partial agonist on TRP channels, it follows that CBD could also be nociceptive in nature. However, the interpretation of behavioural changes can be complex and alternative explanations could exist. For example, the change in behaviour may indicate an alteration in anxiety level instead, with buprenorphine shown to act as an anxiolytic agent in mice (Etaee et al. 2017). It would be interesting to further investigate the mechanism behind the reduction in CBD-induced activity: if the reduction is an indication of CBD being nociceptive, to what extent does CBD lead to pain or discomfort and

how do these side effects interact with its antinociceptive effects, and if the reduction occurs through a different interaction between CBD and opioid agonists, through what mechanism does the interaction occur and how does it impact the antinociceptive effects of CBD?

4.3.3 Mechanism of antinociceptive effects of THC and the synergistic effects of THC and CBD

Unlike CBD, THC was unable to prevent the effects of acetic acid. However, THC demonstrated antinociceptive effects against AITC, but only at a concentration of 1 μM AITC. These findings were curious because higher and lower concentrations of THC were ineffective. As this particular experiment was performed much later into the project, I was unable to investigate the mechanism of action of THC. Previous studies suggested that THC primarily interacts with TRPV2, but also modulates TRPV3, TRPV4, TRPA1, and TRPM8 (Muller et al., 2019). HEK-293 cells with rat or human TRPV2 were examined via electrophysiology and THC was shown to act as an agonist at both (Qin et al., 2008). The effect of THC on TRPV3 and TRPV4 was also shown via calcium assays in HEK-293 cells with rat TRPV3 or rat TRPV4, where THC acted as a partial agonist at both receptors, but with lower efficacy and potency than CBD (De Petrocellis et al., 2012). Calcium imaging revealed that THC acts as an antagonist on rat TRPM8 channels expressed in HEK-293 cells (De Petrocellis et al., 2008). However, THC fails to activate TRPV1 (Ligresti et al., 2006; De Petrocellis et al., 2011; Qin et al., 2008) and this lack of interaction could explain the difference between acetic acid and AITC that allows THC to block the nociception from AITC. It has been shown that the CB_1 agonist, WIN55,212-2 exerts antinociceptive effects by desensitising TRPV1 and TRPA1 (Ruparel et al., 2011). Thus, it

is possible that THC, which also acts as an agonist at CB_1, displays antinociceptive effects through desensitisation of TRPA1 channels.

It is theorised that CBD and THC may act synergistically in what is often referred to as the "entourage" effect (Hanuš and Hod, 2020; Heblinski et al., 2020). This idea was initially applied to the ability of inactive endogenous glycerol esters to enhance the binding of endocannabinoid receptor ligands, anandamide and 2-arachidonoylglycerol, to CB_1 and CB_2 receptors (Ben-Shabat et al., 1998). This was later expanded to include cannabinoids found in the *C. sativa* plants. A study on neuropathic pain in mice explored the synergistic effects of THC and CBD and found that combinations of lower doses of the two compounds enhance the anti-allodynia effects of THC without exacerbating the side effects (Casey et al., 2017). Other synergies between THC and CBD include their effects on locomotion, anxiogenic properties, and c-Fos expression in the corticolimbic regions of mouse brains (Todd and Arnold, 2016). THC and another cannabinoid, cannabichromene (CBC), were also shown to display synergistic effects in their pharmacology including anti-inflammatory effects (DeLong et al., 2010). Several research papers have focused on the entourage effects of cannabinoids, but none were on their direct actions on acute nociceptive pain. My finding in this study suggests the presence of an enhancing effect of the two compounds: when THC is administered with CBD, the antinociceptive effects of CBD was more pronounced (Fig 12A): the combination of 2.5 mg/L CBD and THC had a greater ability to prevent the acetic acid-induced activity compared to 2.5 mg/L CBD alone. This was in spite of THC alone being incapable of preventing acetic acid-induced nociception in this same system.

This disparity in outcomes when applying CBD alone versus CBD with THC could be due to the difference in how each cannabinoid interacts with TRPA1 channels. Both CBD and

THC were shown to be potent antinociceptive agents against AITC (Fig 11). However, the potencies of the two at TRPA1 are quite different: THC has an EC_{50} of 0.23 ± 0.03 μM, whereas CBD has an EC_{50} of 0.09 ± 0.01 μM. This suggests that at comparable effective concentrations, the number of TRPA1 channels that open with CBD is higher than with THC.

My results could be explained if THC and CBD were competing against each other for their interaction with TRPA1. Both have similar efficacies, but THC has a lower potency (De Petrocellis et al., 2011). THC competing for and then occupying binding sites on TRPA1 channels, thereby preventing CBD from binding, could lead to a lower activation of the TRPA1 channels. This would in turn relay fewer signals down pain pathways. Since my results show that both CBD and THC are capable of desensitising TRPA1, both would contribute to the antinociception against acetic acid. If the reduction in nociceptive signals from agonism of TRPA1 by acetic acid remains constant following desensitisation by either CBD only or CBD plus THC treatments, the difference in overall signals propagated down pain pathway would depend on the level of TRPA1 activation by the cannabinoids themselves. The difference in potency between CBD and THC could lead to a potentially significant difference in the TRPA1-activated signal, thereby resulting in the observed differential in activity levels between CBD and THC group and the CBD alone group.

However, the results of my thesis do not necessarily support this: the activity level of zebrafish larvae when either THC or CBD is administered alone is a very similar (Fig 9). If the above theory was correct, the activity level with THC alone would be expected to be lower than CBD. However, there is still a possibility that this theory holds, as the cannabinoid-induced activities could be a compound of various targets of the cannabinoids, such as different TRP channels or endocannabinoid receptors. More experiments into the effects of each target of THC

and CBD must be performed to ascertain their impact on locomotion of zebrafish larvae to truly understand the mechanism of action behind the additive effects of THC and CBD.

Overall, the results of this thesis suggest that the synergistic effect between CBD and THC can enhance the antinociceptive effect of CBD. However, this synergistic effect was only seen in 2.5 mg/L CBD, which displayed some antinociceptive effects even without THC. This indicates that THC was not able to enhance subthreshold effects of CBD against nociception to lead to antinociceptive effects at a lower dosage.

4.3.4 Mechanism of antinociceptive effects of terpenoids

A search of the literature reveals that there are fewer studies on the mechanistic effects of terpenoids on nociception than there are for THC or CBD. *Trans*-nerolidol has also been suggested to display antinociceptive effects, albeit in an essential oil context (Ogunwande et al., 2019; Chan et al., 2016). More specifically, *trans*-nerolidol has been shown to be able to cause calcium influx in HEK cells containing TRPV1 channels (El-Hammadi et al., 2022). Another calcium-assay study on TRPV1 expressing HEK cells and terpenes showed that β-myrcene and *trans*-nerolidol led to calcium influx through TRPV1 channels (Jansen et al., 2019). Interestingly, the results of this study indicated that TRPV1 was activated only by these two terpenes while the remaining 8 terpenes were ineffective and more importantly, the channels were activated mainly by β-myrcene, than by *trans*-nerolidol. This result could lend credence to the theory that *trans*-nerolidol acts on TRPV1 channels as a partial agonist, much like CBD, and desensitised the channel to lead to antinociception against acetic acid.

Previous research on (-)-caryophyllene oxide has demonstrated its analgesic effects on acetic acid-induced writhing behaviour in mice (Chavan et al., 2010). This effect was thought to

be mediated by the inhibition of cyclooxygenase, lipoxygenase, or other inflammatory mediators. No other studies focused on the mechanism of action through which (-)-caryophyllene oxide could exert antinociception. However, other studies using calcium assays show that β-caryophyllene activates TRPV1 channels expressed on HEK cells, though to a lesser extent than *trans*-nerolidol or β-myrcene (El-Hammadi et al., 2022). Another study demonstrated antinociceptive effects of β-caryophyllene against acetic acid-induced writhing in mice and determined that CB_2 receptors and TRPV1 receptors were involved in this process through pharmacological interventions (Venkatakrishna et al., 2022). However, the effects of β-caryophyllene may be different from those of (-)-caryophyllene oxide and it is difficult to deduce the mechanism of antinociception beyond the possibility of interaction with TRPV1.

As mentioned, the literature on the mechanism of action of terpenoids is less established. Therefore, my proposed mechanism for the antinociceptive effects of trans-nerolidol and (-)-caryophyllene oxide is more speculative. My results show that the TRPV1 antagonist A-784168, at 10 μM, altered the antinociceptive effects of *trans*-nerolidol and (-)-caryophyllene oxide (Fig 20C, D). I also found that the TRPA1 antagonist HC-030031 prevented the terpenoids' reduction of acetic acid-induced activity at 50 μM (Fig 21F, G). This could indicate that the antinociceptive effects of the two terpenoids function via TRPV1 and TRPA1.

The interaction between the terpenoids and the TRP channels suggests that the terpenes activate the channels because blocking the TRP channels prevents the increase in activity observed with *trans*-nerolidol and (-)-caryophyllene oxide. A previous study has indicated that *trans*-nerolidol acts as a partial agonist on TRPV1 (Jansen et al., 2019). It is possible that the antinociceptive effects of *trans*-nerolidol is, like CBD, a result of desensitisation of TRPV1. Additionally, both (-)-caryophyllene oxide and trans-nerolidol, and only these two of the seven

terpenoids I tested, are sesquiterpenes. Sesquiterpenes have been documented to display similar pharmacological effects, such as their anticancer properties (Lněničková et al., 2018; Hanušová et al., 2017). The similarities in function between these sesquiterpenes could indicate that the mechanism of action for both terpenoids is similar. If that is the case, (-)-caryophyllene oxide could also be exerting antinociceptive effects through desensitisation, like *trans*-nerolidol could be. However, this is highly speculative, and unfortunately, the scope of the thesis could not conclusively identify the mechanism of actions behind the antinociceptive effect of either of these terpenes. Further research into both terpenes is required to make a more direct inference as to the mechanism.

4.3.5 (-)-Caryophyllene oxide demonstrate a synergistic effect with CBD and phytol demonstrate an anti-synergistic effect with CBD.

The discussion on the "entourage" effect that spread throughout the research into cannabinoids has also been extended to the study of the relationship between terpenoids and cannabinoids (Russo, 2019). However, most discussions around terpenoids have been limited to observations of enhanced effects in cannabis extract compared to pure CBD (Gallily et al., 2015; Berman et al., 2018) and fail to provide specific information such as how the interaction between two specific phytochemicals lead to a certain specific effect. For example, in one study, the antinociceptive effects of CBD in mice reached a ceiling beyond which an increase in dosage did not improve the antinociceptive effects, but with a plant extract containing the same amount of CBD, such ceiling was not observed (Gallily et al., 2015). Another study also demonstrated a similar improvement in the anti-convulsant effect of CBD in mice when given in an extract form

(Berman et al., 2018). However, the overall lack of specificity in these studies make it harder to apply the findings to a mechanistical understanding of these synergistic effects.

In this thesis, instead of an extract of multitudes of compounds, I focused on the interaction between two phytochemicals at a time, mixing CBD with multiple concentrations of each terpenoid. Of the seven terpenoids, only two altered the activity level when co-administered with CBD: phytol and (-)-caryophyllene oxide. (-)-Caryophyllene oxide and CBD enhanced their antinociceptive effects, decreasing the acetic acid-induced activity further than either compound alone (Fig 15G). This could mean that the two phytochemicals are able to function in parallel with each other to complement their antinociceptive effects. However, if the proposed mechanism of action of desensitisation of TRP channels is correct for both (-)-caryophyllene oxide and CBD, a theory of competition between the two phytochemicals that I used to explain the synergistic effects of THC and CBD could also apply here. However, because our understanding of the mechanism of action of (-)-caryophyllene oxide is very poor, further investigation into the interaction as well as the individual mechanisms must be performed for a more complete understanding.

Interestingly, the activity level, when CBD and phytol were administered together, was not different from the acetic acid treatment alone (Fig 15E); the antinociceptive effects of CBD was eliminated when phytol was administered alongside it. As most studies that explored the concept of the “entourage” effect has focused their theories on the additive and enhancing effects (Russo, 2019; Gallily et al., 2015; Berman et al., 2018), they fail to provide much insight into why this anti-synergistic effect may be occurring between phytol and CBD. One potential explanation for this interaction comes from a study done on silkworm excrements: the excrement, used in Chinese herbal medicine, was used to isolate phytol, which was further tested

on HEK-Flp-In cells with TRPA1 and TRPV1 channels (Song et al., 2023). The cells were then recorded via whole cell voltage clamping, and the result showed that phytol acts as a weak antagonist against both TRPA1 and TRPV1 channels. If phytol was able to block the agonistic effects of CBD, this could help explain how the antinociceptive effects of CBD was blocked in the combination treatment: As CBD fails to activate the TRP channels, they fail to be desensitised. If phytol, as a weak antagonist, is unable to prevent the binding of acetic acid with the TRP channels, the acetic acid-induced nociception would cause an increase in activity in the zebrafish larvae. As this theory is entirely based on one study, it would be important to investigate the interaction directly to better understand the mechanism behind the anti-synergistic effects of phytol and CBD.

4.4 Limitations and Future Directions

To fully answer the research question of this thesis, several additional studies must be performed to provide a more complete picture. One of the biggest limitations of this thesis was the focus on one experimental method. Behavioural experiments provide a good basis on which to start an inquiry into a specific topic of interest, especially for a broad project such as this one, where several compounds at different concentrations and combinations must be tested. However, it often fails to distinguish more subtle changes and differences between treatments. Specifically, there were many instances in which changes in the activity level could be attributed to a myriad of different mechanisms that are not nociceptive or antinociceptive in nature. While the use of different experimental methods to study the mechanism of actions of terpenoids and CBD does not eliminate this issue, genetic mutations, immunohistochemistry, and calcium imaging, among other techniques, may provide a more complete answer as to which systems and receptors are

involved in the nociceptive effect of acetic acid or the antinociceptive effects of cannabinoids and terpenoids. For example, specific knock-out of TRP channels, such as TRPA1a, TRPA1b, or TRPV1 in zebrafish larvae could further validate their involvement in the antinociceptive effects of CBD, THC, *trans*-nerolidol, and (-)-caryophyllene oxide. Additionally, an investigation of the expression levels of these TRP channels in specific afferent neurons through immunohistochemistry and/or Western blotting could help identify how cannabinoids and terpenoids interact with the gene expression of these proteins and give us a clue as to which pathway is involved in the antinociceptive effects of these phytochemicals. Lastly, calcium imaging could help validate some of the theories proposed in this thesis for the mechanism of action for the antinociceptive effects of the terpenoids or the synergistic effects of these phytochemicals by observing the amount of calcium that enters the TRP channel-expressing neurons, thus validating whether there is a desensitisation happening in subsequent exposures to acetic acid or AITC.

Additionally, the use of pharmacological tools has its strengths and limitations, with each new compound requiring further verification of specificity, off-target effects, and effective concentrations. This made the investigation into the mechanism behind the antinociceptive effects a weaker aspect of the thesis. In the case of opioid receptor blockers or endocannabinoid receptor blockers, they did not alter the acetic acid-induced activity by themselves, indicating that neither opioid receptors nor endocannabinoid receptors were directly linked to the nociceptive effects of acetic acid. As such, it was possible to isolate the effects they exerted on the cannabinoids and terpenoids. However, in the case of TRP channel blockers, acetic acid was directly affected by them. This made it impossible to test the involvement of TRP channels in the antinociceptive effects of the phytochemicals with acetic acid as the noxious stimulus. A study

by De Petrocellis and colleagues (2011) on HEK cells demonstrated through calcium imaging that exposure to CBD does in fact lead to desensitisation in subsequent agonist-induced agonism of multiple TRP channels. However, no such study has been performed for trans-nerolidol or (-)-caryophyllene oxide. This makes it difficult to speculate on the mechanistic link between TRP channels and these terpenoids. A future study in which a more direct method of investigation, such as calcium imaging or electrophysiology, could be highly beneficial in determining whether the proposed interaction between TRP channels and the antinociceptive effects of these terpenoids is backed by more concrete evidence.

Finally, recent innovations in the field have demonstrated the involvement of different channels such as the voltage-gated calcium channels $Ca_v3.2$, $Ca_v3.1$, and $Ca_v3.3$ in nociception (Gadotti, Huang, & Zamponi, 2021), and it has become more important than ever to consider various options for the mechanism behind the antinociceptive effects of cannabinoids and terpenoids and recognise that the effects are likely not a result of the phytochemicals' interaction with a singular system, but rather their simultaneous interactions with multiple systems.

Through this study, I was able to identify the presence of antinociceptive effects of CBD and two of the tested terpenes, (-)-caryophyllene oxide and *trans*-nerolidol. Brief investigation into the mechanism of action demonstrated the potential involvement of TRP channels, specifically TRPA1 and TRPV1 channels, in this process. While further inquiry into the topic is necessary for a more concrete identification of the mechanism, this thesis served to lay the foundation on which future studies on non-opioid-related methods of pain management can be based and furthered the overall understanding of nociception, pain, and management of such conditions.

Figures

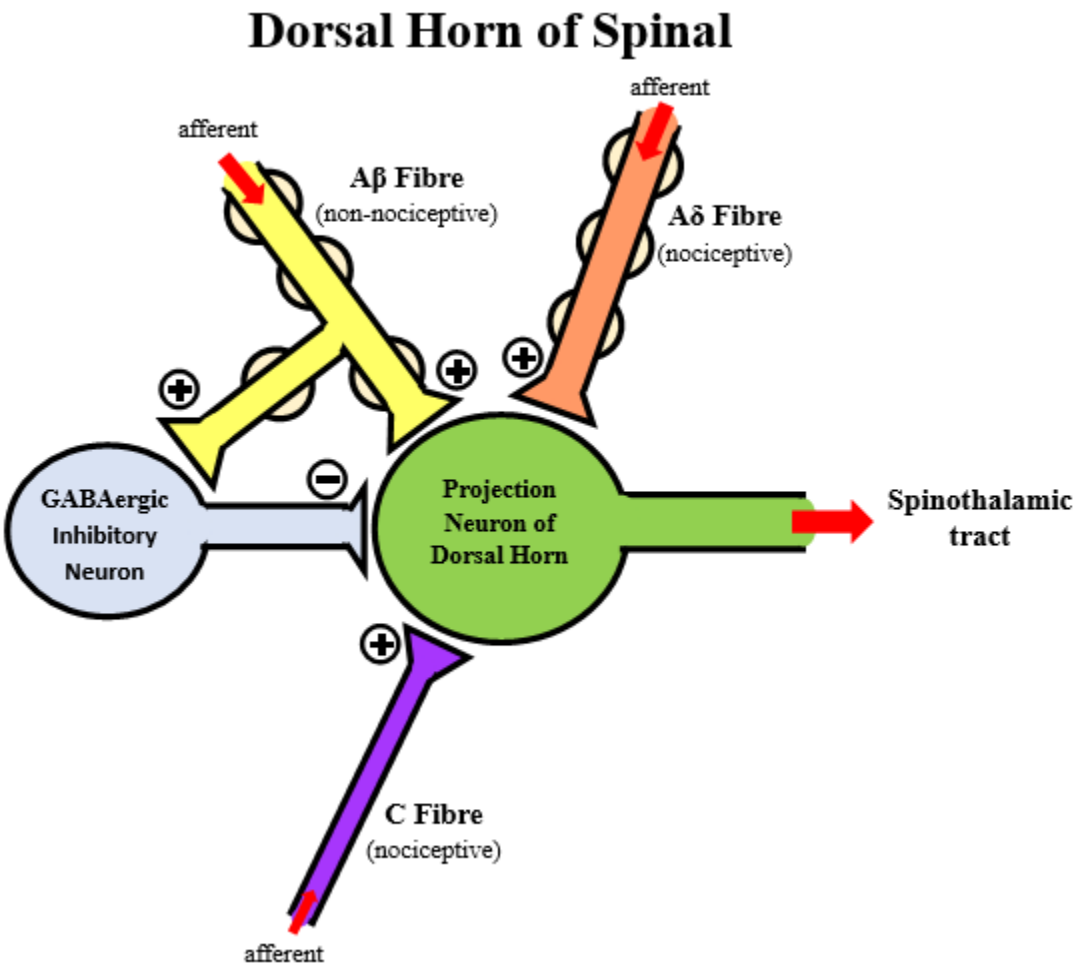


Figure 1. Diagram of the primary afferents and interneurons involved in the pain pathway in the dorsal root of the spinal cord. The faster, myelinated signals from Aδ fibre signals the projection neuron in the dorsal horn, which subsequently propagates the nociceptive signal up the spinothalamic tract to the thalamus and other sensory regions of the brain. The slower, unmyelinated signals from C fibres also send nociceptive signals to the dorsal horn. However, the activation of the non-nociceptive, myelinated Aβ fibres can inhibit the slower signals from the C fibre, acting as the first line of regulating pain.

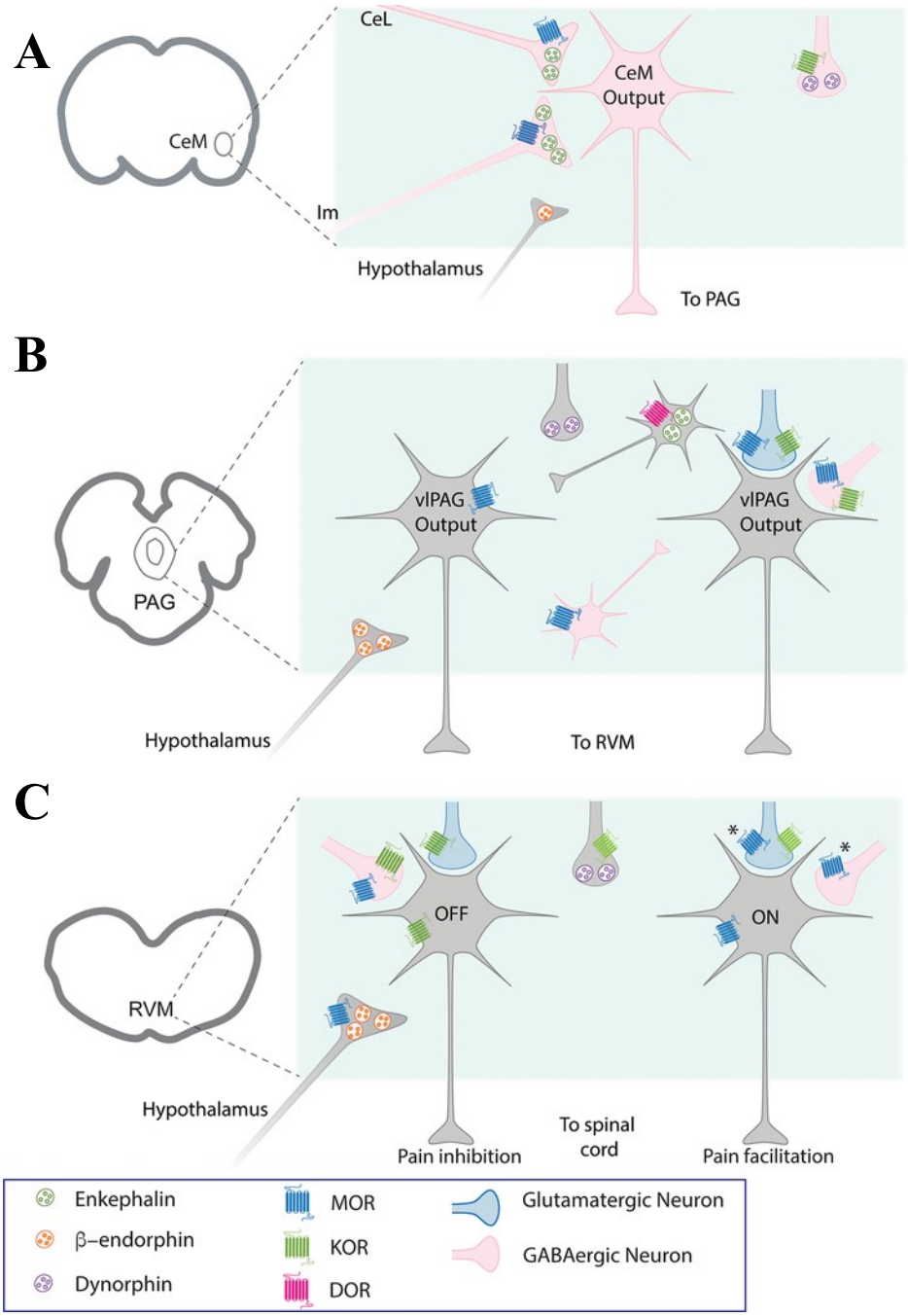


Figure 2. Diagram representing the top-down pain inhibition pathway with the relevant endogenous opioids and receptors in rats from Bagley and Ingram (2021). Each diagram represents cells of: **A.** medial central nucleus of amygdala (CeM), **B.** periaqueductal grey (PAG), and **C.** rostral ventromedial medulla (RVM). **A.** Release of enkephalin, an endogenous opioid, from lateral central nucleus of amygdala (CeL) and main islands of intercalated cells of the amygdala (Im), and release of β-endorphin from hypothalamus regulate the GABAergic signals onto CeM via μ-opioid receptors (MOR). This disinhibition allows for propagation of signals from CeM to PAG. **B.** Opioid ligands enkephalin, β-endorphin, and dynorphin are released at

PAG from cells projecting from hypothalamus and amygdala. Postsynaptic MOR coupled with G protein-coupled potassium channels hyperpolarises the output cells of PAG, while presynaptic MOR regulate glutamatergic and GABAergic signal to the output cell. Other opioid receptors, namely κ- and δ-opioid receptors (KOR and DOR, respectively) are also expressed in the PAG. **C.** There are both on- and off-cells in RVM. On-cells are inhibited by activation of MOR, and some off-cells are inhibited by activation of KOR. Presynaptic projections from PAG are regulated by both, regardless of whether they are GABAergic or glutamatergic. Off-cell projections lead to inhibition of nociception and on-cell projections lead to enhancement of nociception. Both project to the spinal cord.

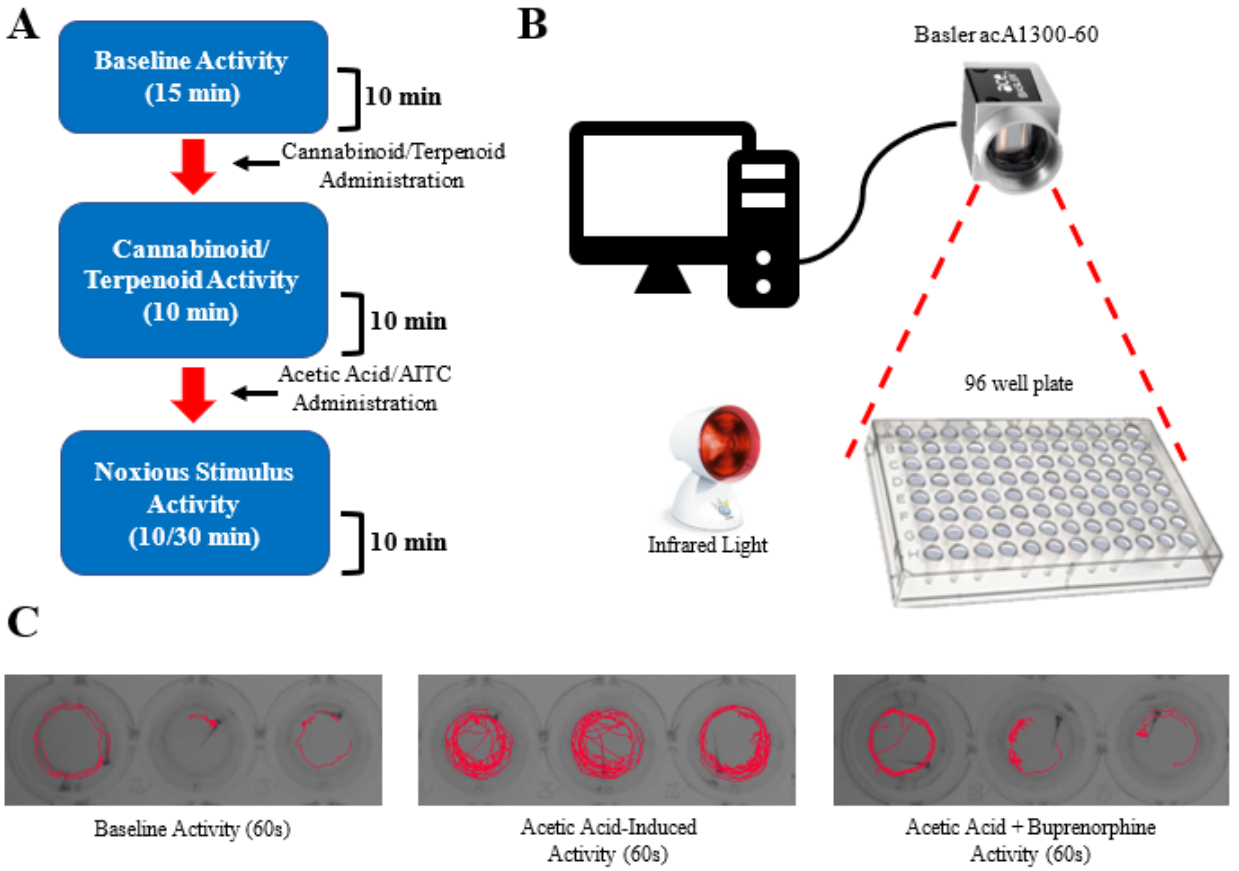


Figure 3. A. Flow chart of the behavioural assay used throughout the study. **B.** A pictorial representation of the equipment used in the behavioural experiments. **C.** Representative figures of the activity seen in zebrafish larvae at 5 dpf over a duration of 60 seconds. The left figure shows the baseline activity in which the larvae were not exposed to any compounds. The middle figure shows the activity of larvae exposed to acetic acid. The right figure shows the activity of larvae pre-treated with buprenorphine for 30 minutes and exposed to acetic acid.

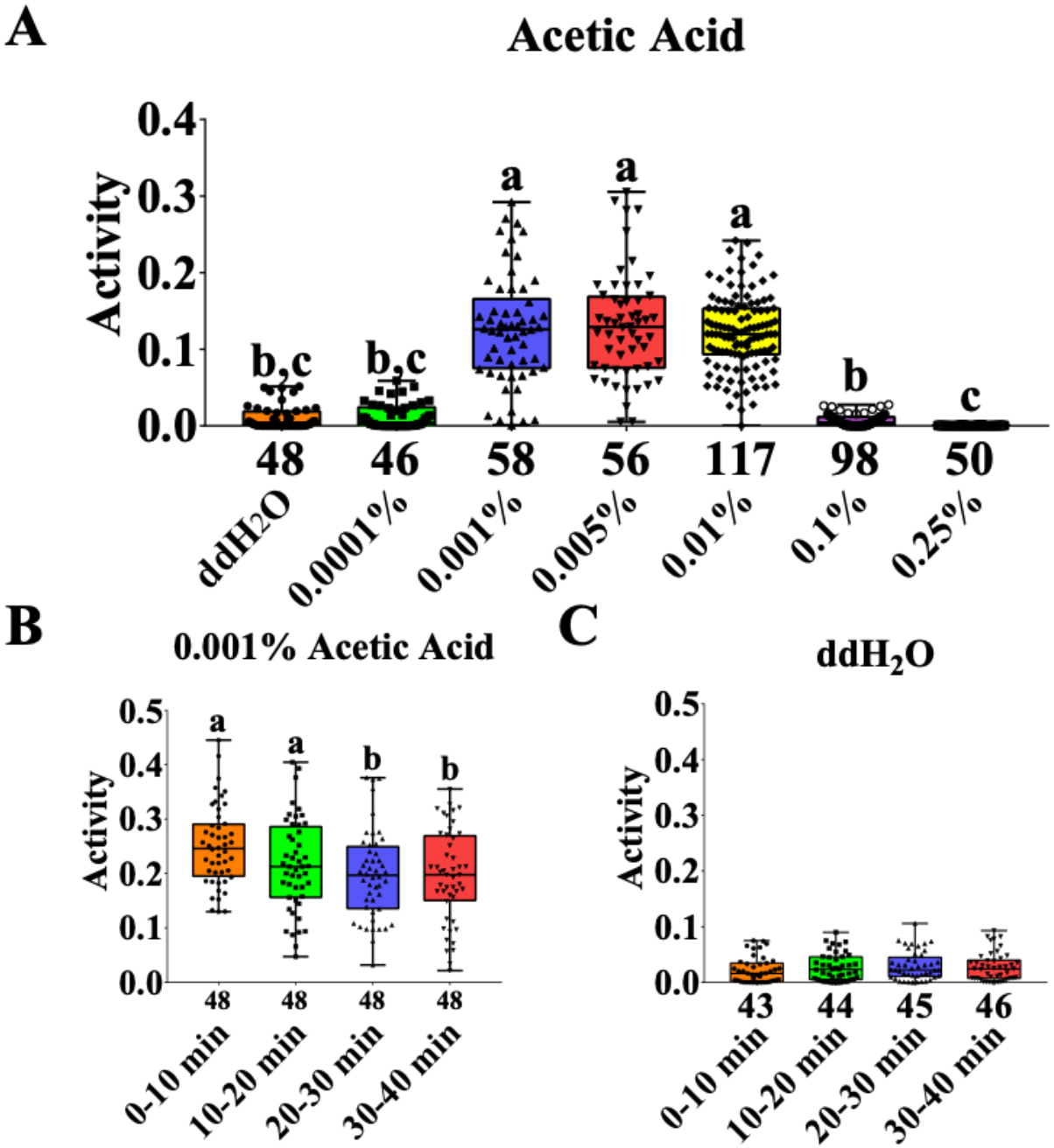


Figure 4. Mean % activity of zebrafish larvae at 5 dpf when exposed to acetic acid. **A**. At concentrations between 0.001% and 0.01%, acetic acid increased zebrafish larval activity ($p < 0.0001$). **B**. Zebrafish larvae were exposed to 0.001% v/v acetic acid over 40 minutes, divided into four 10-minute periods. There was a significant decrease in activity over time, with the period between 20 to 40 minutes after the initial exposure showing a lower activity level than the first ten minutes ($p = 0.0134$ for 20-30 minutes, $p = 0.0147$ for 30-40 minutes). **C**. The activity of zebrafish larvae did not change when exposed to ddH_2O for a period of 40 minutes, divided into four periods of 10 minutes each ($p = 0.3688$). Box and

whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

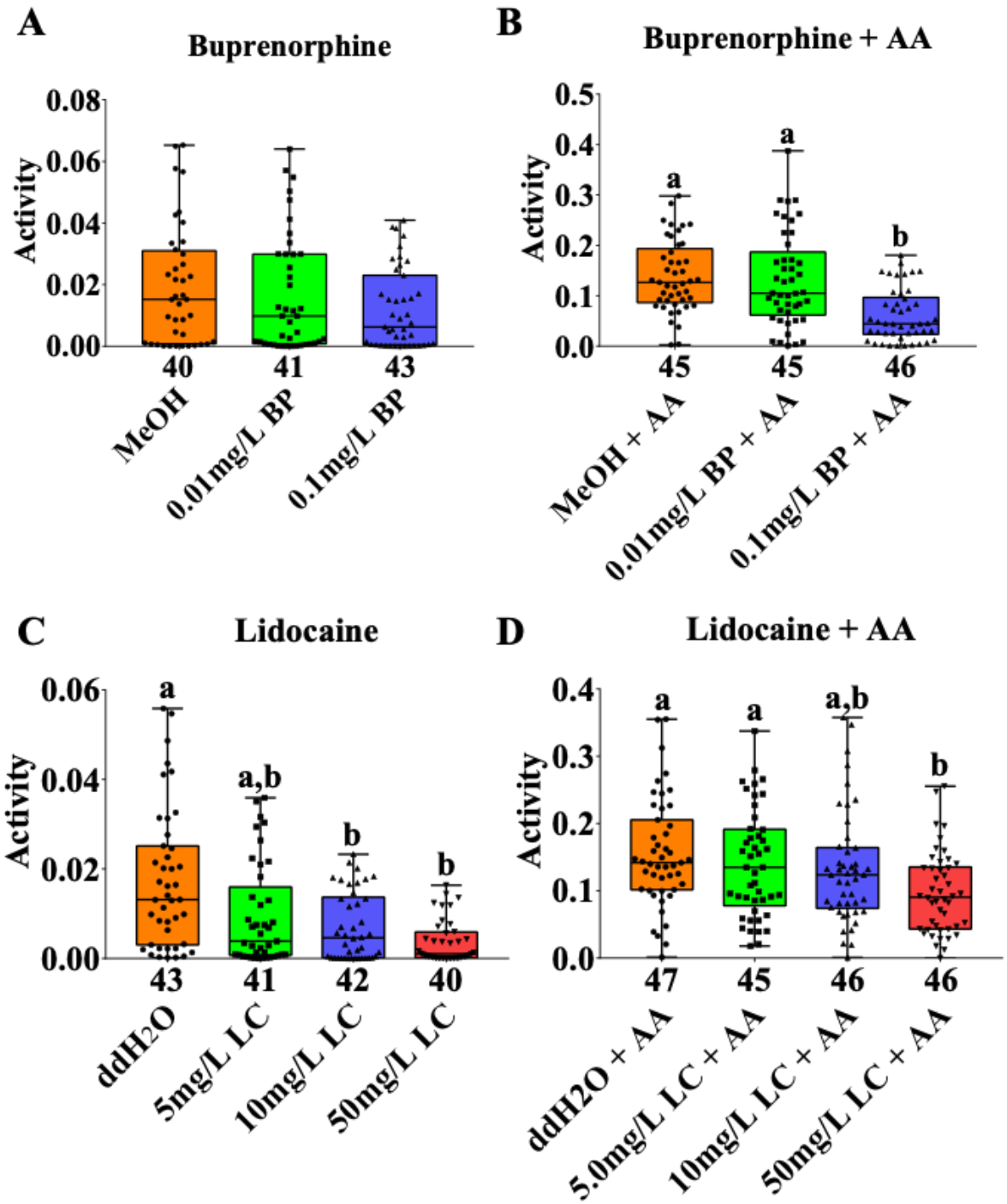


Figure 5. Mean % activity of zebrafish larvae at 5 dpf when exposed to **A**. buprenorphine (BP), **B**. buprenorphine and acetic acid (AA) (0.001% v/v), **C**. Lidocaine (LC), or **D**. Lidocaine and acetic acid (0.001% v/v). **A**. Activity did not change with the introduction of buprenorphine at 0.1mg/L or 0.01mg/L ($p = 0.3019$). **B**. Activity did not change for 0.01mg/L buprenorphine with acetic acid ($p > 0.9999$) but was significantly lower for 0.1 mg/L ($p < 0.0001$). **C.** Compared to vehicle control (ddH_2O), activity decreased with exposure to lidocaine at 10mg/L ($p = 0.0017$) and at 50mg/L ($p < 0.0001$), but not at 5

mg/L ($p = 0.0582$). **D**. At 50mg/L, lidocaine reduced acetic acid-induced activity ($p = 0.0014$). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

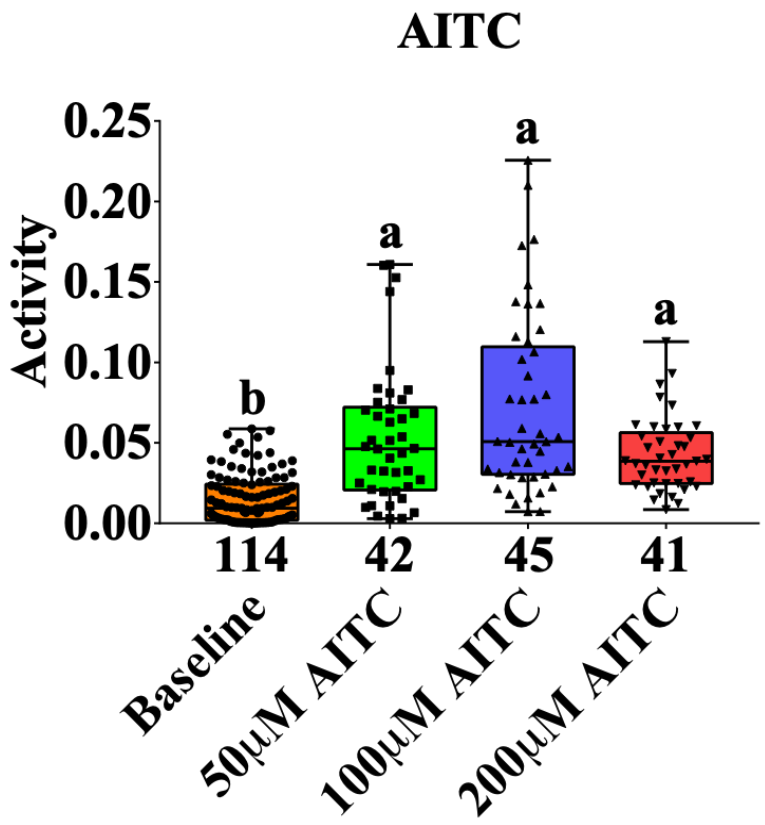


Figure 6. Mean % activity of zebrafish larvae at 5 dpf when exposed to allyl isothiocyanate (AITC) at 50 µM, 100 µM, and 200 µM. Activity increased with the exposure to AITC ($p < 0.0001$ at all three concentrations). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

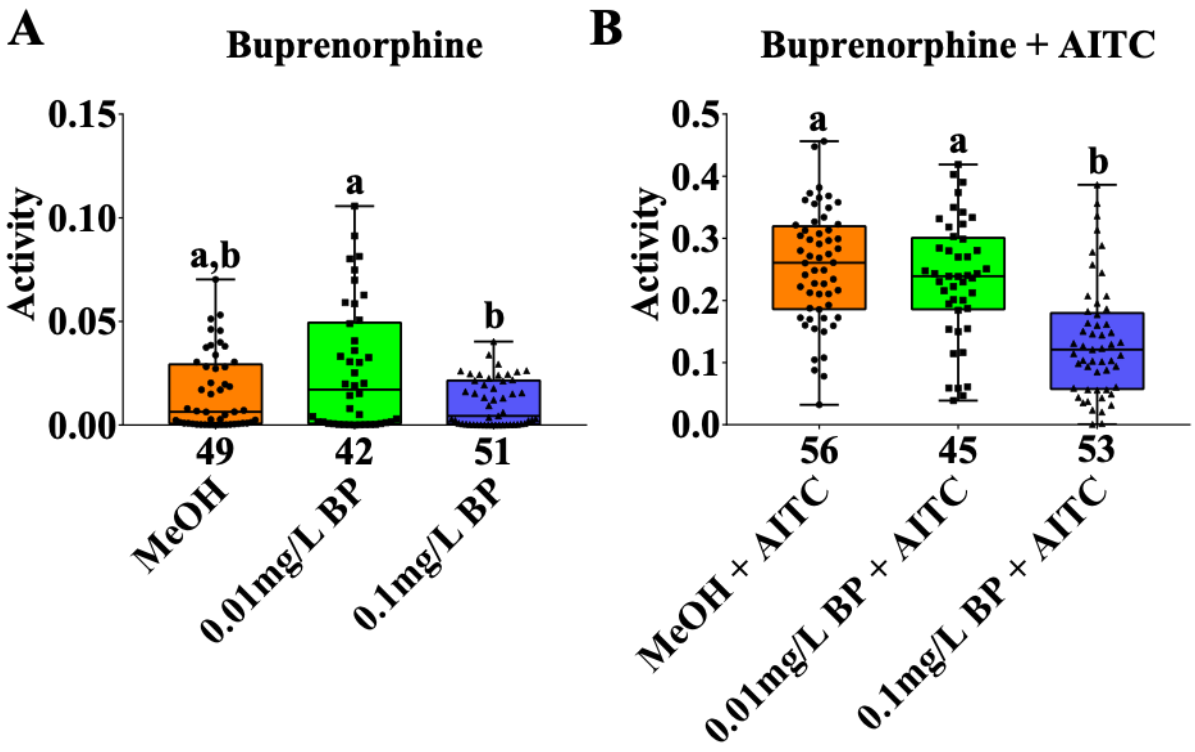


Figure 7. Mean % activity of zebrafish larvae at 5 dpf when exposed to **A**. buprenorphine or **B**. buprenorphine and allyl isothiocyanate (AITC) (50µM). **A**. Activity did not change with the introduction of buprenorphine at 0.1mg/L or 0.01mg/L ($p = 0.6570$ and $p = 0.5714$, respectively). However, activity did differ between the two groups ($p = 0.0375$) **B**. Activity did not change for the 0.01mg/L buprenorphine with AITC ($p > 0.9999$) but was lower for 0.1mg/L ($p < 0.0001$). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

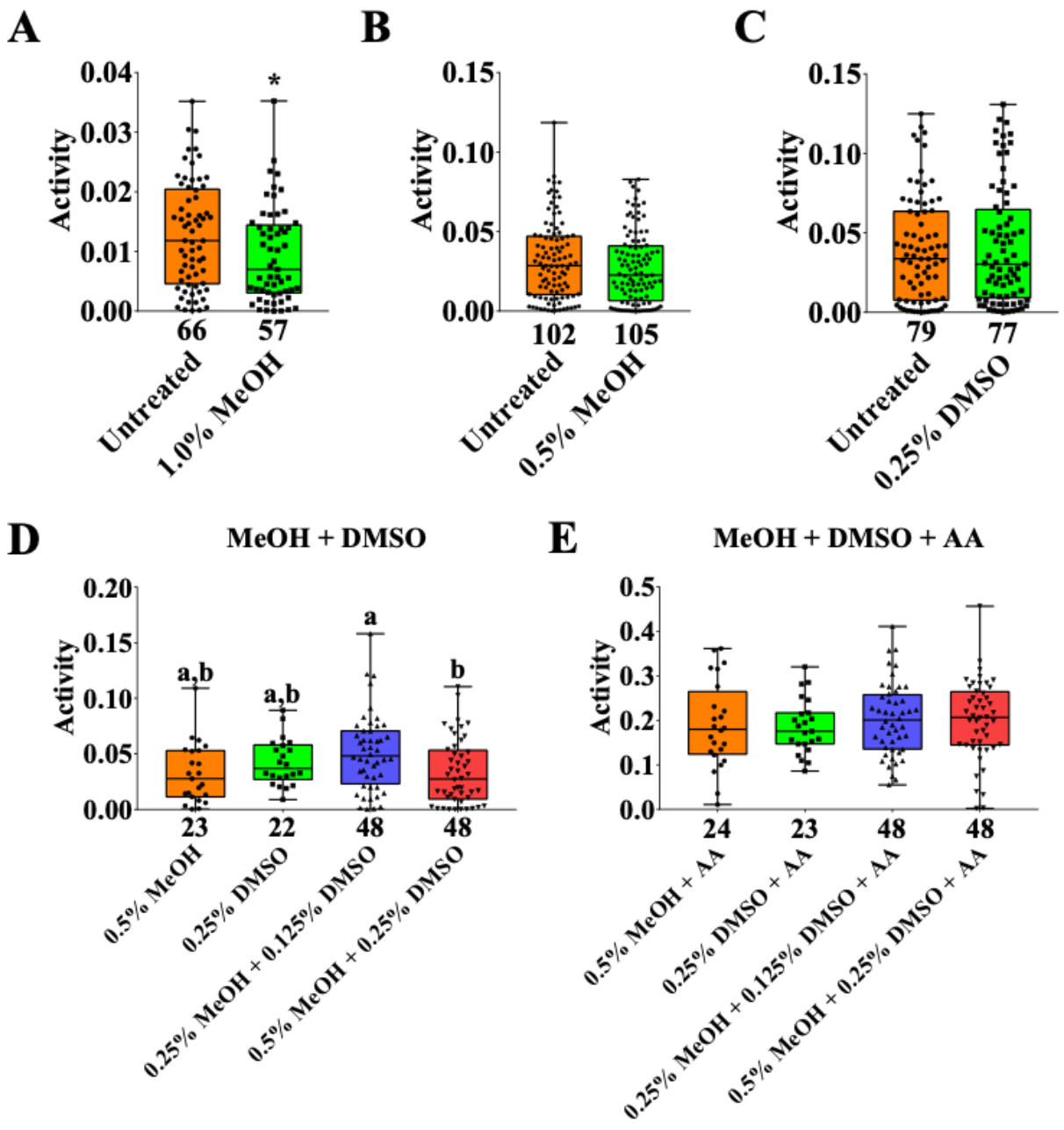


Figure 8. Mean % activity of zebrafish larvae at 5 dpf with exposure to methanol (MeOH), DMSO, or a combination of the two. **A.** Activity with 1.0% methanol was lower than the untreated baseline activity ($p = 0.0248$). **B.** Activity with 0.5% methanol was the same as the untreated control ($p = 0.1630$). **C.** Exposure to DMSO at 0.25% did not alter the activity level from untreated control ($p = 0.8403$). **D.** Combinations of methanol and DMSO was not different from either methanol or DMSO alone. Combination of 0.25% methanol and 0.125% DMSO had a slightly higher activity level than the combination of 0.5% methanol and 0.25% DMSO ($p = 0.0292$). **E.** When exposed to acetic acid (AA), activity of all four treatment groups did not differ from one another ($p = 0.7459$). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

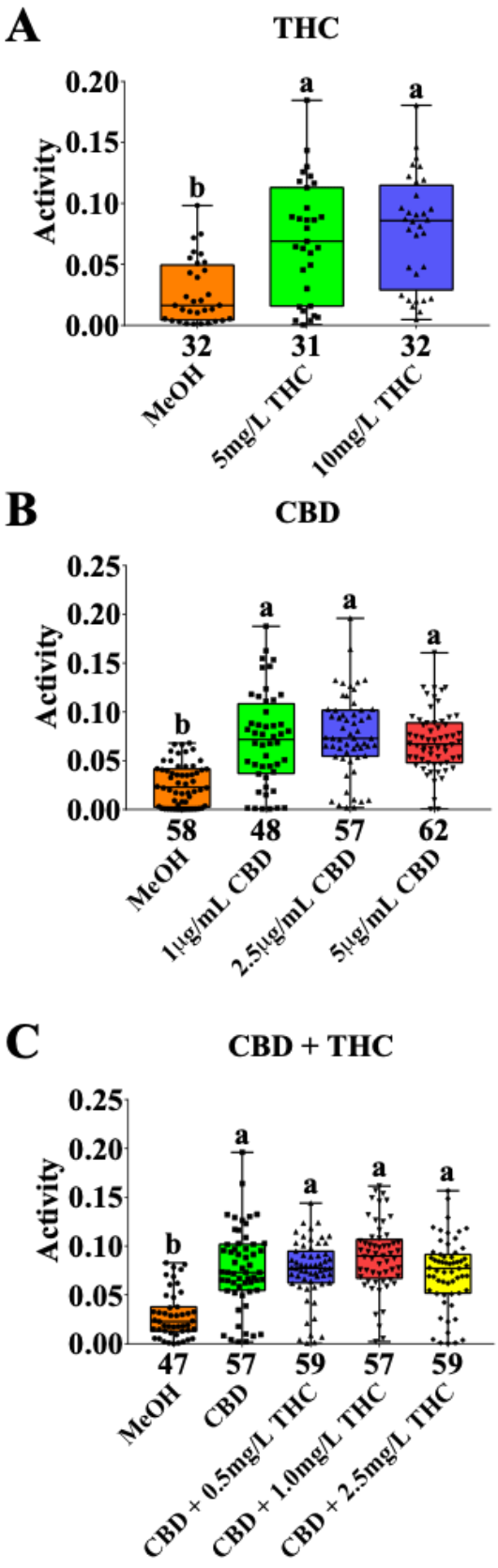


Figure 9. Mean % activity of zebrafish larvae at 5 dpf when exposed to tetrahydrocannabinol (THC), cannabidiol (CBD), or a combination of both without noxious stimuli with methanol (MeOH) as the vehicle control. Each box represents the activity induced by: **A.** THC, **B.** CBD, or **C.** THC and 2.5mg/L CBD. **A.** the administration of 5mg/L and 10mg/L THC dissolved in 1.0% methanol increased activity compared to the control activity ($p = 0.0007$ for 5mg/L and $p < 0.0001$ for 10mg/L). **B**. the administration of 1mg/L, 2.5mg/L, and 5.0mg/L CBD dissolved in 0.5% methanol also increased activity ($p < 0.0001$ for each). **C**. the administration of 2.5mg/L CBD with 0mg/L, 0.5mg/L, 1.0mg/L, and 2.5mg/L THC increased activity from the control ($p < 0.0001$ for each). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

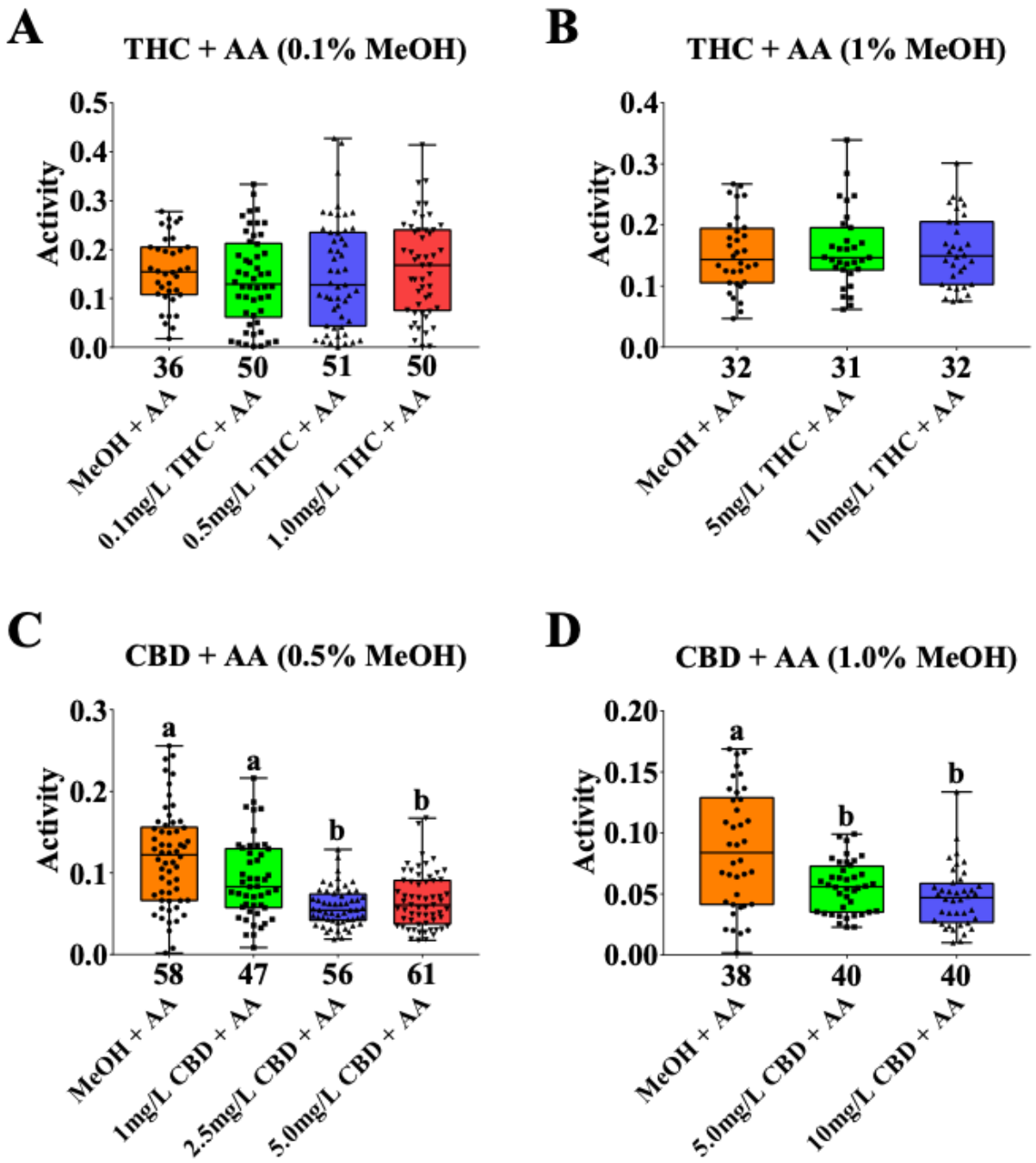


Figure 10. Mean % activity of zebrafish larvae at 5 dpf when exposed to: **A-B**. tetrahydrocannabinol (THC) and acetic acid (AA) or **C-D**. cannabidiol (CBD) and acetic acid. **A**. all three concentrations (0.1mg/L, 0.5mg/L, and 1.0mg/L) of THC dissolved in 0.1% methanol (MeOH) did not affect acetic acid-induced movement ($p = 0.5459$). **B**. both concentrations (5mg/L and 10mg/L) THC dissolved in 1.0% methanol also did not affect acetic acid-induced activity ($p = 0.9676$). **C**. Compared to the methanol + acetic acid group, CBD at 2.5mg/L and 5.0mg/L, dissolved in 0.5% methanol, reduced acetic acid-induced activity ($p < 0.0001$). At 1mg/L, CBD did not affect the acetic acid-induced activity ($p = 0.1956$). **D**. At both 5mg/L and 10mg/L, CBD dissolved in 1.0% methanol reduced acetic acid-induced activity (p

= 0.0472 for 5 mg/L, p = 0.0002 for 10 mg/L). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

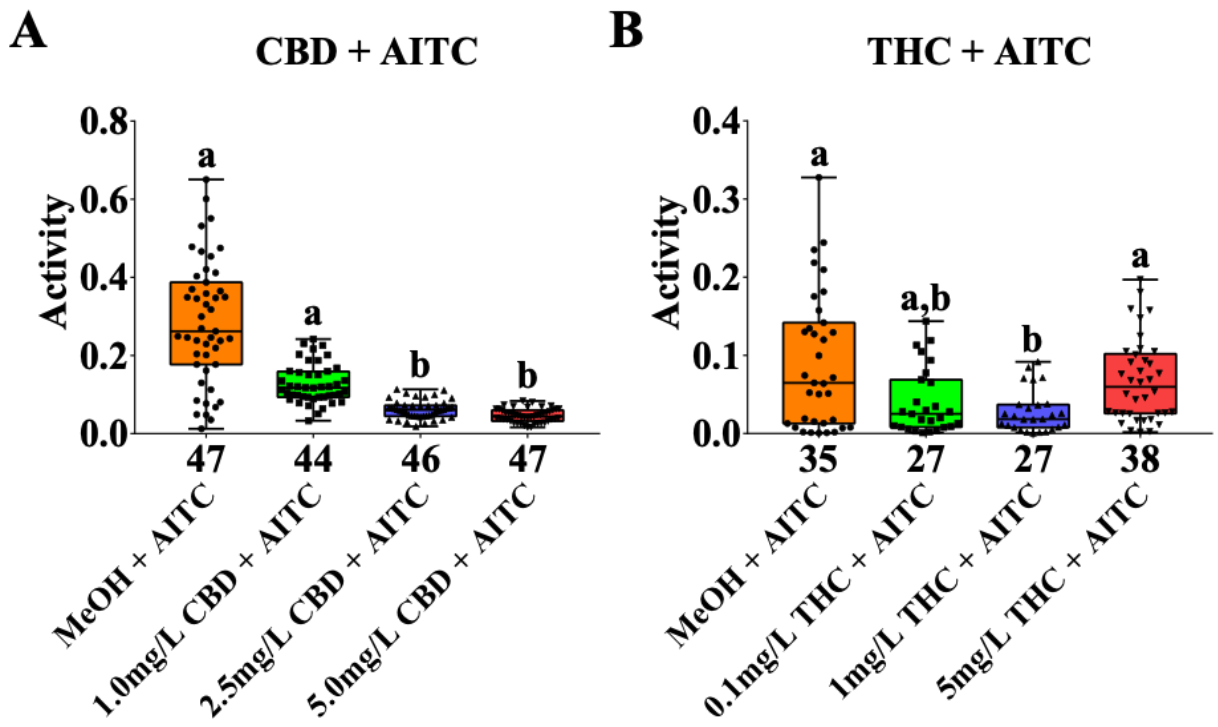


Figure 11. Mean % activity of zebrafish larvae at 5 dpf when exposed to various concentrations of cannabidiol (CBD) or tetrahydrocannabinol (THC) and 50µM allyl isothiocyanate (AITC). **A.** At 1.0mg/L, CBD did not change AITC-induced activity ($p = 0.1036$) but reduced it at 2.5mg/L and 5.0mg/L ($p < 0.0001$ for both). **B.** At 0.1mg/L and 5mg/L, THC did not alter AITC-induced activity ($p = 0.2700$ and $p > 0.9999$, respectively). At 1mg/L, however, THC did reduce AITC-induced activity ($p = 0.0205$). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

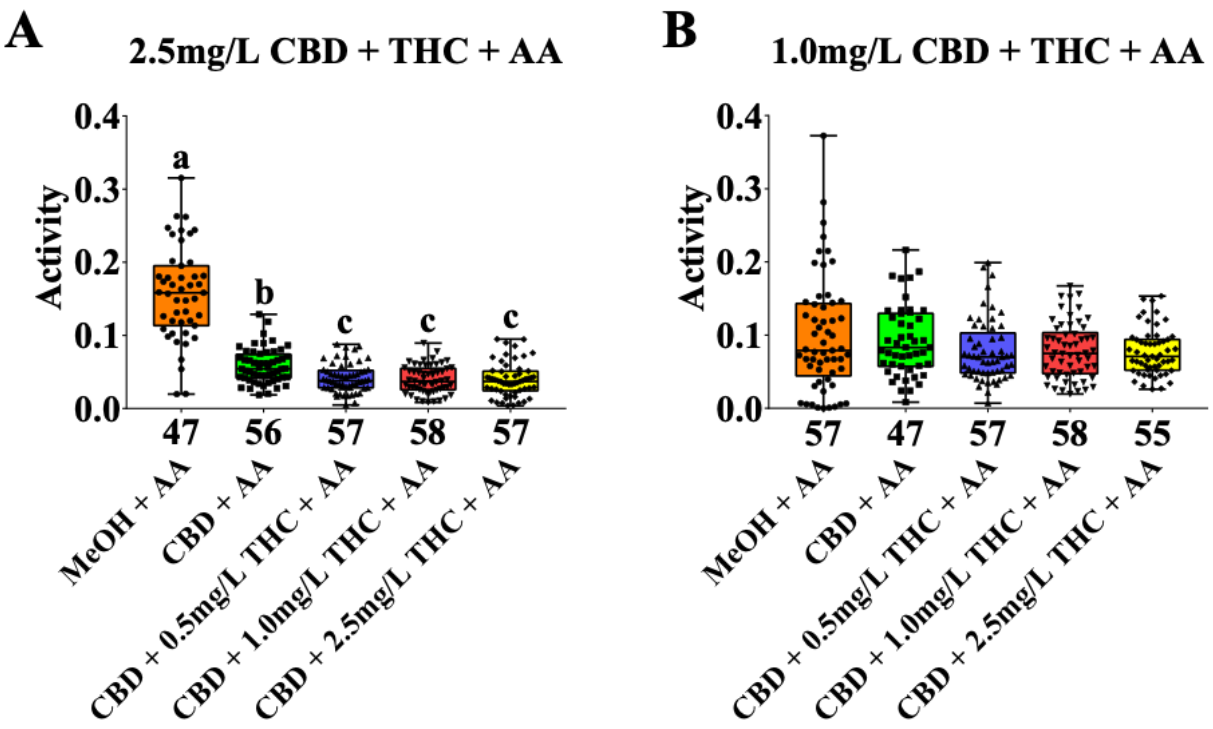


Figure 12. Mean % activity of zebrafish larvae at 5dpf when exposed to acetic acid (AA), 2.5mg/L and 1.0mg/L cannabidiol (CBD), and various concentrations of tetrahydrocannabinol (THC). **A.** When 2.5mg/L CBD is administered alone prior to the administration of acetic acid, activity was reduced compared to the methanol + acetic acid group ($p < 0.0001$). Additionally, when 0.5 mg/L, 1.0 mg/L, and 2.5 mg/L THC were added alongside 2.5mg/L CBD, there was a further decrease in activity ($p < 0.0001$ from control; $p = 0.0158$, $p = 0.0048$, and $p = 0.0019$ from CBD+AA, respectively). **B.** When 1.0mg/L CBD is administered with acetic acid, with or without THC, activity did not change from the methanol (MeOH) + acetic acid group ($p = 0.4001$). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

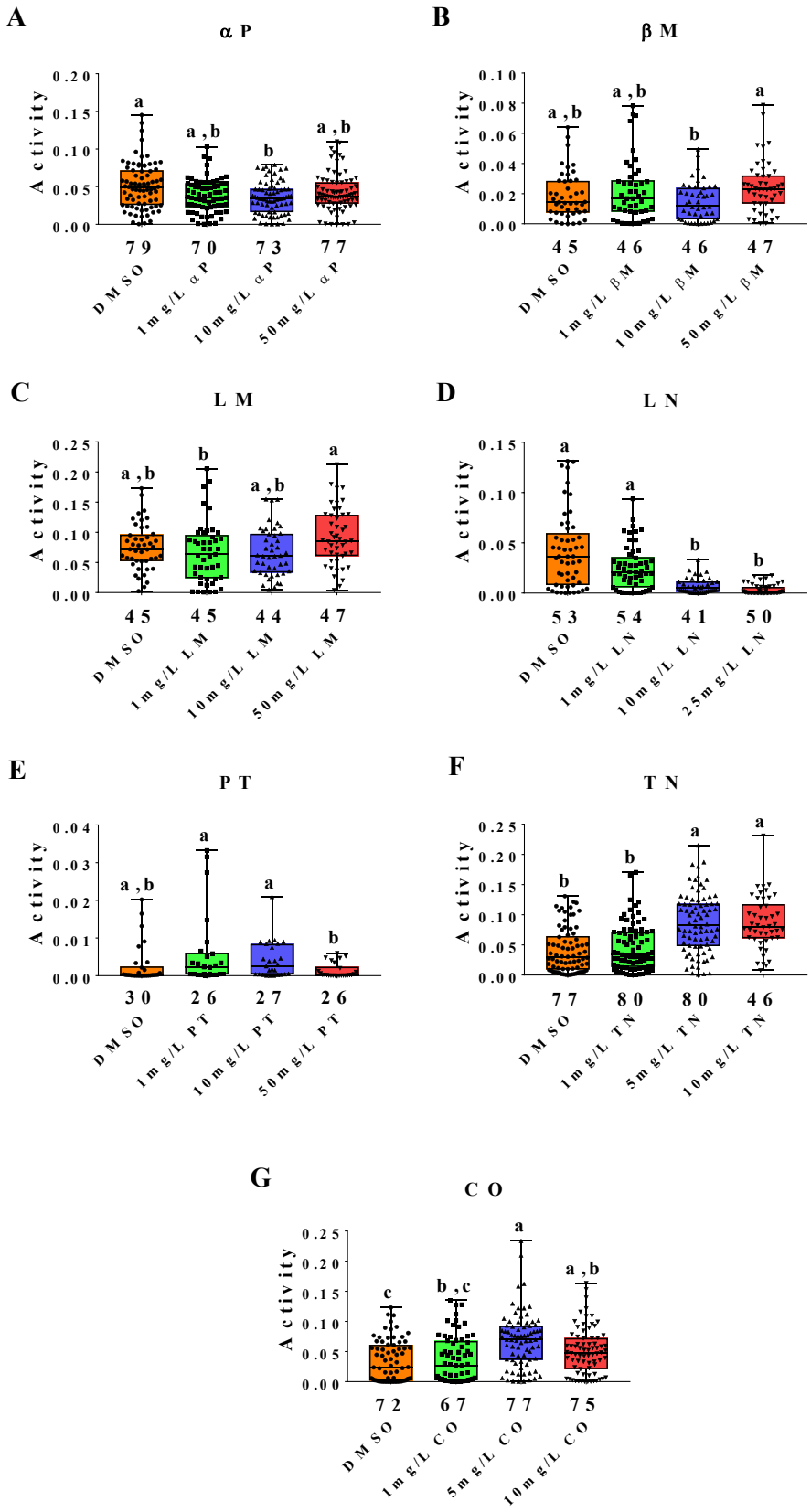

A
α P
Activity
0.20
0.15
0.10
0.05
0.00
a
a , b
b
a , b
79
70
73
77
DMSO
1 mg/L α P
10 mg/L α P
50 mg/L α P
B
β M
Activity
0.10
0.08
0.06
0.04
0.02
0.00
a , b
a , b
b
a
45
46
46
47
DMSO
1 mg/L β M
10 mg/L β M
50 mg/L β M
C
L M
Activity
0.25
0.20
0.15
0.10
0.05
0.00
a , b
b
a , b
a
45
45
44
47
DMSO
1 mg/L L M
10 mg/L L M
50 mg/L L M
D
L N
Activity
0.15
0.10
0.05
0.00
a
a
b
b
53
54
41
50
DMSO
1 mg/L L N
10 mg/L L N
25 mg/L L N
E
P T
Activity
0.04
0.03
0.02
0.01
0.00
a , b
a
a
b
30
26
27
26
DMSO
1 mg/L P T
10 mg/L P T
50 mg/L P T
F
T N
Activity
0.25
0.20
0.15
0.10
0.05
0.00
b
b
a
a
77
80
80
46
DMSO
1 mg/L T N
5 mg/L T N
10 mg/L T N
G
C O
Activity
0.25
0.20
0.15
0.10
0.05
0.00
c
b , c
a
a , b
72
67
77
75
DMSO
1 mg/L C O
5 mg/L C O
10 mg/L C O

Figure 13. Mean % activity of zebrafish larvae at 5 dpf when exposed to **A.** α-pinene (αP), **B.** β-myrcene (βM), **C.** limonene (LM), **D.** linalool (LN), **E.** phytol (PT), **F.** *trans*-nerolidol (TN), or **G.** (-)-caryophyllene oxide (CO). **A.** Compared to the DMSO control, 10 mg/L α-pinene showed lower level of activity ($p = 0.0066$). **B-C, E.** Activity did not differ from the DMSO control for each terpenoid. **D.** At 1 mg/L linalool did not change activity ($p = 0.8223$), but at the higher concentrations, linalool decreased activity significantly ($p < 0.0001$). **F.** At 1 mg/L, trans-nerolidol did not change the activity from the control ($p > 0.9999$). However, at both 5 mg/L and 10 mg/L, *trans*-nerolidol increased zebrafish larval activity ($p < 0.0001$). **G.** Similarly, at 1 mg/L, (-)-caryophyllene oxide did not alter activity levels ($p > 0.9999$). However, activity was increased at higher concentrations ($p < 0.0001$ for 5 mg/L, $p = 0.0394$ for 10 mg/L). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

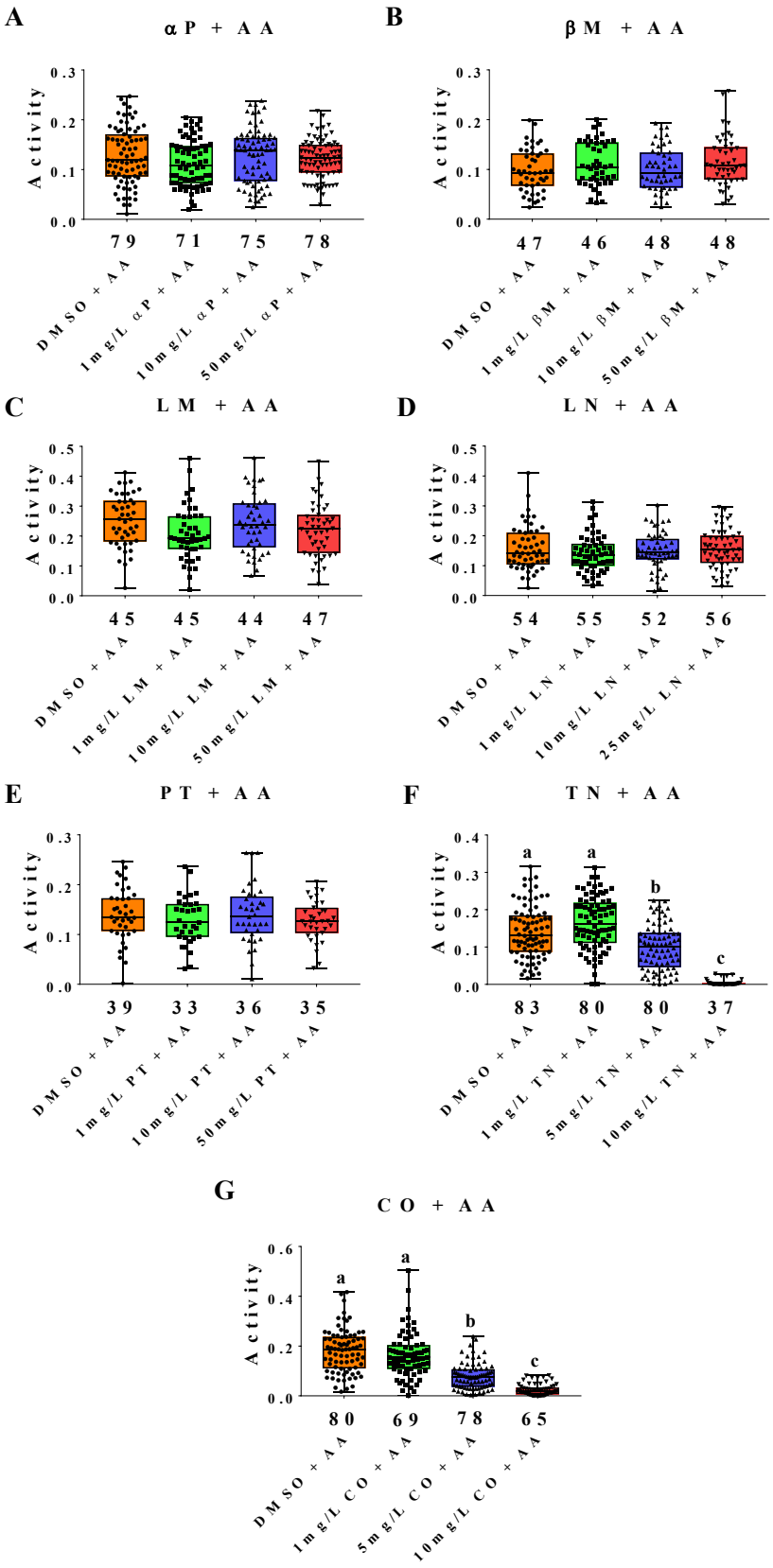

A
αP + AA
Activity
0.3
0.2
0.1
0.0
79
71
75
78
DMSO + AA
1mg/L αP + AA
10mg/L αP + AA
50mg/L αP + AA
B
βM + AA
Activity
0.3
0.2
0.1
0.0
47
46
48
48
DMSO + AA
1mg/L βM + AA
10mg/L βM + AA
50mg/L βM + AA
C
LM + AA
Activity
0.5
0.4
0.3
0.2
0.1
0.0
45
45
44
47
DMSO + AA
1mg/L LM + AA
10mg/L LM + AA
50mg/L LM + AA
D
LN + AA
Activity
0.5
0.4
0.3
0.2
0.1
0.0
54
55
52
56
DMSO + AA
1mg/L LN + AA
10mg/L LN + AA
25mg/L LN + AA
E
PT + AA
Activity
0.3
0.2
0.1
0.0
39
33
36
35
DMSO + AA
1mg/L PT + AA
10mg/L PT + AA
50mg/L PT + AA
F
TN + AA
Activity
0.4
0.3
0.2
0.1
0.0
a
a
b
c
83
80
80
37
DMSO + AA
1mg/L TN + AA
5mg/L TN + AA
10mg/L TN + AA
G
CO + AA
Activity
0.6
0.4
0.2
0.0
a
a
b
c
80
69
78
65
DMSO + AA
1mg/L CO + AA
5mg/L CO + AA
10mg/L CO + AA

Figure 14. Mean % activity of zebrafish larvae at 5 dpf when exposed to terpenoids and acetic acid (AA) (0.001% v/v). **A**. Pre-treatment to 1 mg/L, 10 mg/L and 50 mg/L α-pinene (αP) did not change the acetic acid-induced movement seen in the DMSO control ($p = 0.2860$). **B**. 1mg/L, 10mg/L and 50mg/L β-myrcene (βM) also did not affect acetic acid-induced activity ($p > 0.9999$, $p > 0.9999$, and $p = 0.6207$, respectively). **C**. 1 mg/L, 10 mg/L and 50 mg/L limonene (LM) also did not change acetic acid-induced activity ($p = 0.0586$). **D.** 1 mg/L, 10 mg/L and 25 mg/L linalool (LN) did not change acetic acid-induced activity ($p = 0.5520$). **E.** 1 mg/L, 10 mg/L, and 50 mg/L phytol (PT) did not change acetic acid-induced activity ($p = 0.6032$). **F.** At 1 mg/L, *trans*-nerolidol (TN) did not change acetic acid-induced activity from control ($p = 0.4382$). However, at 5 mg/L, *trans*-nerolidol reduced the activity ($p = 0.0075$) and further reduced it at 10 mg/L ($p < 0.0001$ from both control and 5 mg/L). **G.** At 1 mg/L, (-)-caryophyllene oxide (CO) did not alter acetic acid-induced activity ($p > 0.9999$). However, at 5 mg/L, the activity was significantly reduced ($p < 0.0001$) and further at 10 mg/L ($p < 0.0001$ from both control and 5 mg/L). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

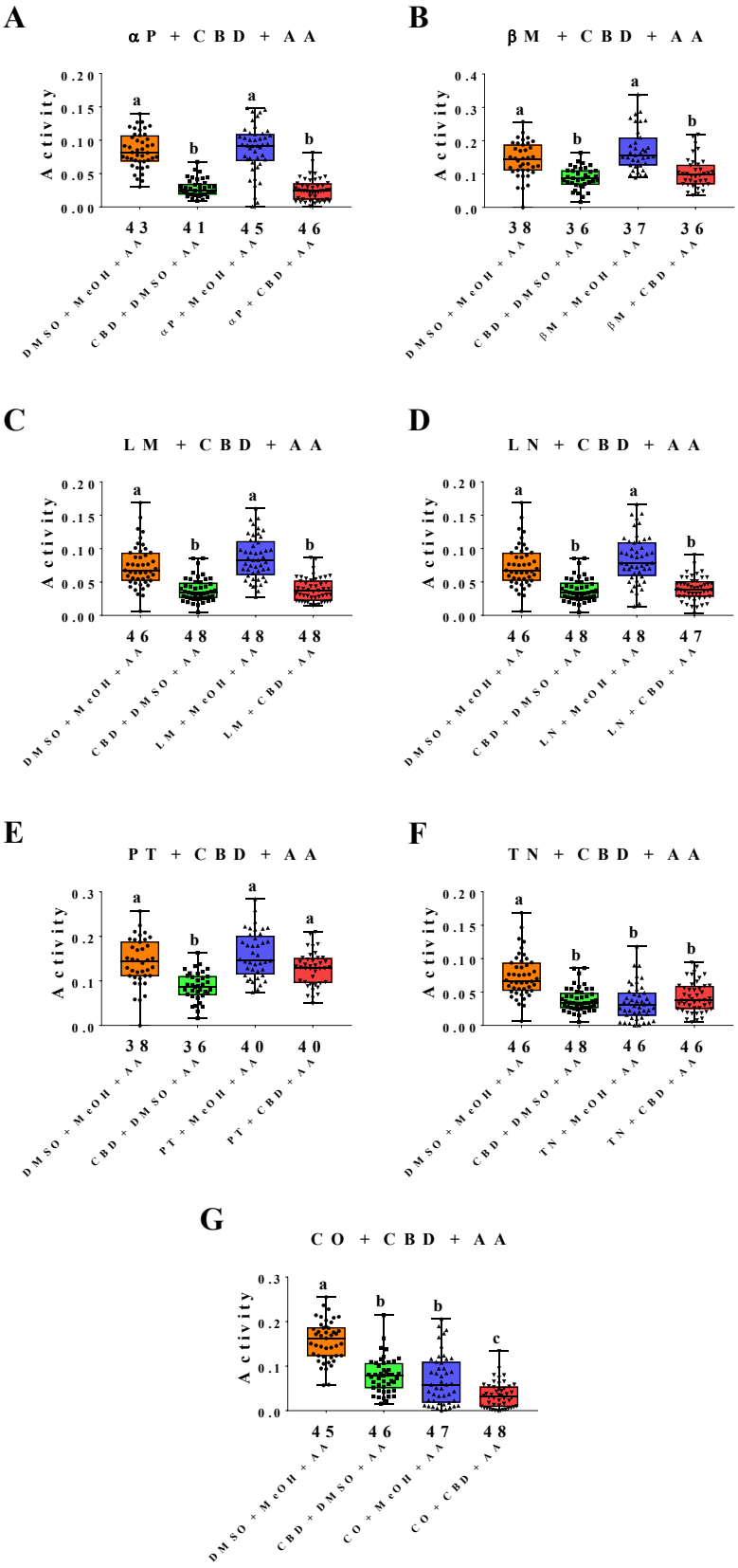

A
αP + CBD + AA
Activity
0.20
0.15
0.10
0.05
0.00
a
b
a
b
43
41
45
46
DMSO + MeOH + AA
CBD + DMSO + AA
αP + MeOH + AA
αP + CBD + AA
B
βM + CBD + AA
Activity
0.4
0.3
0.2
0.1
0.0
a
b
a
b
38
36
37
36
DMSO + MeOH + AA
CBD + DMSO + AA
βM + MeOH + AA
βM + CBD + AA
C
LM + CBD + AA
Activity
0.20
0.15
0.10
0.05
0.00
a
b
a
b
46
48
48
48
DMSO + MeOH + AA
CBD + DMSO + AA
LM + MeOH + AA
LM + CBD + AA
D
LN + CBD + AA
Activity
0.20
0.15
0.10
0.05
0.00
a
b
a
b
46
48
48
47
DMSO + MeOH + AA
CBD + DMSO + AA
LN + MeOH + AA
LN + CBD + AA
E
PT + CBD + AA
Activity
0.3
0.2
0.1
0.0
a
b
a
a
38
36
40
40
DMSO + MeOH + AA
CBD + DMSO + AA
PT + MeOH + AA
PT + CBD + AA
F
TN + CBD + AA
Activity
0.20
0.15
0.10
0.05
0.00
a
b
b
b
46
48
46
46
DMSO + MeOH + AA
CBD + DMSO + AA
TN + MeOH + AA
TN + CBD + AA
G
CO + CBD + AA
Activity
0.3
0.2
0.1
0.0
a
b
b
c
45
46
47
48
DMSO + MeOH + AA
CBD + DMSO + AA
CO + MeOH + AA
CO + CBD + AA

Figure 15. Mean % activity of zebrafish larvae at 5 dpf when exposed to terpenoids (10 mg/L), cannabidiol (CBD) (2.5 mg/L), and acetic acid (AA) (0.001% v/v). In all seven experiments, pre-treatment with CBD decreased acetic acid-induced activity significantly from the respective controls ($p < 0.0001$ for all). **A-D**. Pre-treatment to 10 mg/L of α-pinene (αP) ($p > 0.9999$), β-myrcene (βM) ($p = 0.6073$), limonene (LM) ($p = 0.9742$), or linalool (LN) ($p > 0.9999$) did not change the activity of the DMSO + acetic acid control. While the concurrent treatment of each of these terpenoids with CBD changed the activity level from control ($p < 0.0001$ for AP, $p = 0.0040$ for BM, $p < 0.0001$ for LM, & $p < 0.0001$ for LN), it was the same as the CBD group without alpha-pinene ($p > 0.9999$ for all). **E.** Pre-treatment to 10 mg/L of phytol (PT) did not change the acetic acid-induced activity ($p > 0.9999$). Phytol and CBD, when administered together, also did not differ from the control activity ($p = 0.1078$), and the activity was significantly higher than that of CBD treatment without phytol ($p = 0.0009$). **F.** When pre-treated at 10 mg/L, *trans*-nerolidol (TN) reduced acetic acid-induced activity significantly ($p < 0.0001$), which did not differ from the CBD treatment ($p > 0.9999$). However, the concurrent treatment of both compounds did not result in any further change in activity and did not differ from treatment to either *trans*-nerolidol alone ($p = 0.5590$) or CBD alone ($p > 0.9999$). **G.** Pre-treatment to 10 mg/L (-)-caryophyllene oxide (CO) reduced acetic acid-induced activity ($p < 0.0001$). Additionally, when administered together, CBD and (-)-caryophyllene oxide decreased the activity further ($p < 0.0001$ from control, $p = 0.0001$ from CBD alone, & $p = 0.0170$ from CO alone). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

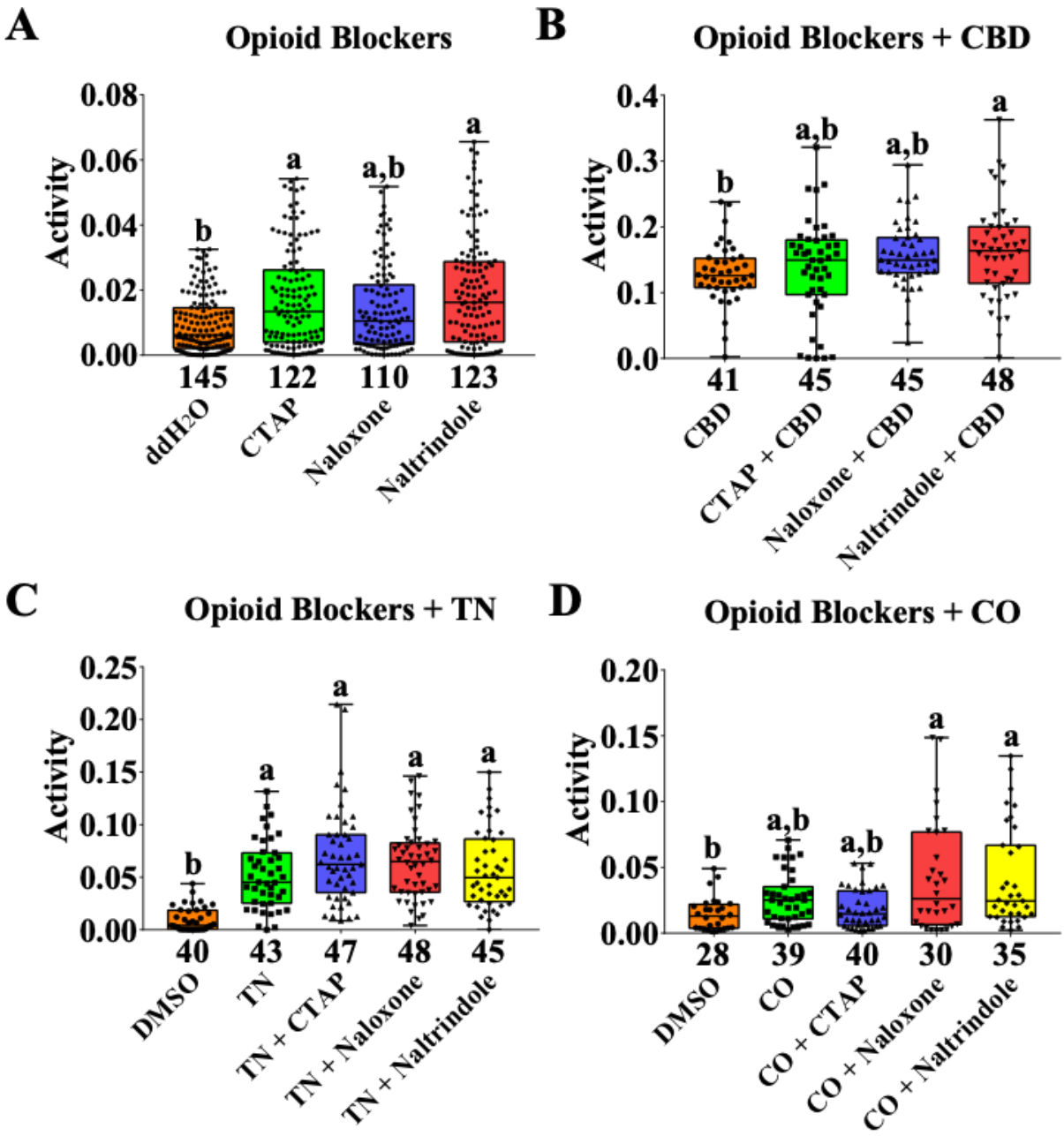


Figure 16. Mean % activity of zebrafish larvae at 5 dpf when exposed to opioid blockers, by themselves or with 2.5 mg/L cannabidiol (CBD), 10 mg/L *trans*-nerolidol (TN), or 10 mg/L (-)-caryophyllene oxide (CO). **A.** CTAP and naltrindole increased activity from ddH_2O control ($p = 0.0004$ and $p < 0.0001$, respectively). **B.** Compared to CBD alone, MOR antagonists CTAP and naloxone did not change the activity level ($p = 0.9831$ and $p = 0.0646$, respectively). Naltrindole, on the other hand, increased the activity from the control ($p = 0.0356$), but still did not differ from the MOR antagonist groups ($p > 0.9999$). **C.** *Trans*-nerolidol increased activity compared to DMSO control ($p < 0.0001$) and the coadministration of opioid receptors did not alter that activity level ($p > 0.9999$). **D.** (-)-caryophyllene oxide did not increase activity from the control ($p = 0.1128$). Coadministration of CTAP did not alter this

($p > 0.9999$), but coadministration of naloxone and naltrindole increased activity significantly ($p = 0.0401$ and $p = 0.0060$, respectively). Activity between (-)-caryophyllene oxide and the opioid receptor blocker groups did not differ ($p = 0.8241$ for CTAP and $p > 0.9999$ for naloxone and naltrindole). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

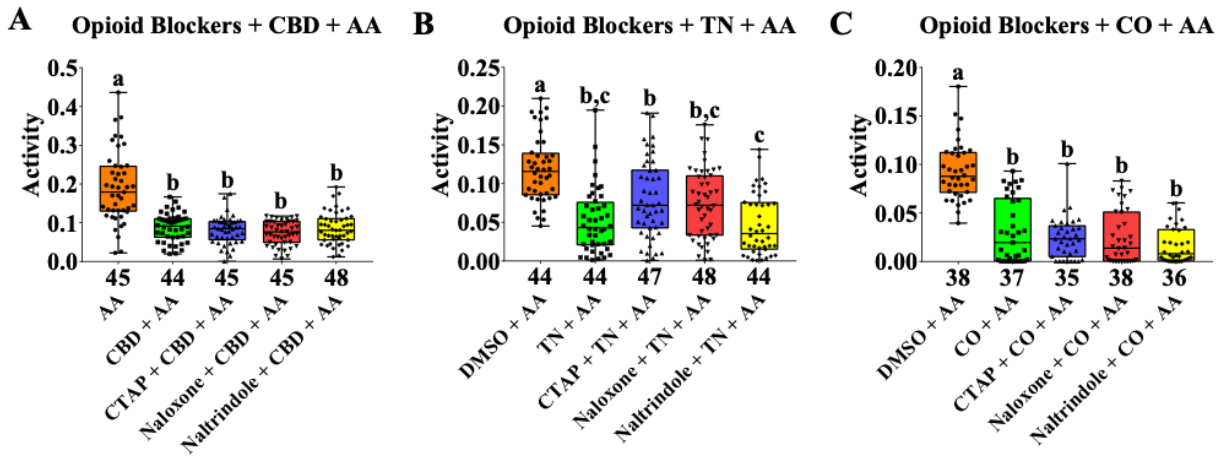
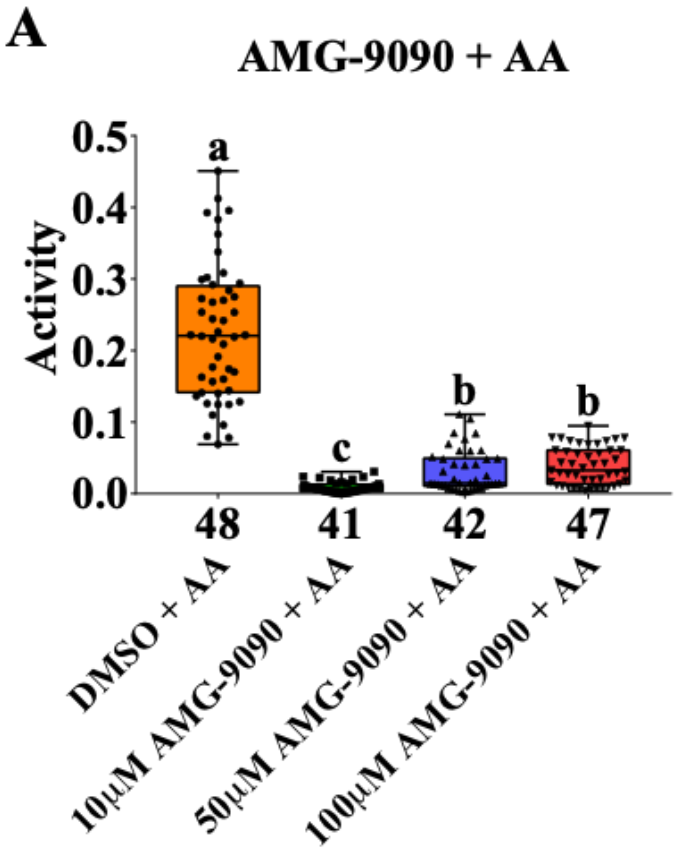


Figure 17. Mean % activity of zebrafish larvae at 5 dpf with **A.** cannabidiol **(**CBD) (2.5 mg/L), **B.** trans-nerolidol (TN) (10 mg/L), or **C.** (-)-caryophyllene oxide (CO) (10 mg/L) and acetic acid (AA) (0.001% v/v), pre-treated with μ-opioid receptor blockers, CTAP (20μM) and Naloxone (0.1 mg/L), and δ-opioid receptor blocker, Naltrindole (20μM). **A.** Treatment with CBD reduced acetic acid-induced activity ($p < 0.0001$). However, pre-treatment with any of the opioid receptor blockers did not alter this reduction in activity ($p > 0.9999$). **B.** *Trans*-nerolidol, when administered before acetic acid, reduced acetic acid-induced activity ($p < 0.0001$). When pre-treated with the opioid blockers, the activity remained significantly lower than the control ($p = 0.0036$ for CTAP, $p = 0.0007$ for naloxone, and $p < 0.0001$ for naltrindole). These activity levels were not significantly different from *trans*-nerolidol treatment without opioid blockers ($p = 0.0735$ for CTAP, $p = 0.2135$ for naloxone, and $p > 0.9999$ for naltrindole). However, the activity level of CTAP treatment group and naltrindole treatment group differed significantly from each other ($p = 0.0182$). **C.** Administration of (-)-caryophyllene oxide, with or without the opioid receptor blockers, significantly reduced the acetic acid-induced activity ($p < 0.0001$ for all). Additionally, each treatment did not differ from one another ($p > 0.9999$ for all). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.


A
AMG-9090 + AA
Activity
0.5
0.4
0.3
0.2
0.1
0.0
a
c
b
b
48
41
42
47
DMSO + AA
10μM AMG-9090 + AA
50μM AMG-9090 + AA
100μM AMG-9090 + AA

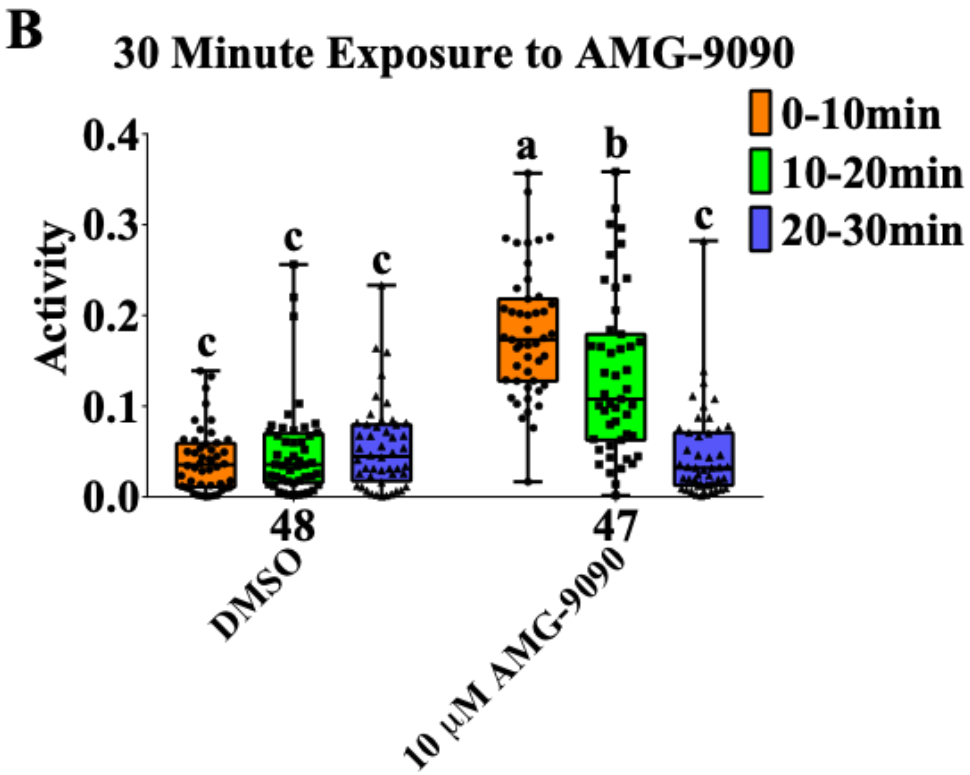

B
30 Minute Exposure to AMG-9090
0-10min
10-20min
20-30min
Activity
0.4
0.3
0.2
0.1
0.0
c
c
c
a
b
c
48
47
DMSO
10 μM AMG-9090

Figure 18. Mean % activity of zebrafish larvae at 5 dpf when exposed to **A.** DMSO (0.25% v/v) or a general TRP channel blocker, AMG-9090 (10 μM, 50 μM, or 100 μM) with acetic acid (AA) (0.001% v/v) or **B.** DMSO (0.25%) or AMG-9090 (10 μM) over a period of 30 minutes. **A.** Exposure to AMG-9090 reduced activity significantly ($p < 0.0001$ for all). Additionally, 10 μM was significantly different from the other two concentrations ($p = 0.0050$ from 50 μM and $p < 0.0001$ from 100 μM). **B.** Each box and whisker plot represents a 10-minute duration. With the exposure to 10 μM of AMG-9090, activity increased drastically upon the initial administration and gradually decreased over time, with the activity level between 10 to 20 minutes being lower than the first ten-minute period ($p = 0.0193$) and the last ten-minute period lower than the second ($p < 0.0001$). On the other hand, with the exposure to DMSO, the activity level remains constant throughout the duration ($p = 0.3718$). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

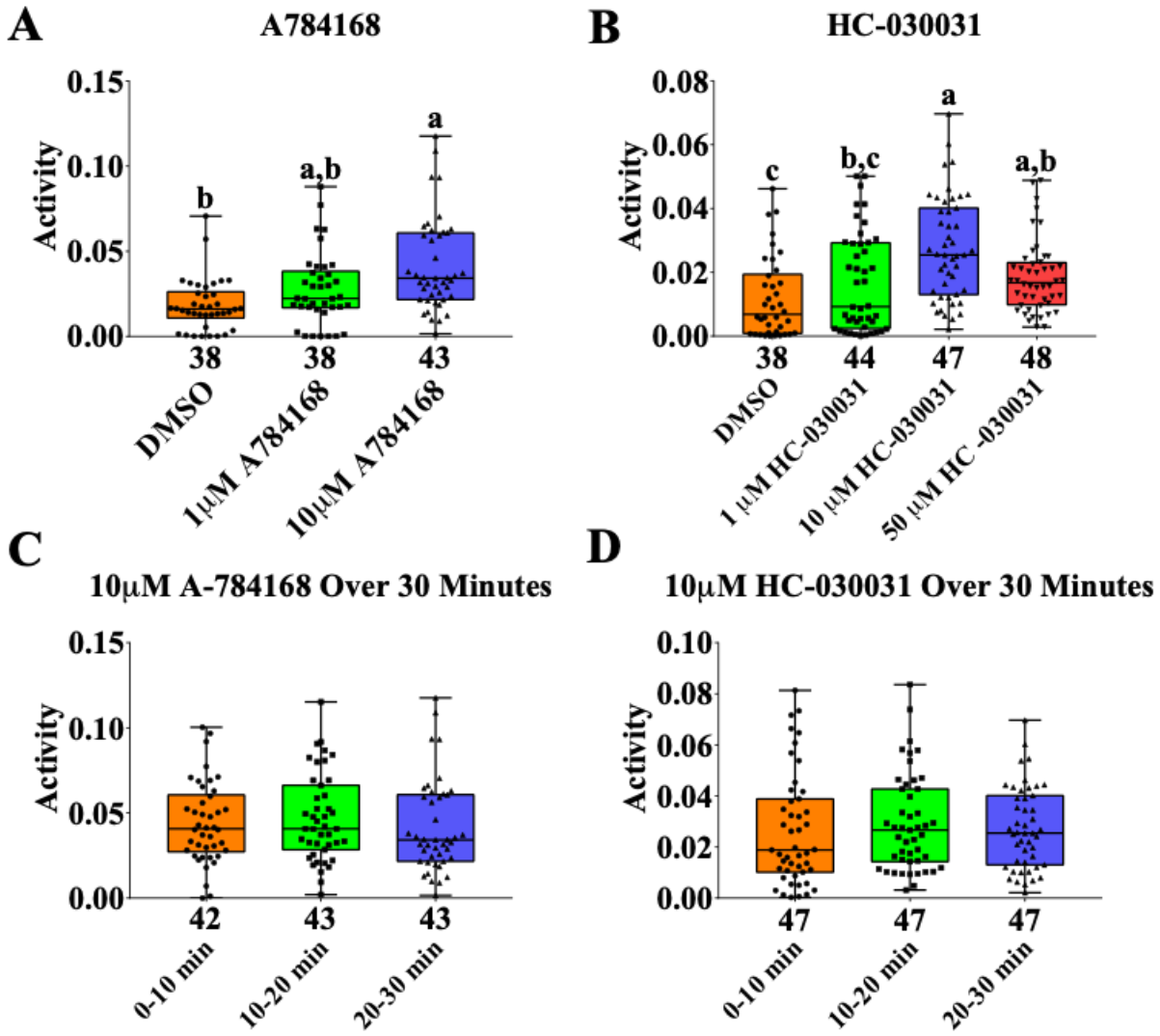


Figure 19. Mean % activity of zebrafish larvae at 5 dpf when exposed to TRPV1 blocker, A784168, or TRPA1 blocker, HC-030031. **A.** A-784168 showed an increase in movement at 10 μM ($p < 0.0001$) compared to control, but not at 1 μM ($p = 0.0670$). **B.** HC-030031 showed an increase in activity at both 10 μM ($p < 0.0001$) and 50 μM (0.0314), but not at 1 μM ($p = 0.6187$). **C-D.** Activity of zebrafish when exposed to **C**. 10 μM A-784168 ($p = 0.4129$) or **D.** 10 μM HC-030031 ($p = 0.5049$) remained constant throughout the thirty-minute exposure. Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

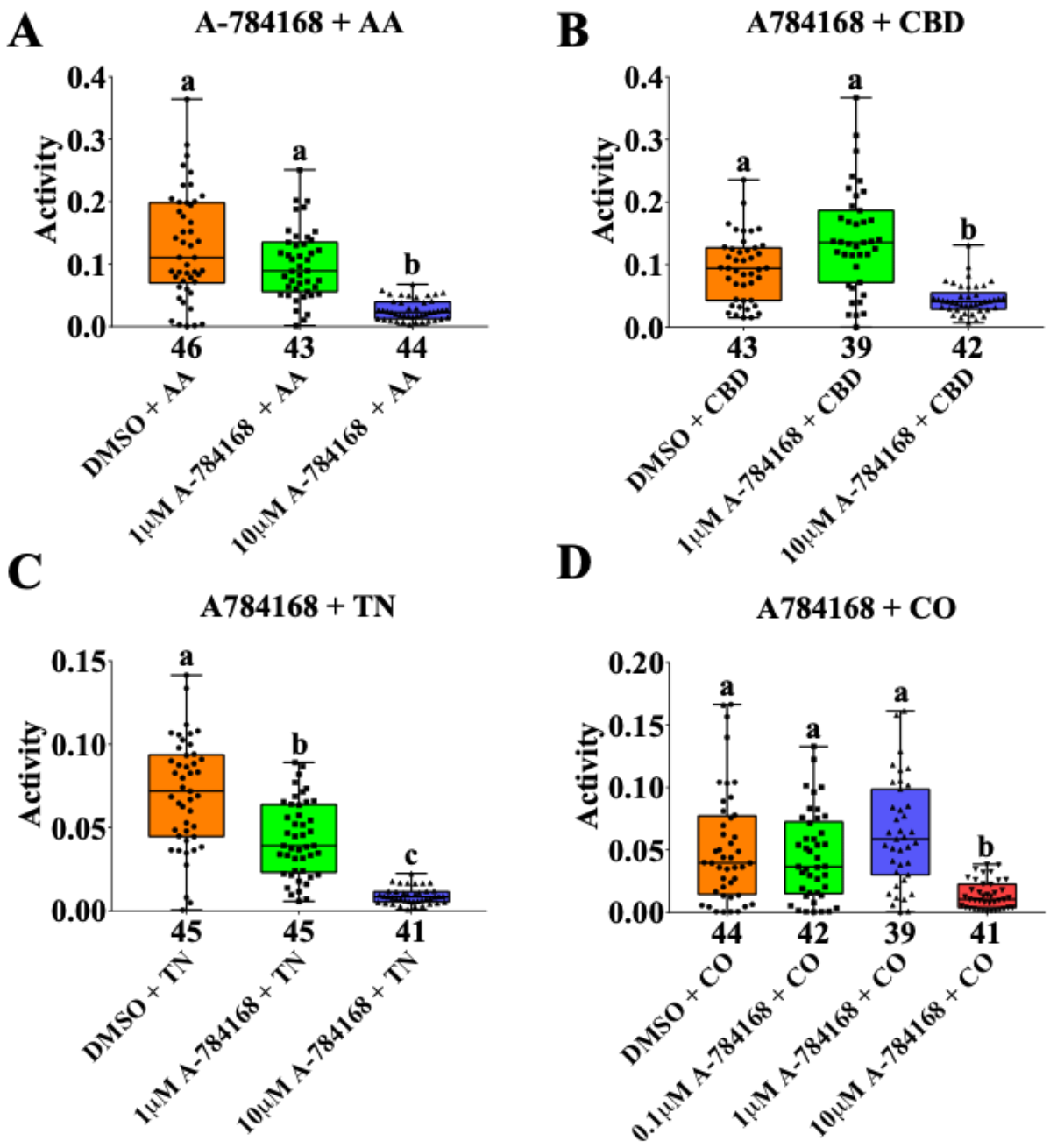


Figure 20. Mean % activity of zebrafish larvae at 5 dpf with TRPV1 blocker, A-784168, and **A.** acetic acid (AA) (0.001% v/v), **B**. cannabidiol (CBD) (2.5 mg/L), **C**. *trans*-nerolidol (TN) (10 mg/L), or **D**. (-)-caryophyllene oxide (CO) (10 mg/L). **A.** Compared to the DMSO + acetic acid control, A-784168 at 10 μM reduced activity significantly ($p < 0.0001$). **B.** Similarly, A-784168 at 10 μM reduced CBD-induced activity significantly ($p = 0.0003$). **C**. With *trans*-nerolidol, A-784168 reduced activity at both tested concentrations ($p = 0.0124$ for 1 μM and $p < 0.0001$ for 10 μM. **D.** A-784168 was able to reduce (-)-caryophyllene oxide-induced activity at 10 μM ($p < 0.0001$) but not at other concentrations ($p > 0.9999$ for 0.1 μM and $p = 0.7542$ for 1 μM). Box and whisker plots show the medium and the inter-quartile

range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

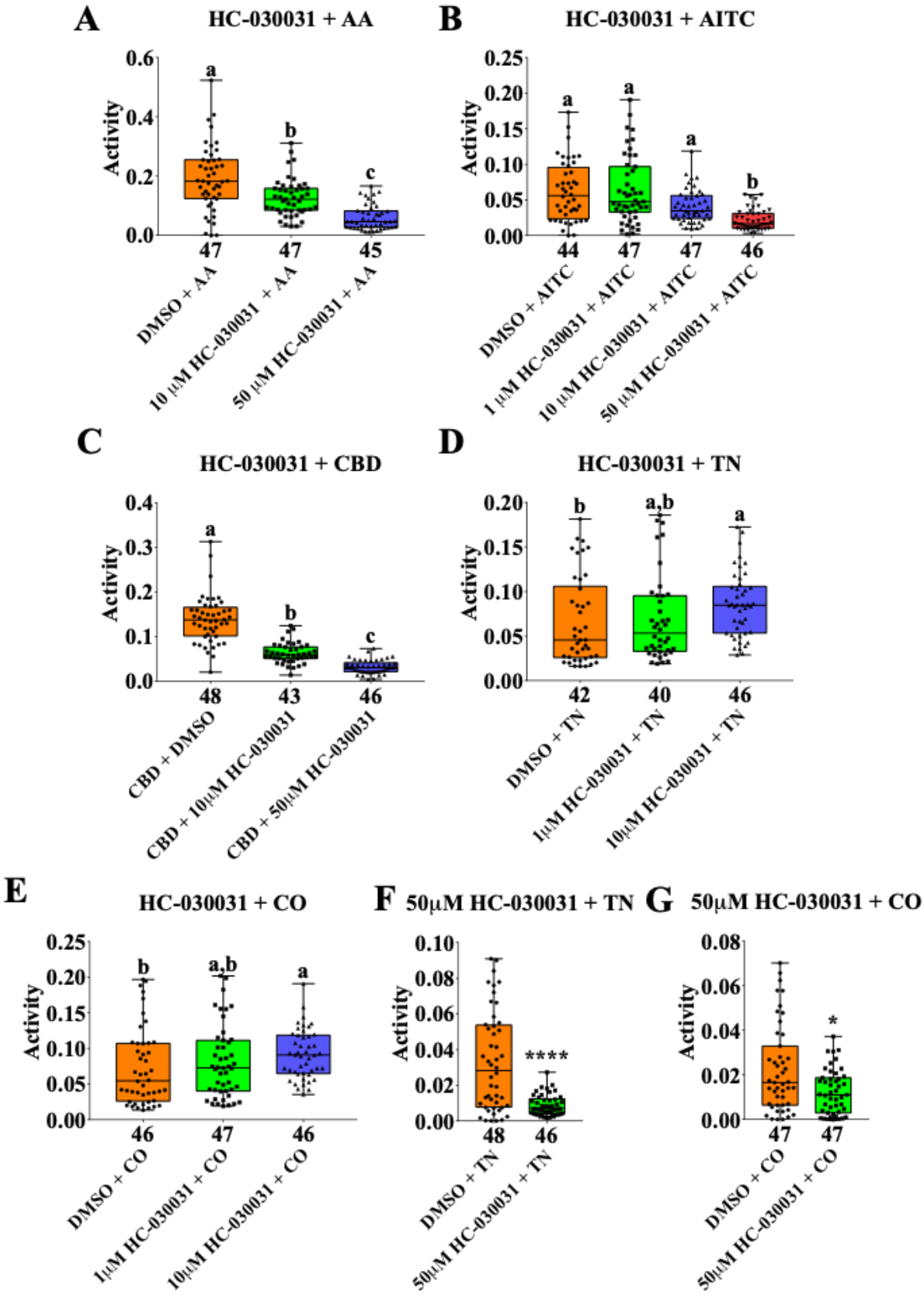

A
HC-030031 + AA
Activity
0.6
0.4
0.2
0.0
a
b
c
47
47
45
DMSO + AA
10 μM HC-030031 + AA
50 μM HC-030031 + AA
B
HC-030031 + AITC
Activity
0.25
0.20
0.15
0.10
0.05
0.00
a
a
a
b
44
47
47
46
DMSO + AITC
1 μM HC-030031 + AITC
10 μM HC-030031 + AITC
50 μM HC-030031 + AITC
C
HC-030031 + CBD
Activity
0.4
0.3
0.2
0.1
0.0
a
b
c
48
43
46
CBD + DMSO
CBD + 10μM HC-030031
CBD + 50μM HC-030031
D
HC-030031 + TN
Activity
0.20
0.15
0.10
0.05
0.00
b
a,b
a
42
40
46
DMSO + TN
1μM HC-030031 + TN
10μM HC-030031 + TN
E
HC-030031 + CO
Activity
0.25
0.20
0.15
0.10
0.05
0.00
b
a,b
a
46
47
46
DMSO + CO
1μM HC-030031 + CO
10μM HC-030031 + CO
F 50μM HC-030031 + TN
Activity
0.10
0.08
0.06
0.04
0.02
0.00
****
48
46
DMSO + TN
50μM HC-030031 + TN
G 50μM HC-030031 + CO
Activity
0.08
0.06
0.04
0.02
0.00
*
47
47
DMSO + CO
50μM HC-030031 + CO

Figure 21. Mean % activity of zebrafish larvae at 5 dpf with TRPA1 blocker, HC-030031, and **A.** acetic acid (AA) (0.001% v/v), **B**. allyl isothiocyanate (AITC) (50 μM), **C.** cannabidiol (CBD) (2.5 mg/L), **D**. & **F**. *trans*-nerolidol (TN) (10 mg/L), or **E**. & **G**. (-)-caryophyllene oxide (CO) (10 mg/L). **A.** HC-030031 at 50 μM showed a lower acetic acid-induced activity than control ($p < 0.0001$) but not at 10 μM ($p = 0.4043$). **B.** Activity induced by AITC was also reduced with 50 μM HC-030031 ($p < 0.0001$), but not with 1 μM or 10 μM ($p > 0.9999$ and $p = 0.3439$, respectively). **C.** CBD-induced activity was also lowered at 10 μM ($p = 0.0002$) and 50 μM ($p < 0.0001$). **D-E.** In contrast to this, at 10 μM, administration of HC-030031 with either *trans*-nerolidol or (-)-caryophyllene oxide led to an increase in activity instead ($p = 0.0123$ and $p = 0.0111$, respectively). **F**-**G**. At 50 μM, HC-030031 reduced the *trans*-nerolidol and (-)-caryophyllene oxide induced activity ($p < 0.0001$ and $p = 0.0177$, respectively). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

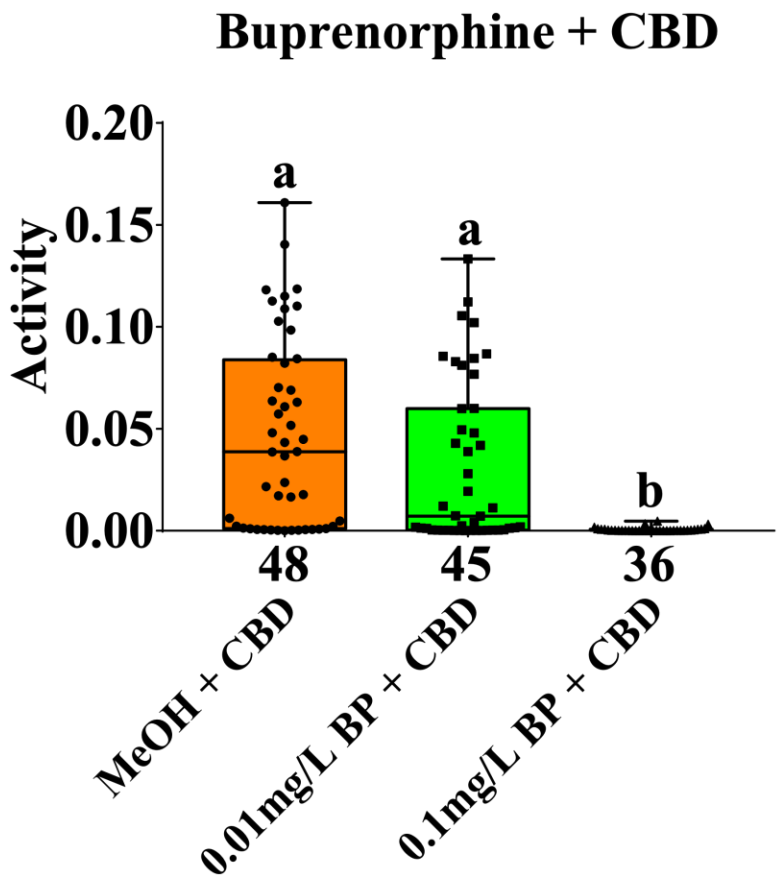


Figure 22. Mean % activity of zebrafish larvae at 5 dpf with cannabidiol (CBD) (2.5 mg/L) and a synthetic μ-opioid receptor agonist, buprenorphine. Buprenorphine at 0.01 mg/L did not change the CBD induced activity ($p = 0.2271$) but did change it at 0.1 mg/L ($p < 0.0001$). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

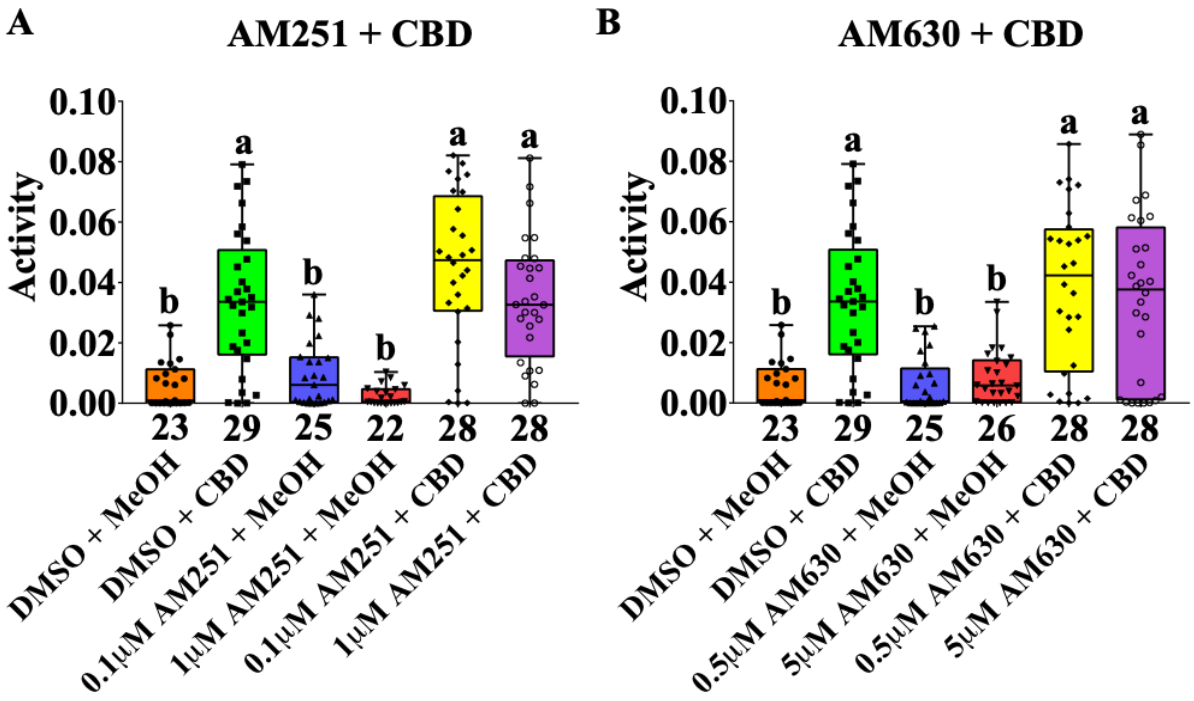


Figure 23. Mean % activity of zebrafish larvae at 5 dpf with CB_1 and CB_2 receptor blockers and 2.5 mg/L cannabidiol (CBD). Administration of CBD had higher activity than the vehicle control (methanol (MeOH)) ($p = 0.0001$). **A.** When administered alone, AM251 did not change the activity level from the control at either 0.1 µM or 1 µM ($p > 0.0001$). The blocker also did not change the activity level from the CBD group when co-administered with CBD at either concentration ($p > 0.9999$). **B.** AM630 also showed the same level of activity as control when administered alone at either 0.5 µM or 5 µM ($p > 0.9999$), and also had the same level of activity as CBD + DMSO group when co-administered with CBD ($p > 0.9999$). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

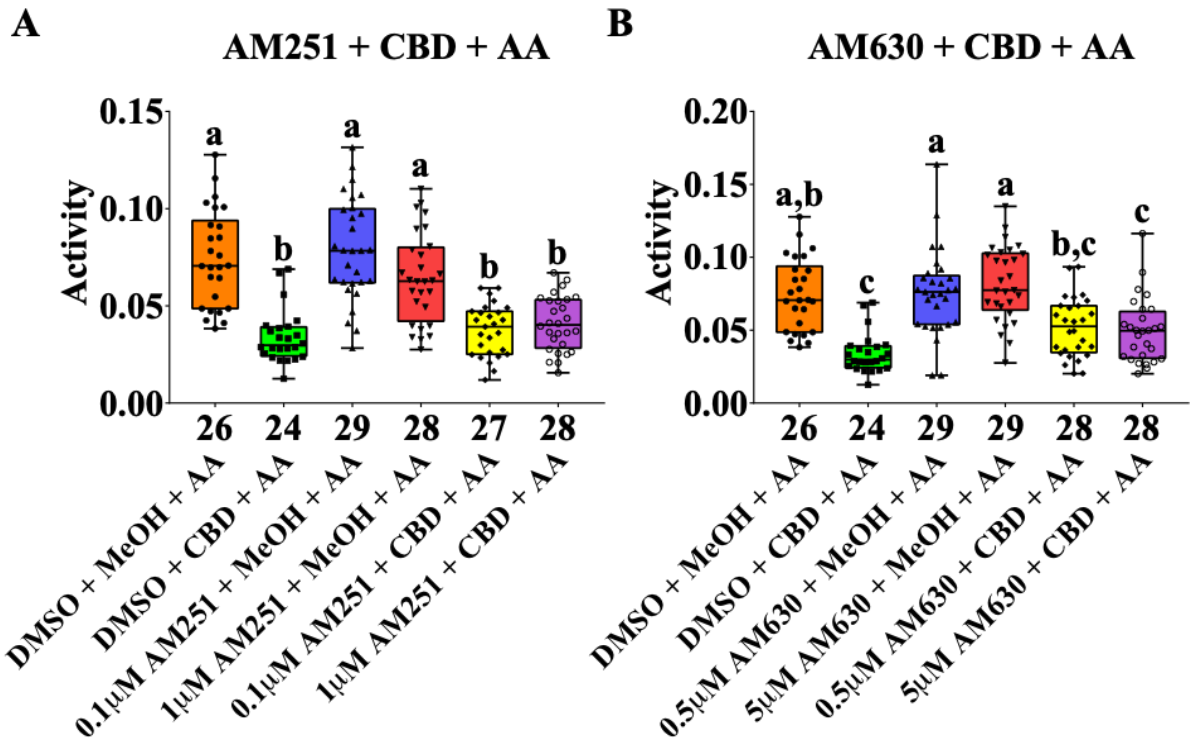


Figure 24. Mean % activity of zebrafish larvae at 5 dpf when exposed to CB_1 and CB_2 receptor blockers, AM251 and AM630, with cannabidiol (CBD) (2.5 mg/L) and acetic acid (AA) (0.001% v/v). **A.** CBD at 2.5 mg/L decreased acetic acid-induced activity ($p < 0.0001$) whereas AM251 at either concentration did not ($p > 0.9999$). Pre-treatment with AM 251 did not change the decrease in activity observed with CBD, regardless of the concentration of AM251 ($p > 0.9999$). **B.** When administered without CBD, AM630 at either 0.5 µM or 5 µM showed the same level of activity as vehicle (methanol (MeOH) and DMSO) + acetic acid control ($p > 0.9999$). However, unlike with AM251, 0.5 µM AM630 and CBD was not different from either the control activity ($p = 0.0548$) or the activity of the CBD group without AM630 ($p = 0.1353$). At 5 µM, on the other hand, AM630 + CBD had a lower level of activity than control ($p = 0.0114$) but was still not significantly different from the activity with CBD alone ($p = 0.4603$). Box and whisker plots show the medium and the inter-quartile range, with the error bars representing the range of minimum to maximum value. Number under each box represents the sample size.

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