University of Reading

The Effects of Phytocannabinoids Cannabidiol and Cannabigerol on Platelet Modulation, Thrombus Formation and Inflammatory Responses

A thesis submitted for the degree of Doctor of Philosophy

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DECLARATION

I confirm that this is my work and the use of all material from other sources has been properly and fully acknowledged.

Signed: Eman Alzahrani
Date: October 2023

ABSTRACT

Cardiovascular disease (CVD) is the leading cause of death worldwide each year. Antiplatelet therapies have been found to reduce the risk of mortality for CVD patients and prevent subsequent occurrences. Platelets are small circulating blood cells that perform critical roles in blood clotting and the maintenance of haemostasis. In pathological settings, inappropriate activation of platelets causes thrombosis in the arterial circulation, obstructing blood flow to key organs such as the heart and brain, resulting in myocardial infarction and stroke, respectively. Anti-platelet medications are currently in use, and while they do help save lives, they are accompanied by several undesirable side effects, including gastrointestinal toxicity, gastrointestinal ulcers, nausea, and bleeding. These adverse reactions are caused by the therapeutic agents themselves. Therefore, the development of improved therapeutic strategies to treat/prevent thrombotic disorders is a pressing concern. Non-psychoactive phytocannabinoids derived from the *Cannabis Sativa* plant, such as cannabigerol (CBG) and cannabidiol (CBD), have been shown to possess several therapeutic benefits in a variety of pathological disorders. As a result, the purpose of this study was to demonstrate the effects of CBD and CBG on agonists-induced platelet activation, thrombus formation under arterial flow conditions, and haemostasis in mice. CBD and CBG were found at the used concentrations (1.56, 3.125, 6.25, 12.5 and 25 μM) to reduce platelet aggregation when stimulated by a variety of agonists, showing their broad impact on platelet signalling pathways. Moreover, each of CBD and CBG at the mentioned concentrations was found to block granule release and intracellular calcium mobilisation in platelets, as well as inside-out signalling of the integrin αIIb- β3 and outside-in signalling that was activated by the same integrin. CBD and CBG also decreased thrombus formation on a collagen-coated surface under arterial flow conditions. In addition, CBD and CBG had modest effects on the haemostasis of mice. Even at high concentrations such as 100 μM, CBD and CBG did not have any cytotoxic effects on platelets.

Their anti-inflammatory properties decrease inflammatory cytokines released by induced platelets and reduce platelet-leukocyte aggregates. It has been found that CBD and CBG impair platelet activity by interacting with a variety of molecules, including AKT, Src, and cAMP. In light of these findings, the study shows that CBD and CBG have the potential to operate as treatments to regulate inappropriate platelet reactivity under a variety of pathophysiological condition.

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PRESENTATIONS

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The Modulating Effects of Phytocannabinoids on Thrombosis and Hemostasis

ABBREVIATIONS

ACD – acid citrate dextrose

ADP – adenosine diphosphate

ANOVA – analysis of variance

ATP – adenosine triphosphate

AU – arbitrary units

BSA – bovine serum albumin

Ca^{+2} – calcium ion

cAMP – cyclic adenosine monophosphate

CD62P – P-selectin

cGMP – cyclic guanosine monophosphate

CHD – coronary heart disease

CO_2 – carbon dioxide

COX – cyclooxygenase

CRP-XL – cross-linked collagen-related peptide

CVD – cardiovascular disease

Cy5 – cyanine 5 dye

C57BL/6 – C57 Black 6 mice

DAG - diacylglycerol

DIC – disseminated intravascular coagulation

DioC6 – 3,3'-dihexyloxacarbocyanine iodide

DTS – dense tubular system

EGTA – ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid eNOS – endothelial NOS

ERK – extracellular signal-regulated kinase

FcR – Fc receptor

FITC – fluorescein isothiocyanate

g – g-force

g – grams

Gads – Grb2-related adaptor protein downstream of Shc

GEF – guanine nucleotide exchange factor

GP – glycoprotein

GPCR – G-protein coupled receptor

GPO – Gly-Pro-Hyp

GTP – guanosine triphosphate

h – hour(s)

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acidIC50 – half maximal inhibitory concentration **Ig** – immunoglobulin

iNOS – inducible NOS

IP3 – inositol triphosphate

ITAM – immunoreceptor tyrosine-based activation motif KCl – potassium chloride

kg – kilogram

LAT – linker for activation of T cells

LTA – light transmission aggregometry

M – molar

MAPK – mitogen-activated protein kinase

mg – milligram

MgCl2 – magnesium chloride

mg/kg – milligrams/kilogram

mL – millilitre

mm – millimetre(s)

mRNA – messenger RNA

µg – microgram

µL – microliter

µM – micro-molar

NaCl – sodium chloride

NaHCO3 – sodium bicarbonate

Na2HPO4 – disodium phosphate

nm – nanometre

nM - nanomolar

NO – nitric oxide

NOS – nitric oxide synthase

OCS – open canalicular system

PBS – phosphate-buffered saline

PE – phycoerythrin

PGI2 - prostacyclin

PH – pleckstrin homology

PIP2 – phosphatidylinositol 4,5-bisphosphate

PIP3 – phosphatidylinositol 3,4,5-trisphosphate

PI3K – phosphoinositide 3-kinase

PKA – cAMP-dependent protein kinase

PKC – protein kinase C

PKG – cGMP-dependent protein kinase

PLC – phospholipase C

PRP – platelet-rich plasma

PVDF – polyvinylidene fluoride

RBC – red blood cell RPM – revolutions per minute

s – second(s)

SDS – sodium dodecyl sulphate

SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM – standard error of the mean

SFK – Src-family kinase

SH2 – Src homology 2

SLP-76 – SH2 domain-containing leukocyte protein of 76 kDa

Syk – spleen tyrosine kinase

TBS-T – Tris-buffered saline with Tween 20

TGX – Tris-glycine eXtended

TP – thromboxane receptor

U/mL – unit/millilitre

U46619 – 9,11-Dideoxy-11a,9a-epoxymethanoprostaglandin

v/v – volume/volume

VWF – von Willebrand Factor

w/v – weight/volume

Y – tyrosine

^{o}C – degrees centigrade

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CHAPTER ONE

1. Introduction

1.1 Introduction

Cardiovascular diseases (CVD) are the most frequent cause of death and morbidity globally. Two-thirds of all fatalities worldwide are due to non-communicable diseases (NCDs). According to the World Health Organisation (WHO), CVD alone accounts for almost 50% of all NCD fatalities and by 2030 costs associated with CVD worldwide are projected to increase from approximately $863 billion to $1.44 trillion (WHO., 2017). Moreover, in the United Kingdom, the leading cause of death for men is heart disease and is considered the second cause of death for women. It costs over £26 billion in the overall cost for treating and preventing CVD according to the government's statistics in 2016. In the United States, adult spending for CVD significantly rose between 1996 and 2016, going from $212 billion to $320 billion (Birger et al., 2021). CVD represents a group of diseases or conditions that affect the cardiovascular system including the heart, blood vessels and blood. Thrombotic diseases are the major CVD that occur due to the formation of blood clots within the circulation unnecessarily under various pathological conditions. Platelets and blood clotting factors play critical roles in maintaining the haemostasis to prevent excessive bleeding upon vascular injury. However, their inappropriate activation results in thrombosis i.e., the formation of blood clots within the vasculature unnecessarily. Inappropriate activation of platelets under pathological conditions such as the rupture of an atherosclerotic plaque results in the development of thrombi under arterial circulation. Here, the clotting factors play a minimal role. However, the activation of clotting factors results in thrombosis in veins with minimal input from platelets. Blood vessel occlusion or restricted blood flow is the outcome of thrombus development. While the currently used anti-platelet drugs help save lives, they are associated with serious side effects such as bleeding. Therefore, there is an urgent need to develop better, efficacious, and safer drugs to treat and prevent thrombotic conditions. Since platelets play critical roles in the modulation of haemostasis, thrombosis, and inflammatory responses, they act as a powerful therapeutic target for thrombotic diseases (Dwivedi and Pomin, 2020).

Platelets are small discoid-shaped cells with a size of around 2 - 4 μm in diameter with a lifespan of 6 - 9 days in humans. The primary function of these cells is to form blood clots to stop bleeding upon vascular injury. As mentioned above, the normal process of forming a blood clot to stop bleeding is called 'haemostasis' (Davì and Patrono, 2007).

It is the physiological state where platelets form clots to prevent bleeding and maintain vascular integrity. The human body on average produces approximately 100 billion new platelets daily to maintain haemostasis. Additionally, platelets play secondary roles in innate immunity and angiogenesis (Von Hundelshausen and Weber, 2007). However, abnormal clot formation, such as thrombosis, is detrimental to the body and is one of the major factors contributing to a range of pathological conditions affecting the heart and blood vessels. Coronary heart disease (CHD), ischemic stroke, and peripheral arterial disease are important CVDs that are caused by abnormal clotting/thrombosis due to inappropriate activation of platelets (Stokes and Granger, 2012). Hence, thrombotic conditions need urgent medical attention to avoid life-threatening conditions.

1.2 Production and Development of Platelets

Bone marrow is the primary site of platelet production where long cytoplasmic extensions of highly specialised progenitor cells called megakaryocytes produce platelets after various stages of maturation and differentiation (**Figure 1.1**) (Von Hundelshausen and Weber, 2007). Platelet production is regulated by thrombopoietin (TPO) that binds to the cMpl receptor on the megakaryocyte surface (Dwivedi and Pomin, 2020). TPO regulates the DNA replication and endomitosis of megakaryocytes to synthesise proplatelets. A single megakaryocyte can produce ten to twenty proplatelets. As the proplatelet grows, it becomes thinner and more branched to develop into platelets while receiving organelles and granules from megakaryocytes. Later, the developing platelets are released into blood sinusoids to release the platelets into circulation (Dwivedi and Pomin, 2020). Each megakaryocyte produces around 10^{11} fully developed platelets per day (Bianchi et al., 2016). Due to this remarkable process, the platelet population is continuously renewed every 6-9 days, counted at roughly one trillion platelets in adults (Machlus and Italiano, 2013).

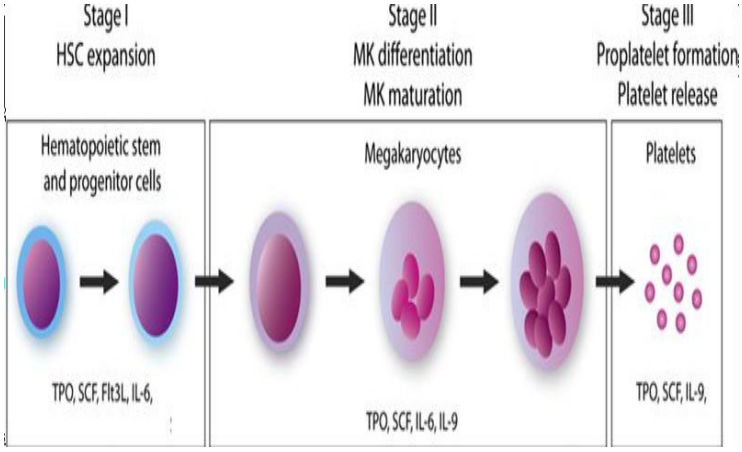


Figure 1.1: Platelet production stages.

It starts with the commitment of hematopoietic stem cells to megakaryocyte (MK) progenitors, their proliferation and differentiation, polyploidization of precursor cells, and maturation of megakaryocytes. Platelets are produced through a process called proplatelet formation, where long pseudopodial elongations break off in the blood flow. The differentiation and proplatelet formation processes are regulated by different transcription factors, such as Thrombopoietin (TPO), GATA-1 and NF-E2. This figure was adapted from (Lee, Godara and Haylock, 2014)

1.3 Structure of Platelets

Platelets contain all essential components that are required to perform their haemostatic and other roles. These components are comprised of membranes, organelles, intracellular signalling proteins and surface receptors as shown in (**Figure 1.2**). Here, these components are briefly discussed for a better understanding of platelets.

1.3.1 Platelet Membrane

The platelet membrane contains glycoproteins that interact with different ligands and induce signalling to regulate platelet function. The membrane is composed of a characteristic bilayer of phospholipids, cholesterol, and membrane proteins (Grozovsky et al., 2015). These phospholipids are selectively permeable for selective molecules to facilitate internal platelet activation and act as a surface for external blood coagulation (Periayah, Halim and Saad, 2017). The open canalicular system (OCS) is a unique feature of platelets and this is a network of numerous membrane folds that connect the platelet interior to the exterior via small pores (Selvadurai and Hamilton, 2018) (**Figure 1.2**). Upon platelet activation, these invaginated membrane folds promote platelet spreading by providing the required membrane surface and thus, platelets extend their surface to around 12 μM (Semple, Italiano and Freedman, 2011). The dense tubular system (DTS) is another important membranous system in platelets, that is developed from the smooth endoplasmic reticulum of megakaryocytes. DTS stores calcium along with other components that support platelet activation (Lam, Vijayan and Rumbaut, 2015). Lastly, actin, filamin, tubulin, and spectrin are proteins that assist in maintaining the structure of the cytoskeleton and connect the cytoplasm to the plasma membrane. These microtubule proteins help platelets retain their discoid shape in resting conditions (Lam, Vijayan and Rumbaut, 2015).

1.3.2 Platelet Organelles

Platelet organelles primarily include α granules, dense granules, and lysosomes (**Figure 1.2**) (van der Meijden and Heemskerk, 2019a). The largest-sized granules in the platelet's cytoplasm are alpha granules (200 - 500nm) and they account for around 50–60 granules per platelet.

They are also responsible for the granular appearance of the cytoplasm, which is mainly due to the presence of platelet factors involved in haemostasis and thrombosis (Jenne and Kubes, 2015). A variety of adhesion molecules and coagulation factors such as fibrinogen as well as growth factors and chemokines are found in these granules (van Gils, Zwaginga and Hordijk, 2009). The dense granules in platelets are the smallest granules (around 100-200 nm) and they contain non-proteinous substances such as calcium, serotonin, adenine nucleotides and phosphate. They appear as condensed masses under electron microscopy (Smyth et al., 2009). Each platelet has 3–8 dense granules (Clemetson and Clemetson, 2019). Each platelet contains 1 to 3 lysosomes, which contain hydrolytic enzymes including acid phosphatase, cathepsin, and galactosidase, among others that help in the clearance of thrombi and phagocytosis of invading pathogens (Dwivedi and Pomin, 2020).

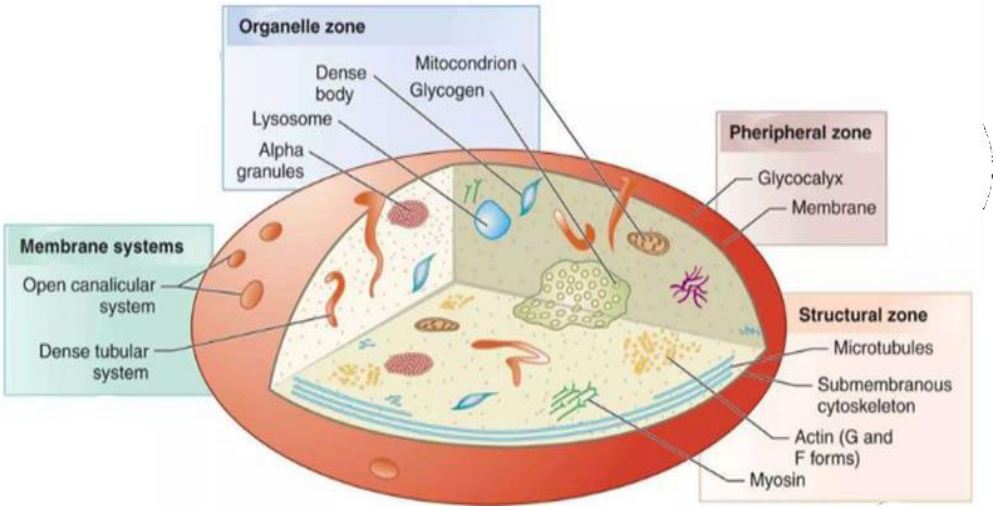


Figure 1.2: Schematic representation of platelet contents.

The platelet plasma membrane has an external coat of glycoproteins and glycocalyx. The cytoplasm is rich in actin, microfilaments, and microtubules to maintain the discoid shape when not activated. To facilitate the flow of molecules in and out of the platelet and to provide additional membrane space for spreading, the open canalicular system which is membrane folds is present in platelets. In addition, there are several lysosomes, mitochondria, glycogen storage, and dense tubular systems in the cytosol. This figure was adapted from (Gunnarsdottir, 2014)

1.3.3 Platelet Receptors

The activation of platelets is an important step in the haemostasis process and is extensively dependent upon various receptors. There are numerous platelet receptors which regulate the functions of platelets as required (Clemetson and Clemetson, 2019). The following sections describe some important receptors that are required for platelet activation (**Figure 1.2**).

The GPIb-V-IX complex includes GPIb as the major receptor that is involved in the initial contact between platelets and the subendothelial matrix upon vessel wall injury. Approximately 25,000 copies of GPIbα are present in each platelet (BERNDT et al., 1985). Its primary function is to tether platelets to the exposed collagen through its interaction with the von Willebrand factor (VWF). Evidence suggests that this complex can also stimulate tyrosine kinase signalling. Upon localisation to lipid rafts, binding of vWF to the complex results in phosphorylation of the Fc Receptor (FcR) γ-chain and adaptor molecules to initiate signalling (BERNDT et al., 1985). This in turn leads to calcium mobilisation, phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) activity.

Integrins are a family of large transmembrane adhesion receptors and are heterodimers comprised of non-covalently associated α and β subunits (Watson et al., 2005). Integrin αIIbβ3 is a fibrinogen receptor; with around 40000 copies of in each platelet (Huang et al., 2019). The integrin αIIbβ3 is a key adhesion receptor found on platelets that plays a crucial role in platelet-platelet interactions and aggregation (Bennett, 2005). It binds to fibrin and VWF along with fibrinogen in order to facilitate the formation of platelet aggregates (Kuijpers et al., 2004). Integrin activation causes it to shift from a low-affinity state to a high-affinity state, allowing efficient binding to fibrinogen (Bennett, 2005). This binding is essential for various platelet functions, including adhesion, aggregation, spreading, and clot retraction (Bye., 2016). The integrin also serves as a link between the platelet's external environment and its cytoskeleton (Gibbins, 2004). Activation of the integrin triggers intracellular signaling events, leading to increased calcium levels, granule secretion, and further platelet activation. Due to its important role in platelet function regulation, the integrin αIIbβ3 has become a target for therapeutic agents used in the treatment and prevention of thrombotic diseases (Watson et al., 2005).

Integrin α2β1 with 2000-4000 copies/platelet is a key adhesion receptor involved in the binding of collagen to platelets (Saboor et al., 2013). In its resting state, integrin α2β1 is in a low-affinity state with its extracellular domain in a closed conformation (Jokinen et al., 2009). However, activation of the GPVI receptor leads to a rapid shift of integrin α2β1 into a high-affinity state (Watson et al., 2005). This activation induces conformational changes in the extracellular domains, transitioning them from a closed to an open conformation that allows binding to collagen (Sarratt et al., 2005). This binding of integrin α2β1 to collagen is crucial for platelet adhesion and binding to the damaged blood vessel walls (Nieswandt et al., 2001). It plays a critical role under low shear conditions, whereas the interaction between GPIb-V-IX and VWF is more important under high shear conditions (Varga-Szabo, Pleines and Nieswandt, 2008a).

Collagen-GPVI binding and subsequent increased affinity of integrin α2β1 for collagen leads to the formation of filopodia and lamellipodia in platelets (Nieswandt et al., 2001). Evidence has shown that in platelets deficient in β1, GPVI signalling is diminished (Kuijpers et al., 2004). GPVI is a platelet and megakaryocyte-specific 62kDa type I transmembrane collagen receptor, with approximately 3000-6000 copies per platelet. GPVI binds collagen through the recognition of specific Gly-Pro-Hyp (GPO) sequences in collagen (Kuijpers et al., 2004). Platelet activation upon collagen binding to GPVI results in calcium mobilisation, granule secretion and integrin activation (Watson et al., 2005). This activation is also demonstrated by the GPVI-specific ligand, collagen- related peptide (CRP-XL), which potently activates and aggregates platelets. The phosphorylation of the FcR γ-chain ITAM results in the binding of Syk via its Src homology 2(SH2) domains, which is subsequently auto-phosphorylated and phosphorylated by Src kinases (Nieswandt et al., 2001). Syk proceeds to phosphorylate linker for activation of T cells (LAT), which leads to the formation of a signalosome structure composed of adaptor and effector proteins. Two such adaptor proteins key to the activatory process are Gads (Grb2-related adaptor protein downstream of Shc) and SLP-76 (SH2-domain containing leukocyte protein of 76kDa) (Smith-Garvin, Koretzky and Jordan, 2009). The activation of platelets through collagen binding to GPVI results in the secretion of platelet granules, which contain numerous molecules including ADP that act to drive the process forward (Nieswandt and Watson, 2003).

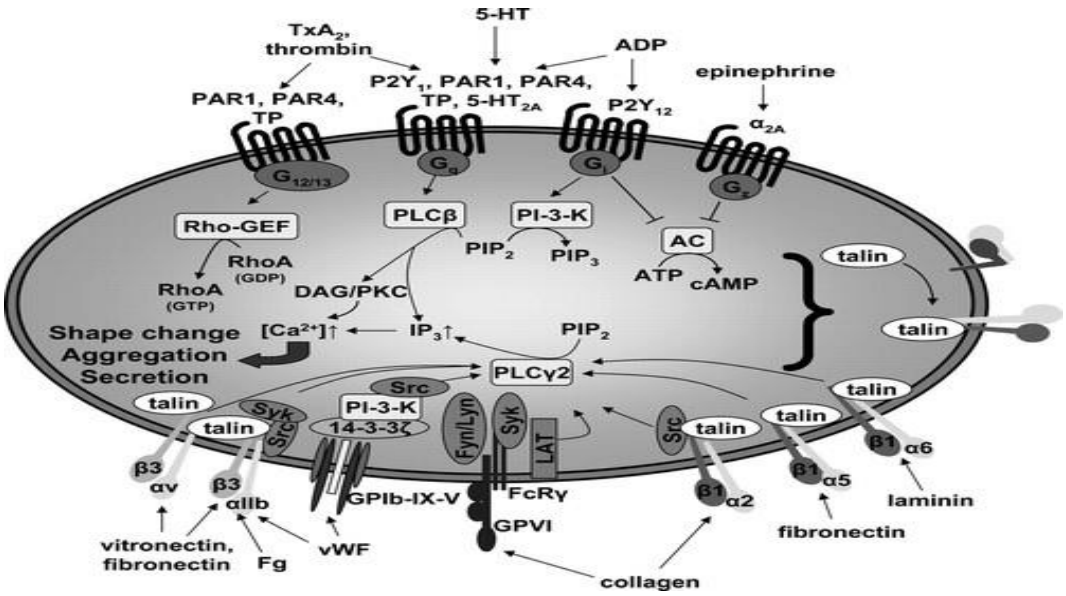
In addition, thromboxane A2 is synthesised and released from activated platelets, and it can also active platelets. These agonists stimulate platelet activation via binding to G-protein coupled receptors

(GPCRs).

Proteinase-activated receptor 1 (PAR1) is a high-affinity receptor for thrombin, with approximately 1500-2500 copies per platelet, while PAR4 contributes to the activatory process at higher concentrations. Thrombin does not bind to PAR1/4 in a typical receptor- ligand manner, instead, it cleaves the extracellular N-terminal region of the receptor. This cleavage serves to unmask a new peptide, which acts as a tethered ligand, binding to the receptor's second extracellular loop (Clemetson and Clemetson, 2019). Heterotrimeric G proteins Gαq and Gα13 subunits activate phospholipase C isoforms, leading to the release of Ca^{2+} from intracellular stores and the activation of protein kinase C (PKC).

ADP binds two different purinergic receptors known as $P2Y_1$ with 400 copies/platelet and $P2Y_{12}$ with 150 copies/platelet (Zhao et al., 2022). These two receptors may be required for ADP-induced platelet activation by the dissociation of the G-protein into α and βγ dimer subunits and activation of the Gαq subunit. It also activates phospholipase Cβ (PLCβ), which acts to cleave phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), with the downstream mobilisation of Ca^{2+} from intracellular stores. ADP-induced activation of PI3K leads to the production of PIP3, resulting in the downstream activation of Akt, a serine/threonine kinase, and Rap1B, which subsequently leads to the activation of integrin αIIbβ3 and promotion of granule secretion (Hemmings and Restuccia, 2012). It has been observed that upon stimulation of platelets with ADP, platelets become unresponsive to subsequent ADP stimulation; this is thought to be due to receptor desensitization and/or internalisation(Campbell, Cohn, and Savage, 2014). This requirement for concomitant activation is also demonstrated using drugs such as clopidogrel, which selectively inhibits $P2Y_{12}$, demonstrating significant anti-platelet effects (Cattaneo, 2004).

Thromboxane A2 (T_XA2) is a potent agonist for platelet activation and vasoconstrictor that is produced within the activated platelets. T_XA2 diffuses across the plasma membrane where, due to its short half-life, it acts in an autocrine or paracrine manner to provide positive feedback activation and aggregation. Thromboxane receptors are coupled to Gαq and Gα12/13 G-proteins and stimulate the activation of RhoA, Rho kinase and PLCb(Hemmings and Restuccia, 2012).



<u>Figure 1.3:</u> Major platelet receptors.

Platelet activation occurs when adhesion receptors interact with their ligands, such as collagen and von Willebrand factor. These receptors include integrins α2β1 and αIIbβ3, as well as the GP Ib–V–IX complex. At this stage, signaling involves small G-protein regulators, Src-family kinases, and serine/threonine-protein kinases. Soluble agonists like ADP and thromboxane A2 are then released, activating $P2Y_{12}$ and $P2Y_1$, as well as the TP receptor. These receptors stimulate various signaling pathways, leading to the release of calcium, activation of protein kinases, and downstream events. GPVI and C-type lectin-like receptor 2 also induce strong signaling through protein tyrosine kinase pathways. Thrombin further contributes to platelet activation by activating Gαq-coupled receptors PAR1 and PAR4. This figure was adapted from (Varga-Szabo, Pleines and Nieswandt, 2008).

1.4 Mechanism of Activation of Platelets

Activation of platelets is initiated by exposure to collagen revealed from the sub- endothelial matrix at injured blood vessels (Smyth et al., 2009). Platelets bind to immobilized adhesive proteins, such as collagen, that are exposed to the subendothelial matrix during vascular damage (Watson et al., 2005). The VWF attaches to GPIb-V-IX on the platelet's surface, causing platelets to roll across the endothelium surface at the location of the injured vessel (Semple, Italiano and Freedman, 2011). Platelet binding to VWF allows platelets to interact with collagen via GPVI and integrin, facilitating the creation of a platelet monolayer at the injury site (Bennett, 2005).

Following the interaction of collagen with GPVI, results in the release of pro- thrombotic substances such as ADP and T_XA2 (produced by oxygenation of arachidonic acid by cyclooxygenase-1 and T_XA2 synthase) (Montalescot, 2010). So, these chemicals activate and attract more circulating platelets around the injury site via specific platelet surface receptors, subsequently a platelet plug formed. Following the creation of a platelet plug, active platelets engage in intracellular signalling to help stabilise the thrombus (Nieswandt and Watson, 2003). This is initiated by outside-in signalling caused by fibrinogen attachment to αIIβ3, whereas intracellular signalling stimulates the transition of integrin IIβ3 from a low-affinity state to a high-affinity state (Moroi and Jung, 2004). The activation of integrin IIβ3 activates the fibrinogen receptor, which increases fibrinogen affinity/binding and facilitates the formation of fibrinogen cross-links between platelets, resulting in a stable platelet aggregate with cytoskeletal reorganisation and clot retraction, which contributes to hemostasis (Huang et al., 2019).

Furthermore, platelet activation generates thrombin, as part of the coagulation cascade. To avoid blood loss, the coagulation cascade involves the conversion of blood from the liquid phase to the thick structural phase (Periayah, Halim and Saad, 2017). This process involves more than 30 proteins known as coagulation factors where these proteins act through different pathways. However, both intrinsic and extrinsic pathways generate activated factor X, which leads to the cleavage and transformation of prothrombin (Factor II) to thrombin (Factor IIa). Factor IIa works as a catalyst for the conversion of soluble fibrinogen (Factor I) to insoluble fibrinogen (Factor Ia), resulting in a mesh-like network of aggregated platelets. This cascade results in the creation of a fibrin-rich haemostatic block, which prevents blood loss from wounded arteries (Semple, Italiano and Freedman, 2011).

Finally, fibrinolysis is a controlled enzymatic system that stops thrombi from growing unnecessarily by preventing additional fibrin accumulation by activating fibrinolysis (Wu et al., 2016). Plasmin is generated from plasminogen by the action of tissue plasminogen activator on the surface of the fibrin clot, and it is essential for clot dissolution (Whyte et al., 2015). This procedure prevents the formation of additional thrombi while also dissolving the clot to restore blood supply and promote wound healing.

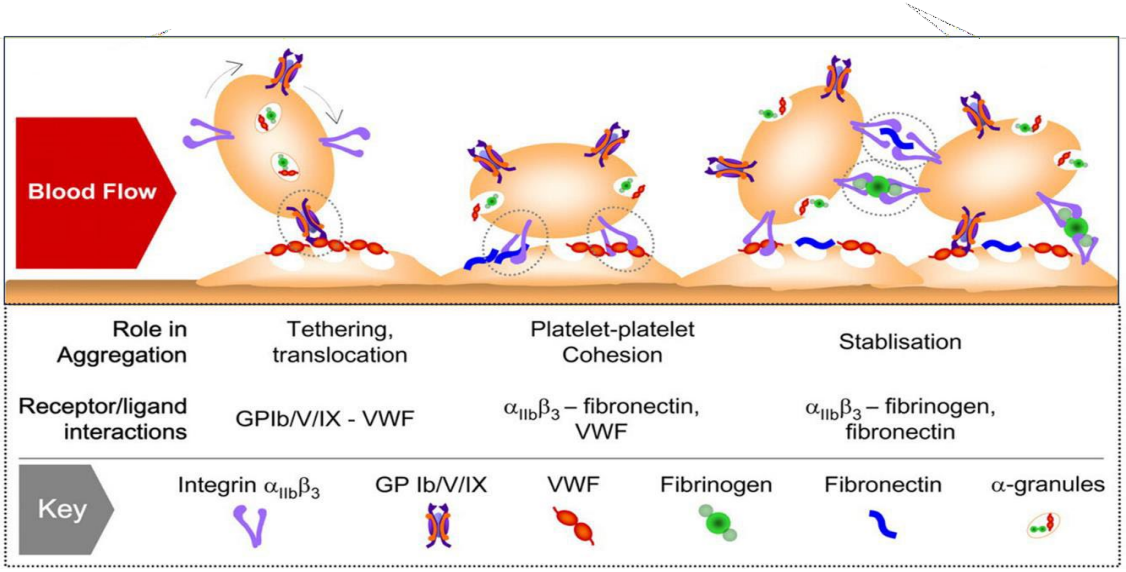


Figure 1. 4: Schematic representation of platelet activation.

After an injury to a blood vessel, platelet adhesion and aggregation in order to form a plug starts with an interaction of von Willebrand factor (VWF) with platelet receptors. Platelets release substances like ADP and thromboxane A2 (T_XA2) to attract more platelets to the injury site. Intracellular signaling pathways are then activated to stabilize the clot, involving the attachment of fibrinogen to platelet receptors and cytoskeletal reorganization. Thrombin is crucial for the formation of a blood clot after tissue damage. As a hemostatic plug or thrombus develops, the fibrin and fibronectin matrix typically form at the interface between the injured vessel wall and the platelet plug. This matrix helps to stabilize the clot and promote further coagulation. Overall, the interaction between fibronectin, platelets, and the injured vessel wall is a key component of the hemostatic process. This figure was adapted from (Jackson, 2007).

1.5 Role of platelets

1.5.1 Haemostasis

Platelets in a resting state circulate normally in the bloodstream. At the resting state, healthy endothelial cells release various vasoactive factors, such as nitric oxides (NO) and prostaglandin (PGI2) that inhibits adhesion and aggregation (McRae, 2011), by this means promoting blood fluidity and preventing thrombosis. So, platelet activation is critical to maintain the vascular integrity and haemostasis. Platelet function by detecting vascular damage and rapidly aggregate together to seal the site of damaged tissue and prevent bleeding. As result of platelet activation, thrombin is produced and then activates and recruits more platelets and eventually coagulation cascade (Ruggeri, 1997). The coagulation cascade leads to form a fibrin-rich haemostatic plug, that leads to a stable thrombus which prevents the blood loss from injured vessels (Smyth et al., 2009).

1.5.2 Thrombosis

Thrombosis is the process that involves the formation of a blood clot or thrombus inside a blood vessel inappropriately (Montalescot,2010). This occurs due to an underlying health condition such as the rupture of atherosclerotic plaque results in the formation of thrombi within the circulation (Ruggeri, 1997). Thrombosis is classified by their site; venous or arterial thrombosis, i.e., whether it is in a vein or an artery (Eisinger, Patzelt and Langer, 2018). Arterial thrombosis is a clot formed in a high shear flow artery which can obstruct or stop the flow of blood to major organs, such as the heart or brain, causing myocardial infarction and stroke (Baaten et al., 2018). Inhibition of platelet activation and aggregation is the strategy to treat arterial thrombosis such as Aspirin. Venous thrombosis affects deep and superficial veins of lower limbs which includes deep vein thrombosis, renal vein thrombosis, portal vein thrombosis, cerebral venous sinus thrombosis, etc (Branchford and Carpenter, 2018). The pathophysiology behind thrombus formation in vein includes hypercoagulability of blood, injury to blood vessel walls and abnormal flow of blood in the vessels. Consequently, venous thrombosis is mainly treated with anti-coagulant therapies targeting proteins mediating coagulation (Palta, Saroa and Palta, 2014).

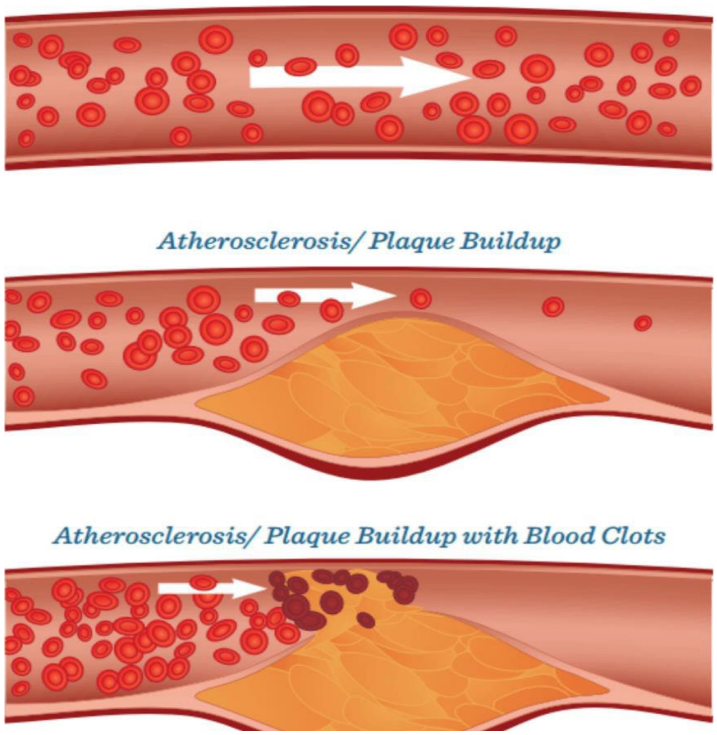


Figure 1.5: Schematic representation of atherosclerosis.

Atherosclerosis is hardening of the arteries. It begins with damage to the endothelium of an artery. This damage leads to the accumulation of blood cells and plaque at the site of injury. This figure was adapted from (Saveljic et al., 2018)

1.5.3 Immunity

Platelets play an important role in the regulation of immune and inflammatory responses, as well as wound healing, and pathogen surveillance in addition to their well- known role in haemostasis(Ali, Wuescher and Worth, 2015). These roles have been elegantly demonstrated due to the discovery of numerous immune receptors in platelets, including Toll-like receptors (TLRs) such as TLR2, 4, and 9, immunoglobulin receptors, including the Sig-lec familyand formyl peptide receptors (FPRs) (Crocker, Paulson and Varki, 2007).

Activated Platelets release a variety of chemokines including CXCL1, PF4 (CXCL4), CXCL5, CXCL7, IL-8 (also known as CXCL8), CXCL12, macrophage inflammatory protein-1 (also known as CCL3), and RANTES (CCL5)(Nagareddy and Smyth, 2013; Barbara et al., 2016). These molecules regulate leukocyte recruitment, their migration from the vasculature into tissues, and other proinflammatory functions such as phagocytosis and ROS generation (Wachowicz et al., 2002). It has been postulated that the proinflammatory cytokine IL-1β generated by activated platelets has a significant role in the development of vascular inflammation (Yun et al., 2016). Platelets expose P-selectin upon activation, and this plays a critical role in mediating the interactions between haemostasis and inflammation. Heterodimeric transmembrane receptors in the integrin family communicate with other cells through the extracellular matrix (ECM) and cell adhesion molecules. Platelets express a variety of β1 and β3 integrins, including α2β1, α5β1 (VLA-5, fibronectin receptor), α6β1 (VLA-6, laminin receptor), αIIbβ3 (fibrinogen receptor), and αvβ3 (vitronectin receptor). Under normal shear settings, these adhesion molecules and ligands complex permit and accelerate platelet adhesion to a variety of distinct cellular and structural targets (Lam, Vijayan and Rumbaut, 2015; Clemetson and Clemetson, 2019).

Due to their ability to bind and react to pathogen-associated molecular patterns (PAMPs) as well as damage-associated molecular patterns (DAMPs), platelets serve as critical mediators of innate immunity (Alharbi et al., 2019). The surface ligands CD62P, CD40L, and integrins are all found on activated platelets, and inflammatory mediators.

such as IL-1β or chemokines are secreted. These molecules enable the platelets to interact with leukocytes and trigger innate immune responses (Iba and Levy, 2018). When platelets are activated, several antimicrobial peptides present in them are also released including platelet factor 4, platelet basic protein (PBP) and its derivatives, connective tissue activating peptide 3, and thymosin B-4 (TB-4) (Semple, Italiano and Freedman, 2011; Koupenova et al., 2018). They are involved in supporting the clearance of invading pathogens. Kinocidins (antimicrobial chemokines) and cationic host defence peptides (CHDPs) are examples of other antimicrobial molecules released from platelets. Kinocidins and CHDPs, in addition to their antibacterial capabilities, have diverse biological activities that broaden platelet participation in several physiological and disease-related processes (Ali, Wuescher and Worth, 2015; Jenne and Kubes, 2015). Additionally, platelets stimulate B lymphocyte isotype switching and the molecular machinery necessary for antigen presentation to cytotoxic T lymphocytes (CTL), including the immunoproteasome, the 2-microglobulin, and major histocompatibility complex (MHC) class I molecules (Koupenova et al., 2018).

1.6 Anti-platelet Agents

Antiplatelet drugs work through preventing platelet sticking together and decrease their ability to form blood clots. There are now four types of anti-platelet medications available: 1- Platelet aggregation inhibitors such as Aspirin and related cyclooxygenase inhibitors, Oral thienopyridines such as clopidogrel, ticagrelor, ticlopidine, and prasugrel, 2- Glycoprotein platelet inhibitors (e.g., abciximab, eptifibatide, tirofiban), 3- Protease- activated receptor-1 antagonists (e.g., vorapaxar). 4- Miscellaneous (e.g., dipyridamole - a nucleoside transport inhibitor and phosphodiesterase type 3 (PDE3) inhibitor, cilostazol also a PDE3 inhibitor) those that are used alone, those that are used in combination, and those used for research (Chua and Nishi, 2013). The mortality and morbidity associated with arterial thrombosis have been lowered primarily as a result of these drugs (**Figure 1.6**). However, inhibiting platelet activity is intrinsically related to a higher risk of bleeding and intolerance in some patients, which may end in severe cardiovascular outcomes and, in rare circumstances, death (Ito, Miyakoda and Mori, 2004; Ahrens et al., 2007; Totani et al., 2012).

Recently, there has been a surge in the production of natural substances derived from plants that have historically been used to treat platelet problems, which is a promising development. Natural compounds originating from plants have long been a source of medicine development. WHO estimates that roughly 80% of people worldwide still utilize herbal medication (Ekor, 2013).

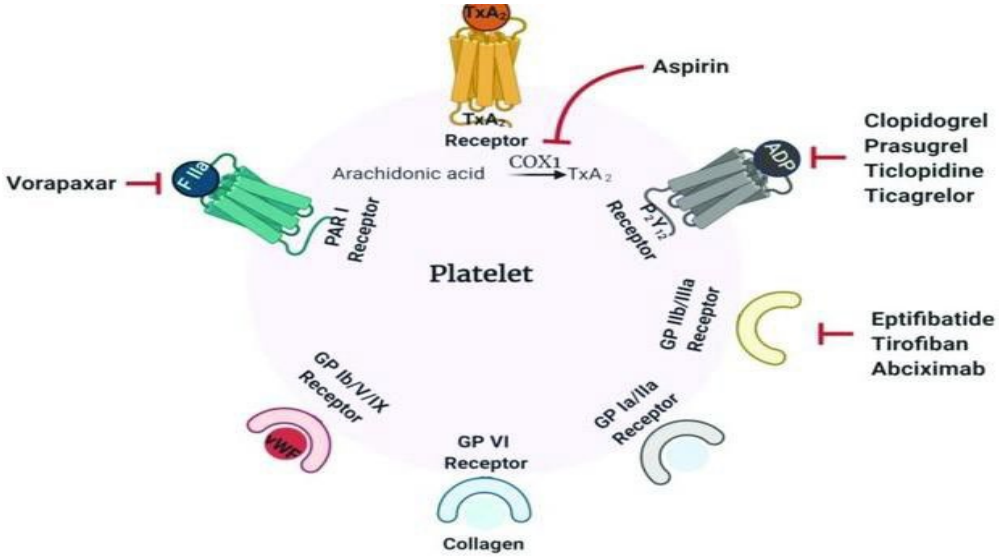


Figure 1. 6: The mechanism of action of commonly used antiplatelet medications.

COX inhibitors, ADP receptor antagonists, PAR 1 inhibitors, and GP IIb/IIIa inhibitors. Aspirin, one of the oldest and most often given antiplatelet medicines. ADP receptor antagonists include clopidogrel, prasugrel, ticagrelor, and ticlopidine. Inhibiting thrombin-mediated PAR 1 cleavage, Vorapaxar reduces platelet activation. The GP IIb/IIIa receptor is responsible for platelet aggregation. Inhibitors like eptifibatide, tirofiban, and abciximab block fibrinogen from binding to the GP IIb/IIIa surface receptor, reducing platelet aggregation and thrombus formation. This figure was adapted from (Dwivedi and Pomin, 2020).

1.7 Phytocannabinods: Introduction

Many chemicals have been extracted from medicinal plants and they are recognized for their diverse therapeutic properties. Some of them have been utilised by humans for thousands of years (Prasathkumar et al., 2021). These compounds were also used as templates for the development of innovative pharmaceutical products. For instance, marijuana (Cannabis) has been used for a range of medical, recreational, and cultural purposes since prehistoric times, according to ancient documents from Mesopotamia, India, and China (Andre, Hausman and Guerriero, 2016). *Cannabis* is mostly derived from the herbaceous plant, *Cannabis sativa* (commonly known as *Indian hemp*), which is a member of the mint family (Andre, Hausman and Guerriero, 2016). This plant contains more than 525 active natural chemicals. They include alkanes, sugars, nitrogenous compounds (such as spermidine alkaloids or muscarine), flavonoids, non- cannabinoid phenols, phenylpropanoids, steroids, fatty acids, and around 140 different terpenes that are predominant in cannabis, which are all beneficial to the health (Cavalli and Dutra, 2021). The cannabinoids are the most active chemicals of these classes, and they are a class of natural molecules classified based on their C21 terpenophenolic backbone which are found mostly in the trichomes of female flowers (Radwan et al., 2021). Over 100 cannabinoids, generally referred to as phytocannabinoids, have been identified from the *cannabis* plant so far.

Most studied cannabinoids are as follows 9-tetrahydrocannabinol (9-THC), cannabidiol (CBD), cannabidivarin (CBDV), cannabigerol (CBG), cannabinol (CBN), and 9-tetrahydrocannabivarin (**Figure 1.7**) (Andre, Hausman and Guerriero, 2016). Their effects are primarily mediated through modification of the endogenous cannabinoid system, which is involved in the regulation of the brain, immune, and endocrine functions. As a result, many chemical compounds that can influence the endocannabinoid system have been manufactured. These chemicals are called synthetic cannabinoids (Pacher, Bátkai and Kunos, 2006). This project will focus exclusively on two phytocannabinoids, CBD and CBG due to their clinical significance.

1.7.1 Endocannabinoid system (ECS)

Endocannabinoids are widespread neuromodulator systems that plays important roles in central nervous system (CNS) development, synaptic plasticity, and the response to endogenous and environmental signals (Lu and MacKie, 2016). It is a well-efficient system since it is responsible for the production and breakdown of all the required enzymes and endocannabinoids. The two main endocannabinoids are anandamide and 2- arachidonoylglycerol (2-AG) (Pacher, Bátkai and Kunos, 2005b). Cannabinoid receptors (CB) are responsible for the effects of cannabinoids which are widely distributed. CB1 receptors are primarily in the brain and central nervous system, while CB2 receptors are mainly in the immune system and peripheral tissues. Studies show that CBD interacts with CB1 and CB2 receptors in a different manner than CBG which has a higher affinity for CB2 (Lu and MacKie, 2016). Development of cannabinoid-based therapies was coordinated with the better understanding for ECS using both cannabis and synthetic cannabinoids that has new possibilities for a wide range of medical conditions in order to help in chronic pain, inflammation, anxiety, and neurodegenerative disorders (De Laurentiis et al., 2010). ECS has numerous cardiovascular effects including decrease blood pressure and myocardial contractility (Pacher, Bátkai and Kunos, 2005a), relax coronary and other arteries and decrease cardiac work (Wagner et al., 2005), and direct vascular actions. It is also involved in a protective manner in response to stressors such as arrhythmias (Krylatov et al., 2002).

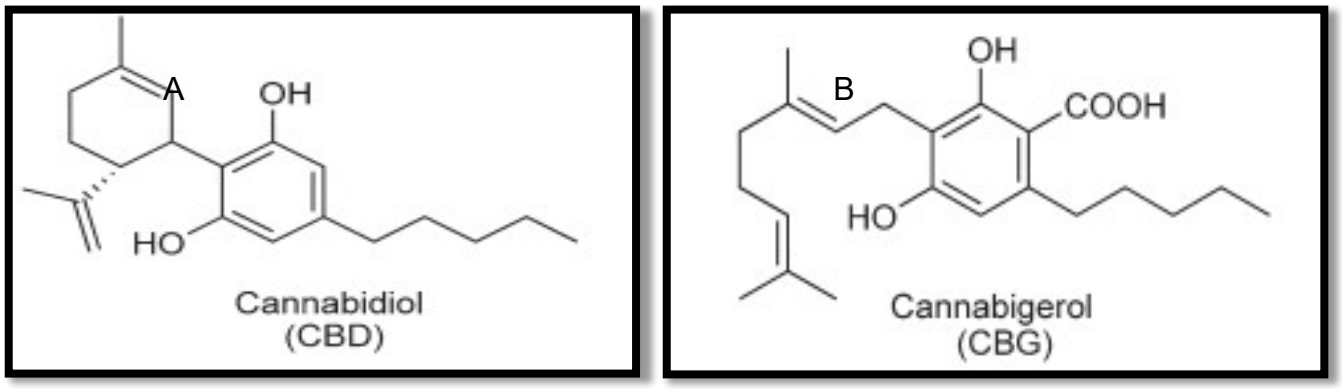


Figure 1.7: Chemical structures of CBD (A) and CBG (B).

1.8. Phytocannabinoids

1.8.1 Cannabidiol (CBD)

CBD is a non-psychoactive constituent of *cannabis* that has shown significant promise in the treatment of most common neurodegenerative disorders. It is a 21-carbon terpene phenolic molecule that is produced by decarboxylation of cannabidiolic acid precursor. CBD can also be produced synthetically. There is currently no evidence of recreational use of CBD or any public health risks linked with its usage. CBD is safe and well tolerated, and its safety profile is satisfactory. CBD has also been reported to act as a negative allosteric modulator of cannabinoid receptors (Thomas et al., 2007), acting as an antagonist of the GPR55(Ryberg et al., 2007), and the equilibrative nucleoside transporter (ENT) at sub- to low-micromolar concentrations. However, it acts as an agonist of the TRPA1 channel and the 5-HT1α receptor (De Petrocellis et al., 2011). CBD activates the peroxisome proliferator-activated receptor (PPAR) at higher micromolar concentrations (De Petrocellis et al., 2011). CBD also prevents adenosine reuptake by inhibiting the equilibrative nucleoside transporter (De Petrocellis et al., 2011). Cannabidiol has been found to exhibit positive effects on neurological disorders such as epilepsy, anxiety, and schizophrenia. CBD has demonstrated the ability to decrease both the frequency and intensity of seizures in certain individuals diagnosed with epilepsy, leading to the approval of a CBD-derived medication for specific forms of epilepsy (McPartland and Russo, 2012). Anxiety disorders had a great potential in improving symptoms such as excessive worry, fear, and tension in cannabidiol-treated-rats. Also, researchers suggested that CBD may play in the regulation of body's response to stress and enhancing overall mood (Kaplan et al., 2003). Some studies also proposed that CBD could help moderate psychotic symptoms and enhance cognitive function in schizophrenia. Furthermore, CBD neuroprotective characteristics offers potential benefits for conditions such as alzheimer's disease and parkinson's disease that may slow the progression of these neurodegenerative conditions (Esposito et al., 2007).

1.8.1.1 Effects of CBD on the cardiovascular system

Under physiological conditions, CBD has minimal impact on the cardiovascular system as it does not carry an increased cardiovascular risk in contrast to the psychoactive, THC (Mishima et al., 2005). Animal and human studies have shown that CBD reduces anxiety under stressful situations. CBD has been shown to alter stress- related changes in the cardiovascular system through 5-HT1 receptors

(Russo et al., 2005). In another study, stimulated public speaking and cold stress did not affect blood pressure or heart rate in people when CBD was administered (Durst et al., 2007a). Accordingly, CBD may have better haemodynamic effects on the cardiovascular system when stress is associated.

It has been shown that CBD has a range of beneficial impacts in the treatment of myocardial infarction. In one study, CBD was given before ligation of the coronary artery and immediately before reperfusion, which resulted in a reduction in also affected the number of ventricular arrhythmias that occurred as well as the amount of collagen-induced platelet aggregation (Walsh et al., 2010). It has been hypothesised that the mechanism of CBD's antiarrhythmic action is that it inhibits platelet release of arthritogenic molecules by promoting adenosine A1 receptors (Gonca and Darici, 2015).

Cannabidiol has been demonstrated to lower the risks of myocardial infarction in a rat model of ischemia and reperfusion. Chronic CBD therapy significantly reduced infarct size and leukocyte infiltration. These effects were correlated with lower IL6 levels in the blood. Anti-inflammatory characteristics of CBD may play a role in CBD's cardioprotective benefits (Durst et al., 2007a). The effects such as reduced cardiac malondialdehyde, nitric oxide, tumour necrosis factor-(TNF-α), and calcium ion levels, increased cardiac reduced glutathione, selenium, and zinc ions, decreased expression of NF-κB, and inducible NOS were all linked to CBD's cardioprotective effects on animal studies. Another research found that CBD administration for five days reduced doxorubicin-induced ventricular dysfunction and lowered cardiac damage markers (Feng et al., 2015). Similarly, Fouad et al. found that treatment with CBD significantly reduced oxidative and nitrative stress (Fouad et al., 2013). Biochemical examinations confirmed a decrease in the expression of proinflammatory cytokines and indicators of oxidative and nitrative stress as well as an improvement in inflammation symptoms (Feng et al., 2015).

CBD may help prevent diabetes-related heart disease although it did not influence glycaemic control or insulin sensitivity in people who had type 2 diabetes (Feng et al., 2015). Nevertheless, CBD had little effect on blood glucose levels in diabetic animals (El- Remessy et al.,2006.; Rajesh et al., 2010). Excessive glucose exposure has been demonstrated to negatively affect human coronary artery endothelial cells (El-Remessy et al., 2006). High glucose levels promote mitochondrial superoxide production, 3- nitrotyrosine synthesis, NF-κB activation, inducible NOS and adhesion molecule expression, and monocyte trans-endothelial movement and adherence to endothelium.

CBD pre-treatment prevented these adverse effects and improved the endothelial barrier function disturbance caused by elevated hyperglycemia (Rajesh et al., 2010a).

1.8.1.1 Anti-inflammatory effects of CBD

CBD has immunosuppressive properties, including the suppression of IL-2 synthesis in response to certain T-cell activation stimuli. It also decreases TNF-α production in lipopolysaccharide (LPS)-treated mice (Kaplan et al., 2003). This action has traditionally been linked to the cannabinoid receptors, CB1 and CB2, which are both expressed in various mouse cells. However, CB1and CB2 receptor antagonists had no effect on CBD's ability to reduce IL-2 production driven by L-phorbol ester/calcium ionophore (PMA/Io), which was inhibited by cannabinoids but not attenuated by CB1 and CB2 antagonists in this investigation. CBD negatively affects the regulation of TNF-α, IL- 1, and IFN- γ production by human peripheral blood mononuclear cells and the reduction of chemokine synthesis by a human B-cell line (Formukong, Evans and Evans, 1988; Watzl, Scuderi and Watson, 1991).

Lymph node cells (LNC) from mice treated with CBD showed a reduction in the release of IFN- γ (Pertwee, 2004). CBD reduced the collagen-type-II-specific proliferation of LNCs from arthritic animals in a dose-dependent manner in separate *in vitro* experiments. TNF-α was considerably reduced in synovial cells from mice given an optimum dosage of CBD (5 mg/kg i.p., daily, for 10 days) compared to controls. According to this study, TNF-α, a pro-inflammatory cytokine implicated in arthritis pathogenesis, is suppressed by CBD treatment effects (Malfait et al., 2000).

Braida et al. (2003) investigated CBD's antioxidant and anti-inflammatory capabilities in minimising cerebral ischemia-induced damage. In this study, CBD was given to gerbils (5 minutes after a 10-minute bilateral carotid-artery occlusion in a free- moving environment). CBD-treated gerbils had 100% survival of their CA1 neurons, according to the histological investigation, suggesting that CBD may have a therapeutic effect in the treatment of cerebral ischemia. The highest level of CBD neuroprotective activity was seen at 5 mg/kg (Braida et al., 2003). In another study, El-Remessy et al. (2006) demonstrated that treatment of CBD to female NOD mice (non-obese diabetic), either in a latent diabetes stage (after 14 weeks) or with first signs of diabetes (showing up to 14 weeks after the administration of CBD), significantly reduces the onset of diabetes. Compared to the untreated groups, only 32% of the CBD-

treated mice were diagnosed with diabetes. Furthermore, CBD administration resulted in a substantial decrease in the production of the pro-inflammatory cytokine IL- 12 by splenocytes, while the production of the anti-inflammatory cytokine IL-10 was dramatically increased (El-Remessy et al., 2006).

Several experiments were used to evaluate retinal cell mortality, blood-retinal barrier function, and oxidative stress. Oxidative stress, retinal neuronal cell loss, and vascular permeability increased in experimental diabetes (Weiss et al., 2008). These effects were correlated with elevated TNF-α, vascular endothelial growth factor (VEGF), intercellular adhesion molecule-1, and p38-MAP kinase activation. CBD treatment dramatically reduced oxidative stress lowered TNF-α, VEGF, and intercellular adhesion molecule-1 levels, and protected diabetic retinal cells from death and hyperpermeability. According to these findings, CBD therapy dramatically reduced the activity of the p38-MAP kinase in the retina. Cardiomyocyte dysfunction is facilitated by increased p38-MAPK signalling, which inhibits the proliferation of individual cardiomyocytes and contributes to damage during myocardial ischaemia (Coulthard et al., 2009). These findings indicate that CBD therapy lowers neurotoxicity, inflammation, and blood-retinal barrier disruption in diabetic mice through mechanisms that may include p38-MAP kinase suppression.

CBD significantly reduces the volume of the infarct, caused by blocking the middle cerebral artery (MCA), in a bell-shaped curve. A serotonin (5-HT1α) receptor antagonist, WAY100135, was shown to reduce the neuroprotective effect of CBD. CBD enhanced cerebral blood flow but was partly reduced by WAY100135. These findings imply that CBD's neuroprotective properties may be linked to an increase in cerebral blood flow via the 5-HT1α receptor (Mishima et al., 2005). CBD and 5-hydroxytryptamine (serotonin agonist) both lower the cAMP concentration in Chinese hamster ovary cells (CHO) (Mishima et al., 2005). Costa *et al.* (2017) found that CBD decreased PGE2, nitric oxide (NO), and malondialdehyde generation, as well as COX activity, in a carrageenan-induced inflammatory model in rats (Pellati et al., 2018a). In LPS-stimulated RAW264.7 macrophages, CBD completely inhibited the production of TNF-α (Ben-Shabat et al., 2006). Furthermore, in mitogen-activated human PBMC, a decrease in IL-1β and TNF-α levels has been reported (Watzl et al., 1991). In an *in vitro* model of LPS-stimulated rat microglia, CBD decreased TNF-α, IL-1β, and IL-6 release by inhibiting NF-κB phosphorylation, as well as COX and iNOS activation, in a CB2-dependent manner (Kozela et al., 2010). CBD has been shown to inhibit the Akt and ERK pathways in human glioma cells (Solinas et al., 2013).

CBD has been hypothesized to reduce microglia activation by inhibiting ATP-induced intracellular calcium increase in conjunction with suppression of NO generation. CBD decreased NO, IL-1β, and TNF-α- induced production in rat primary astrocytes via activating PPAR and reducing NF-κB nuclear translocation (Martín-Moreno et al., 2011; Esposito et al., 2011). In conclusion, CBD's anti-inflammatory effect might be likely mediated by adenosine A2A receptors, TRPV1 receptors, GPR55 receptors, and 5HT(1A) (Peters, Murillo-rodriguez and Hanus, 2007; Pellati et al., 2018a).

1.8.2 Cannabigerol (CBG)

Cannabigerol (CBG) was initially discovered in 1964 and has since been widely used in cancer therapy (Rajesh et al., 2010a)(Rock et al., 2011). Both 9-THC and CBD may be traced back to cannabigerol (CBG), a precursor generated from cannabigerovarin (CBGV)(Radwan et al., 2021). CBGA is generally present in relatively low concentrations in Cannabis since it acts as the precursory molecule to the other cannabinoids. All enzymatically generated cannabinoids (including CBG) begin in their acidic state and are subsequently decarboxylated by heat to produce the "active" form (Izzo et al., 2009). *In vitro* pharmacodynamic investigations of CBG revealed that it is a potent 2-adrenoceptor agonist and a moderate 5-HT1α R competitive antagonist and that it can weakly bind but not activate CB1R (Cascio et al., 2010; Cascio, Pertwee and Marini, 2017). Furthermore, it interacts with a variety of transient receptor potential (TRP) channels, serving as an agonist at TRPA1, TRPV1, and TRPV2, as well as a powerful antagonist at TRPM8(De Petrocellis et al., 2011). Also, CBG easily crosses the blood-brain barrier (Deiana et al.,2012) and is known to have a role in the regulation of eating and energy balance (Borrelli et al., 2013a; Brierley et al., 2016; Kogan et al., 2021).

1.8.2.1 Impact of CBG on cardiovascular diseases and inflammation

Cannabigerol has been explored as a potential therapeutic for gastrointestinal diseases such as colorectal cancer and colitis using mouse models. Orally administered CBG reduced colonic inflammation as measured by significantly reducing myeloperoxidase (MPO) activity, IL-1β levels, and serum fluorescein isothiocyanate (FITC)-dextran (Borrelli et al., 2013a; 2014). In 2020, Pagano et al. used a mouse model of dinitrobenzene sulfuric acid–induced colitis to study the effects of CBG on inflammatory activity (Pellati et al., 2018b).

CBG has been proven to boost eating in rats and prevent weight loss associated with cisplatin treatment. CBG has not been shown to have antiemetic properties and seems to be antagonistic to those of CBD and D9-THC. Taken together, these findings suggest that CBG may play a role in chemotherapy-induced weight loss and appetite reduction. While CBG does not have the euphoric effect associated with nabilone and dronabinol, it may play a significant role in patients undergoing chemotherapy (Parker, Rock and Limebeer, 2011). In 2020, Echeverry et al. investigated the effects of CBG and CBD on

neurotoxicity in brain cell cultures. The protective properties of CBD and CBG were distinct in two separate oxidative damage models (the H_2O_2 model and the rotenone model), suggesting that the protective effects are derived from a receptor-mediated pathway. The findings revealed that when these drugs were administered to subjects exposed to a 5-HT1α antagonist, WAY-100635, the protective impacts were diminished (Echeverry et al., 2021).

CBG and VCE-003.2 have been proven to inhibit the pro-inflammatory cytokines TNF-α and IL-1β. In rat microglial cells treated with lipopolysaccharide (LPS), IL-6, MIP- 1α, and PGE2 levels are reduced, as is glutamate-induced oxidative cell death in the mouse hippocampus. Valdeolivas et al. (2015) demonstrated improvement in Huntington's disease symptoms in CBG-treated animals using two mouse models. CBG, protected striatal cell loss and decreased inflammatory markers in mice treated with 3-nitro propionate. Indeed, CBG avoided striatal neuron loss, decreased inflammation, and improved motor performance (Valdeolivas et al., 2015). In response to oxidative stress, CBG and CBGA reduced COX activity even at high micromolar doses. In LPS-stimulated macrophages, CBG reduces NO generation via activating and desensitizing TRPV4 (Ruhaak et al., 2011). CBG therapy in macrophages inhibited neuronal cytotoxicity by lowering inflammation markers (i.e., IL-1β, TNF-α, and IFN-γ production) and oxidative stress (Gugliandolo et al., 2018).

1.9 Aim of the Study

As highlighted above, the currently used anti-platelet drugs are associated with serious side effects and tolerance in some individuals. Therefore, there is an urgent need to develop better, safer, and efficacious drugs to control platelet-mediated thrombotic and inflammatory conditions. Since CBD and CBG have been shown to be safer and efficacious with no toxic effects in treating several other pathological conditions, this study aims to determine their impacts on the modulation of platelet reactivity, haemostasis, thrombosis and platelet-mediated inflammatory responses. The outcomes of this study will demonstrate the beneficial effects of these compounds in tackling thrombotic diseases.

1.10 Objectives of The Research

The specific objectives of this study are to:

1. Determine the impact of CBD and CBG in the modulation of various platelet functions upon stimulation with diverse agonists,
2. Analyse the effects of CBD and CBG in the modulation of platelet-mediated inflammatory responses, and
3. Establish the molecular mechanisms through which CBD and CBG modulate platelet function.

CHAPTER TWO

2. Materials and Methods

2.1 Materials

2.1.1 Phytocannabinoids

Clinical grade CBD and CBG were obtained from STI Pharma UK. Each compound was dissolved in DMSO. Then, dilutions were carried out in PBS to ensure that the final concentrations of DMSO were kept at 0.01%, which did not affect platelet function.

2.1.2 Platelet Agonists

Different platelet agonists were employed in this study to assess the role of phytocannabinoids in the modulation of platelet reactivity. Cross-linked collagen-related peptide (CRP-XL) was obtained from Professor Richard Farndale (University of Cambridge, UK). Horm Collagen was purchased from Nycomed in Austria. Thrombin and ADP were purchased from Sigma-Aldrich (UK). U46619, a thromboxane A2 receptor agonist, was obtained from Tocris (UK). Unless otherwise noted, all other chemicals were purchased from Sigma-Aldrich (UK).

2.1.3 Antibodies

Anti-human fibrinogen polyclonal antibodies conjugated to FITC were bought from Dako, UK. PE-conjugated anti-human CD62P antibodies, anti-CD45 antibodies, and FITC-conjugated anti-human CD42b antibodies were bought from BD Biosciences UK. Phosphorylation-specific primary antibodies such as anti-human AKT, Syk, PKC, LAT, Src/Y527 and VASP pS157 were obtained from Abcam, UK. Rabbit anti-human 14-3-3ζ antibody was obtained from Santa Cruz Biotechnology (USA). DiOC6 (3,3'-Dihexyloxacarbocyanine Iodide) was purchased from Sigma Aldrich, UK. Fluo-4 AM calcium-sensitive dye was obtained from Life Technologies and was used to monitor platelet intracellular calcium levels.

2.1.4 Animals

12 weeks old C57BL/6 mice (both males and females) were obtained from Envigo (UK) to conduct the tail bleeding assay.

2.2 Methods

The University of Reading Research Ethics Committee approved all the experimental approaches that included the use of human blood from healthy subjects. A signed informed consent was obtained before blood samples were drawn from healthy, aspirin-free volunteers. Blood was collected using vacutainer blood collection tubes that contained 3.2% (w/v) sodium citrate.

2.2.1 Isolation Platelets From Human Blood

Platelet-rich plasma (PRP) was obtained by centrifuging the blood samples at 100g for 20 minutes at room temperature. Before usage, the PRP was rested at 30°C for 30 minutes. Blood was combined with acid citrate dextrose (ACD) [2.5% (w/v) sodium citrate, 2% (w/v) D-glucose, and 1.5% (w/v) citric acid] at a 1 (ACD):9 (blood) ratio and centrifuged at 100g for 20 minutes to separate platelets. PRP was collected, mixed with the required amount of ACD, and centrifuged at 1413g for 10 minutes at room temperature. A platelet pellet was obtained, and the supernatant was discarded. The pellet was resuspended in a modified Tyrodes-HEPES buffer (134mM NaCl, 2.9mM KCl, 0.34mM Na_2HPO_4 ,12mM $NaHCO_3$ 20mM HEPES, and 1mM $MgCl_2$, pH 7.3) with a suitable amount of ACD and centrifuged at 1413g for 10 minutes at room temperature. The resulting platelet pellet was resuspended in modified Tyrode's-HEPES buffer to a final density of $4x10^8$ cells/mL and allowed to rest for 30 minutes at 30°C before use.

2.2.2 Platelet Aggregation Assay

Platelet aggregometry is a gold standard technique to determine and measure the impact of various molecules on platelet activation. This technique makes use of an optical platelet aggregometer (Chrono-Log, USA) and measures the transmission of light in resting and activated samples. For 5 minutes, isolated platelets or PRP (450 µL) were activated in the presence of an agonist (50 µL) with continuous stirring (1200 rpm at 37^oC) and the level of aggregation was assessed in terms of an increase in light transmittance. To determine the effects of phytocannabinoids on platelet aggregation, isolated platelets or PRP were incubated with 1 µl of desired phytocannabinoid concentrations (1.56 µM, 3.125 µM, 6.25 µM, 12.5 µM and 25µM) dissolved in DMSO or with vehicle control [0.01% (v/v) DMSO] alone for

5 minutes (with an initial 10 seconds stirring to distribute phytocannabinoids evenly in the tube) before the addition of an agonist.

2.2.3 Whole Blood Aggregation

Whole Blood impedance aggregometry quantifies the changes in electrical impedance between two electrodes when an agonist induces platelet aggregation as the platelet plug formed between the electrodes affects the transmission of current. According to the manufacturer's guidelines, 445 µL sodium-citrated human blood samples were diluted with 500 µL saline and then treated with (5 µL) vehicle control [0.01% (v/v) DMSO] or different concentrations (1.56 µM, 3.125 µM, 6.25 µM, 12.5 µM and 25 µM) of phytocannabinoids. Three minutes after the incubation at 37°C, activation using collagen (5 µg/ml) was initiated. Platelet aggregation results were evaluated by calculating an area under the curve and the percentage of aggregation.

2.2.4 Adenosine Trisphosphate (ATP) Secretion Assay

The adenosine triphosphate (ATP) assay is a method used to measure and quantify the amount of ATP secreted by activated platelets. It works by utilizing a luminescence technique. ATP, which is found in high levels in platelets, is used as energy in a reaction involving luciferin and luciferase. Luciferin is converted to oxyluciferin through the oxidative enzyme luciferase, resulting in the release of luminescence (Gomi and Kajiyama, 2001). The lumi-aggregometer (model 700, Chronolo-Log, PA, USA) was used to measure the amount of ATP released from dense granules upon platelet activation. In a glass cuvette, 4 X 10^8 cells/mL of isolated platelets (395 µL) were incubated for 2 minutes at 37°C in the dark with 50 µl of chrono-Lume reagent (Chronolo- Log, PA, USA). When the luminous gain was adjusted to the manufacturer's suggested range of 20%-60%, an increase in luminescence was seen using AggroLink 8 software (Chrono-Log, PA, USA). These parameters have been recorded and are being used in this study. Phytocannabinoids or the vehicle control [0.01% (v/v) DMSO] were incubated with isolated platelets for 5 minutes, and Chronolume reagent (50 µL) for 3 minutes. These two reagents were added before the addition of CRP-XL (0.5 µg/mL) and the establishment of a baseline. After agonist addition, dense granule secretion was measured for 3 minutes using the AggroLink 8 software (Chrono-Log, PA, USA) to monitor the level of luminescence released. Then ATP response ranges were calculated according to the manufacturer's instructions.

2.2.5 Intracellular Calcium Mobilisation

Fluo-4 AM calcium-sensitive dye (Life Technologies, UK) was used to determine the intracellular calcium levels in platelets. Two milliliters of human PRP were loaded with 2 µL of Fluo-4 AM dye and incubated in the dark for 45 minutes at 30°C. After centrifugation at 350 g for 15 minutes, the PRP was resuspended in 500 µL of modified Tyrodes-HEPES buffer. Before activating the isolated platelets with 0.5 µg/ml CRP-XL, they were incubated with vehicle control [0.01% (v/v) DMSO] or various doses of phytocannabinoids (1.56 µM, 3.125 µM, 6.25 µM, 12.5 µM and 25 µM). The fluorescence intensity was determined using a NOVOstar plate reader (BMG Labtech, Ortenberg, Germany) at a temperature of 37°C for 5 minutes at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The results were analysed by calculating the proportion of peak calcium levels released at about 90 seconds. This is the time during which the highest levels of calcium were released in the positive control samples.

2.2.6 Platelet Static Adhesion Assay

Static adhesion is performed to assess the ability of platelets to adhere to a surface under static conditions, without any flow. This assay is commonly used to study platelet adhesion and activation *in vitro*. To begin, we added 100 µL of fibrinogen to 96-well plates and incubated them at room temperature for 1 hour. Then we removed the excess and blocked the wells for 1 hour with 150 µL of 1% bovine serum albumin (BSA) in Tyrode's- HEPES. Then, the plates were washed three times with 200 µL Tyrode's-HEPES in each well. Following that, we add the incubated platelets [1×10^8 cells/mL, 50 µL each well with various concentrations] and incubate them for 1 hour at room temperature. Later, we discard non-adherent platelets and wash each well three times with 200 µL Tyrode's-HEPES. Then, 100 µL of citrate lysis buffer was added and incubated at room temperature for 1 hour. Finally, we stopped the reaction by adding 100 µL of 2M NaOH, and the absorbance was measured by using Fluostar Optima plate reader Spectrofluorimeter (BMG Labtech, Germany) at 405nm.

2.2.7 Platelet Spreading Assay

In a 24-well plate, glass coverslips were coated for 45 minutes with 300 µl of fibrinogen (100 µg/ml) dissolved in modified Tyrodes-HEPES buffer. Then the unbound materials were discarded, and 1% (w/v) bovine serum albumin (BSA) was added to the coverslips to minimise the non-specific platelet binding to the glass surface. Washed the coverslips three times with PBS (10 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl, pH 7.4). After 5 minutes of exposure to vehicle control or various concentrations of phytocannabinoids, the platelets were added onto coverslips and incubated at 37°C for 45 minutes. Coverslips were washed three times with PBS to eliminate unbound platelets. Formyl saline 0.2% (v/v) was used to fix the adhered platelets for 10 minutes. To remove any remaining fixative solution, the coverslips were washed three times. The bound platelets were permeabilised for 5 minutes in PBS containing 0.2% (v/v) Triton X-100. After incubation, the coverslips were washed three times with PBS. To label platelet filamentous actin, the adhered platelets were stained with Alexa-Fluor 488-labelled phalloidin on coverslips for 30 minutes in the dark at room temperature. The coverslips were then mounted onto microscope slides using Prolong Gold Antifade [Life Tech (USA)] to retain fluorescence and then preserved at 4°C until observation. The Nikon A1-R confocal microscope was used to image the slides through a 100X oil immersion objective (Nikon, Japan). The amount of platelet spreading was assessed by counting the number of platelets on 10 random photographs of each population (obtained from separate donors) that were recorded. Platelets were classified as 'adhered but not spreading' or spreading when cells expanded filopodia, and as spreading completely' when lamellipodia appeared.

2.2.8 Clot Retraction

Clot retraction is a process that happens after the clot has been formed. The goal of this process is to draw the wound edges closer to facilitate the healing process. The integrin αIIbβ3-mediated outside-in signalling lowers the size of the blood clot. To measure the impact of CBD and CBG on clot retraction, this assay was performed. PRP (200 µL) was incubated with phytocannabinoids or vehicle control [0.01% (v/v) DMSO] for 10 minutes in a glass test tube. 950 µL of modified Tyrodes-HEPES buffer was added (to make the final amount 1 ml) to test tubes, along with 5µL of red blood cells, to enable visualization of the clot. To start clotting, 50 µL thrombin (1 U/mL) was added to the test tubes. A blunt glass pipette was inserted into the middle of each test tube, around which the clot would form at room temperature. Photographs were taken every 30 minutes, and the experiment was terminated at 2 hours when the vehicle-treated samples' clots had retracted totally. Clots were taken from the glass pipettes and put into assigned tubes, which had been individually pre-weighed. Clot weights (without liquids) were calculated. To assess the amount of clot retraction, the clot mass was determined for each sample.

2.2.9 Tail Bleeding Assay

The tail bleeding experiment in mice is often used to examine haemostasis under physiological conditions. The tail tip is trans-sectioned followed by a measurement of the bleeding volume and duration. The experimental protocols employed in the tail bleeding test in mice have been authorized and approved by the British Home Office and University of Reading. Xylazine (5 mg/kg) and ketamine (80 mg/kg) were administered intraperitoneally to C57BL/6 mice (12 weeks old; Envigo, UK) weighing around 20-25 g and put on a heated mat (37°C). After roughly 10- 15 minutes of anaesthetisation, the vehicle control [0.01% (v/v) DMSO] or phytocannabinoids (6.25 and 12.5 µM CBD and CBG 12.5 µM) [final concentrations were calculated based on the estimated volume of blood concerning their weight] were delivered through femoral artery and incubated for 5 minutes. A distal (3 mm) piece of the tail tip was dissected using a scalpel blade and the tail tip was immersed in sterile saline. The time required for the bleeding to stop was measured up to 20 minutes before the experiment ended.

2.2.10 *In vitro* Thrombus Formation Under Arterial Flow Conditions

The *in vitro* thrombus formation test can assess several characteristics of thrombi that develop under arterial flow circumstances on a collagen-coated surface at a physiological shear rate. Human whole blood was treated with 5 µM of a lipophilic dye, DiOC6 (3,3'-Dihexyloxacarbocyanine Iodide) (Sigma Aldrich, UK) at 30°C for 30 minutes. For one hour, collagen (400 µg/ml) was coated onto Vena8 BioChip microfluidic channels (Cellix Ltd, Ireland). Channels were gently washed with modified Tyrodes-HEPES buffer after blocking. Before perfusion across the collagen-coated microfluidic channels at a shear stress of 20 dynes/cm^2 for 10 minutes, the fluorescent-labelled human whole blood was pre-incubated for 5 minutes with a vehicle or different doses of phytocannabinoids (6.25 and 12.5µM). The amount of thrombus development was evaluated using a Nikon A1-R confocal microscope with a 20X objective. Fluorescence pictures of thrombi were recorded every 30 seconds continuously for 10 minutes. The mean fluorescence intensity, volume, and the number of thrombi were computed using NIS Elements software (Nikon, Japan) and the pictures were processed using ImageJ (National Institute of Health, USA) (National Institute of Health, USA).

2.2.11 Lactate Dehydrogenase (LDH) Cytotoxicity Assay

The amount of LDH produced from CBD or CBG-treated human isolated platelets was measured using the LDH Cytotoxicity Assay Kit (Pierce, UK) according to the manufacturer's instructions to evaluate if phytocannabinoids had cytotoxic effects on platelets. In summary, the platelets were incubated at 37°C under 5% CO_2 for 30 minutes. The vehicle or a range of concentrations of phytocannabinoids was applied in triplicates to separate wells of a 96-well plate and incubated for 10 minutes. One set of wells was treated with the lysis buffer given from the kit as a positive control for maximal LDH release, and another set was treated with a modified Tyrode ’s-HEPES buffer for the detection of spontaneous LDH release. To obtain the percentage of LDH release relative to the maximal LDH-releasing positive control, the values are represented as means of triplicate absorbance measurements.

2.2.12 cAMP Levels In Platelets

The total cellular cAMP levels in human platelets were measured using a cAMP ELISA kit (Cambridge Bioscience, UK). Before activation with 0.5 µg/ml CRP-XL, human- isolated platelets were pre-incubated with various concentrations of phytocannabinoids or vehicle control. The platelets were lysed with 0.1M HCl, and the levels of cAMP were determined according to the manufacturer's procedure using the standard curve plotted using the kit's control samples.

2.2.13 SDS-PAGE And Immunoblotting

At a density of 4 x 10^8 cells/ml, human-isolated platelets were treated with phytocannabinoids or vehicle control for 5 minutes at 37°C. Following the incubation, platelets were activated by the addition of 0.5 µg/mL CRP-XL for 5 minutes. A reducing sample treatment buffer (RSTB) was used to lyse the samples. Samples were then heated at 95°C for 5 minutes, before storing at -20°C until use.
Using 4-15% Mini-PROTEAN TGX precast protein gels (Bio-Rad, UK), 10 µL of the samples were run through an sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 150 V for around 50 minutes (until the dye front reaches the bottom of the gel). The gels were then transferred to polyvinylidene difluoride (PVDF) membranes using a Semi-Dry Transfer System (Bio-Rad, UK) at 15V for 2 hours. Then, the membrane was blocked by incubation in 10 mL 5% (w/v) BSA in TBS-T (20mM Tris, 140mM NaCl and Tween 20, pH 7.6). TBS-T was used to wash the blots, which were then incubated for an hour at room temperature with secondary Cy5-conjugated goat anti-rabbit IgG antibodies in 2.5% (w/v) BSA (1:1000) (Invitrogen, UK) after an overnight incubation with the primary antibody (1:1000) in 2.5% (w/v) BSA (Abcam, UK). Following washing in TBS-T for one hour at room temperature, the blots were examined using a Typhoon 9400 Variable Mode Imager system (GE Healthcare, UK) (GE Healthcare, UK). Anti-human 14-3-3ζ antibodies (1:1000) were used to determine the equal loading of proteins in each lane (Santa Cruz Biotechnology, USA).

2.2.14 Measurement Of Reactive Oxygen Species Production

As an indicator for ROS, we used 2′,7′-Dichlorofluorescin diacetate (H_2DCFDA) (Cambridge Bioscience, UK) cell-permeable non-fluorescent, and oxidatively transformed to a highly fluorescent signal by intracellular esterases and oxidation was used to assess ROS levels in CRP-XL-stimulated platelets. Using CRP-XL as a stimulus, we investigated the phytocannabinoids' effect on ROS generation in platelets. Human isolated platelets (2×10^8 cell/mL) were treated with 10 μM H_2DCFDA for 30 minutes in the dark at 37°C. After incubation, platelets were treated for 5 minutes with various concentrations of phytocannabinoids at 37°C before being stimulated for 10 minutes with 1 μg/mL CRP-XL. A modified Tyrode 's-HEPES buffer containing 0.1% (w/v) BSA was used to dilute the samples after stimulation, and the samples were promptly analysed on an Accuri C6 Flow cytometer (BD Biosciences, UK). The amount of reactive oxygen species (ROS) was estimated using the median fluorescence intensity (MFI) values and shown as a fold change compared to the resting platelets. The amount of ROS produced by CRP-XL- stimulated platelets was set to 100% to assess any inhibitory effects of phytocannabinoids.

2.2.15. Platelet-Derived Inflammatory Cytokines

The amounts of inflammatory cytokines and chemokines secreted from human platelets were all measured using appropriate ELISA kits which were purchased from Theromfisher Scientific (UK). For instance, human sP-Selectin ELISA was used to determine the concentration of sP-selectin in platelets. The provided microplate had been pre-coated with an anti-human sP-selectin. The immobilised (capture) antibody is then bound to samples and standards in these wells. Finally, a second antibody conjugated with an enzyme (HRP) is added to the sandwich to generate the final product. A substrate solution (depending on the enzyme conjugate employed) is added, which combines with the enzyme-antibody-target complex to create a quantifiable signal. The strength of this signal is related to the target concentration in the original material. Human isolated platelets were treated with a vehicle or phytocannabinoids, CBD (6.25 and 12.5 μM) or CBG (6.25 and 12.5 μM) for five minutes before stimulating platelet activation using CRP-XL (1 μg/mL) for five minutes under stirring conditions to obtain resting or activated platelets, respectively. The platelets were centrifuged at 1000g for 10 minutes at room temperature. The supernatant was collected to be used in the assay. A sample diluent of 100 μL was added to duplicates to both standard and blank wells. Then, 100 μL of freshly prepared sP-selectin

standards with dilutions of 0 - 40 ng/ml was added to the standard wells. Also, 90 µL of sample diluent was added to the sample wells, as well. Either the resting or treated platelet samples were used at a 1:10 ratio. Approximately 10 µL of the obtained supernatant was added to sample wells containing 90 µL of the sample diluent supplied with the kit. Human sP-selectin present in samples or standards binds to antibody adsorbed to the microwells, and 50 µL of HRP-conjugated anti-human sP-selectin antibody was added to the wells, where it binds to human sP- selectin captured by the first antibody. The plate was then incubated at room temperature for 2 hours on a microplate shaker. The plate was then rinsed three times with wash buffer [PBS with 1% (v/v) Tween 20], and 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added before incubating in the dark for 15 minutes at room temperature until a colour developed. After that, the reaction was terminated by adding 100 µL of stop solution (1 M HCl). An ELISA microplate reader was used to measure the amount of absorbance at 450 nm, which corresponded to the wavelength of light (EMax precision plate reader, Molecular Devices, UK). Human tumour necrosis factor- α (TNF- α), vascular endothelial growth factor (VEGF), human interleukin beta (IL- β), and Human Regulated on Activation Normal T Cell Expressed and Secreted (RANTES) ELISA kits which are available commercially (Theromfisher, UK) and followed the same preparation protocol.

2.2.16. Flow Cytometry Analysis Of Platelet-Leukocyte Interactions

Healthy human whole blood was used for flow cytometry analysis. This measures side scatter and causes light to be released at a greater angle when it hits complex structures such as granules and nuclei inside the cell. However, forward scatter measures the cell size. Monocytes are bigger than lymphocytes and display more intense forward scatter in the flow cytometer. Platelets and leukocytes were labelled with the appropriate antibodies conjugated with fluorescent dyes. A 100 µl aliquot of whole blood was diluted by adding 400 µl of Ca^{+2}-free HEPES buffer. After dilution, the samples were incubated for 20 minutes at room temperature with FITC-conjugated anti-human CD42b platelet antibodies and PE-conjugated anti-human CD45 leukocyte antibodies. Following incubation, the samples were treated for 5 minutes in the dark at 37°C with various concentrations of phytocannabinoids or a vehicle control [0.01% (v/v) DMSO]. To activate platelets, 10 µM thrombin receptor activator peptide 6 (TRAP-6) was added and incubated at room temperature for 5 minutes. Afterwards, paraformaldehyde was used to fix the samples for 10 minutes at room temperature. The red cells were then lysed for 10 minutes in 2 ml of 1x RBC lysis solution covered in foil and stored at 4°C until flow cytometry analysis.

Leukocytes were counted at a total of 20,000 events using forward and side scatter parameters. Fluorescence levels in negative controls were determined using appropriate isotype controls tagged with the same fluorophores as main antibodies. CD45+ cells with fluorescence from CD42b were used to determine the total platelet leukocyte aggregations. A BD Accuri C6 flow cytometer was used to analyse the samples, and C6 Sample Plus software was used to analyse the results.

2.2.18 Statistical Analysis

Data from this study was analysed using GraphPad Prism Version 8. To consider the individual variation of platelets reaction, data were normalised to the positive controls for each donor and are given as mean SEM in bar graphs. Analysis of data from platelet aggregation, dense granule secretion and spread, intracellular calcium mobilization, thrombus formation and cytotoxicity, flow cytometry-based experiments, cytokine secretion, platelet-leukocyte interactions, as well as immunoblot analysis, was performed using one-way ANOVA followed by Bonferroni's correction.

CHAPTER THREE

3. Effects Of PHYTOCANNABINOIDS, CBD and CBG on the Modulation of Platelet Function

3.1 Introduction

Platelets are the most important cell type involved in blood clotting to prevent excessive bleeding upon injury. They are small, anucleate cells that play critical roles in vascular/endothelial injury by initiating blood clotting processes. Upon injury, platelets undergo four phases of activation: initial adhesion, release of various factors, activation of more platelets and aggregation of activated platelets (i.e., formation of a platelet plug). The platelet plug seals the damaged area and then gets stabilised by fibrin mesh (Li et al., 1998), which is produced due to the actions of clotting cascades (Ed Rainger et al., 2015a). This is an essential process under physiological conditions to prevent excessive bleeding and this is commonly known as 'haemostasis' (Ali, Wuescher and Worth, 2015). However, inappropriate activation of platelets under various pathological conditions results in clot formation within the circulation, and this is known as 'thrombosis'(Davì and Patrono, 2007). Thrombosis results in a blockade of blood flow to major organs such as the heart and brain leading to fatal myocardial infarction and stroke, respectively. Arterial thrombosis is largely mediated by platelets, whereas venous thrombosis is mostly initiated by clotting factors (Yeung, Li and Holinstat, 2018). While the currently used anti-thrombotic drugs help save lives, they are associated with several undesirable side effects such as bleeding.

A deeper understanding of platelet reactivity and its importance in the cardiovascular system has led to some of the most successful pharmacological therapies in cardiovascular medicine. Previous research has emphasised that targeting platelets is a highly sustainable approach to lowering the risks of mortality for CVD patients and preventing recurrent thrombotic events. Numerous research studies focusing on platelet activation pathways led to the development of currently used antiplatelet drugs such as aspirin and clopidogrel as well as other novel therapies (Jourdi et al., 2021). Aspirin or acetylsalicylic acid is widely used worldwide, as an effective inhibitor of platelet aggregation (Cattaneo, 2004). Its mechanism relies on preventing the conversion of arachidonate to thromboxane A2, a potent platelet agonist and vasoconstrictor, by irreversibly blocking cyclooxygenase which is a critical enzyme in this pathway (Krötz, Sohn and Klauss, 2008). Clopidogrel is a thienopyridine, a class of ADP receptor ($P2Y_{12}$) inhibitor, that is used as a potent antiplatelet agent (Cattaneo, 2004). They are mostly used in high-risk patients and have proven efficacy in preventing thrombotic events including myocardial infarction and ischaemic stroke as well as resulting deaths (Coccheri, 2010). However, these drugs are associated with significant side effects including gastrointestinal toxicity, ulcers, nausea, and bleeding (Wolfe,

Lichtenstein and Singh, 1999; Cryer and Mahaffey, 2014). Therefore, there is an urgent need to develop better, efficacious, and safer drugs to tackle thrombotic conditions.

Phytocannabinoids extracted from the *Cannabis sativa* plant include over 70 active compounds. The two most abundant compounds are the psychoactive Δ9- tetrahydrocannabinol (THC) and the non-psychoactive cannabidiol (CBD). These compounds received excellent attention in scientific research and have numerous beneficial properties such as appetite stimulation, analgesic, anti-glaucoma, anti-emetic, neuroprotective, anti-inflammatory, and immunomodulatory effects (Turner et al., 2017). In contrast to THC, CBD has been investigated in diverse clinical applications due to their non-psychoactive and excellent pharmacokinetic properties (Morales, Hurst and Reggio, 2017). CBD has also been briefly demonstrated to have positive effects on the cardiovascular system (Stanley, Hind and O'Sullivan, 2013). Some research studies have shown CBD's ability to reduce infarct size in the heart (Durst et al., 2007b). This broad range of pharmacological actions and their beneficial effects project CBD as a potential candidate to treat several human diseases.

Cannabigerol (CBG), is another non-psychoactive phytocannabinoid and is the precursor of CBD (Kogan et al., 2021). This has also been receiving increased interest in scientific research due to its pharmacological effects. Recent research has shown that CBG can reduce inflammation in a mouse model of inflammatory bowel disease (Borrelli et al., 2013b). Several cell-based studies have shown that CBG reduces the development of tumours during cancer (Nahler, 2022). This has been demonstrated by the ability of CBG to inhibit cancer cell growth and induce programmed cell death in human colon, breast, and prostate cancer cells. A limited number of preliminary studies have demonstrated the inhibitory effects of CBD and CBG in platelets via aggregation experiments (Formukong, Evans and Evans, 1989). However, the effects of these compounds in the modulation of platelet activation, thrombosis and haemostasis and their underlying molecular mechanisms in platelets have not been underpinned in detail. Therefore, in this study, we investigated the effects of non-psychoactive phytocannabinoids, CBD and CBG in the modulation of platelet function using various experimental approaches.

3.2 Results

3.2.1 CBD and CBG inhibit agonists-induced platelet aggregation

Platelet aggregation is a gold standard assay to determine the response of platelets upon stimulation with a known agonist in the presence and absence of any test compound. This provides details such as platelet shape change and the kinetics of the aggregation. To determine if CBD and CBG can affect platelet aggregation, assays were performed using optical aggregometry upon activation with multiple agonists that stimulate platelets via different receptors.

Human isolated platelets were pre-incubated with vehicle control [0.01% (v/v) DMSO] or either CBD or CBG at various concentrations (1.56, 3.125, 6.25, 12.5 and 25 μM) for five minutes before stimulation with 0.5 μg/ml CRP-XL. The level of aggregation was monitored for a further five minutes. The data demonstrate that CBD can reduce platelet aggregation in a dose-dependent manner with around 90% inhibition achieved at 25 μM (**Figure 3.1A**). Similarly, CBG has shown strong inhibition at 25, 12.5, and 6.25 μM with around 90%, 85%, and 80% inhibition, respectively (**Figure 3.2A**). As CRP-XL is a GPVI-selective agonist, we then used collagen to activate platelets through both GPVI and integrin α2β1. Human isolated platelets were treated similarly with various concentrations of CBD and CBG and then stimulated with collagen (2 μg/ml,). Both CBD and CBG inhibited collagen-induced platelet aggregation in a dose-dependent manner with around 95% inhibition achieved at 25 μM (**Figure 3.2B**). Compared to CBD, CBG has shown slightly stronger inhibition at 25, 12.5, and 6.25 μM, as each of these concentrations was found to inhibit more than 70% of aggregation (**Figure 3.2B**).

To determine if the effects of CBD and CBG are similar when other receptors (e.g., GPCRs)-mediated signalling pathways were activated in platelets, platelet aggregation assays were performed using different GPCR agonists. A thromboxane A2 analogue, U46619 (5 μM) was used to activate platelets that were pre-treated with various concentrations of CBD and CBG. Both compounds inhibited U46619-induced platelet aggregation in a dose-dependent manner with around 85% inhibition achieved at 25 μM (**Figure 3.1C and 3.2C**). As with the above results, CBG has appeared to show a slightly higher inhibition in platelet activation at specific concentrations (25 and 12.5 μM). Similarly, to determine the effects of CBD and CBG on thrombin-mediated platelet activation via protease-activated receptors (PARs), 0.1 U/ml thrombin was used as an agonist. CBD (**Figure 3.1D**) and CBG (**Figure 3.2D**)

completely inhibited thrombin- induced platelet aggregation at 25 μM. These data demonstrate that both CBD and CBG can modulate agonists-induced platelet activation, mostly in a dose-dependent manner.

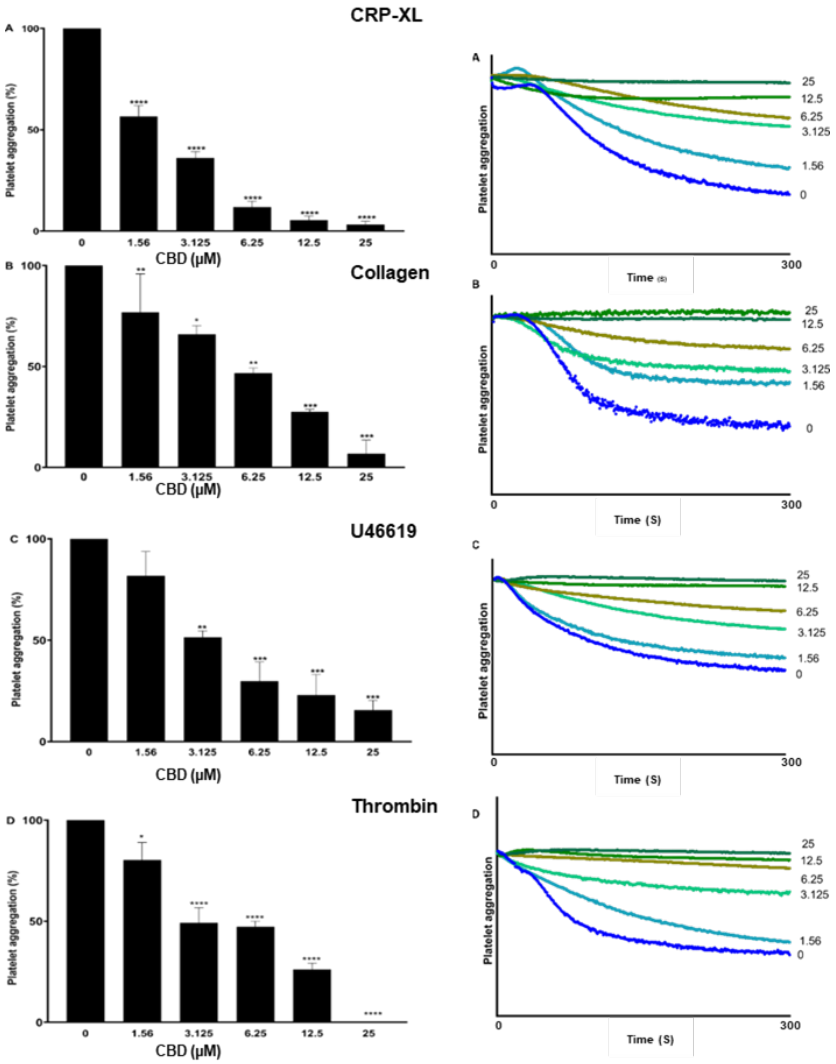


Figure 3.1: Effects of CBD on agonists-induced aggregation in human isolated platelets. Various concentrations (1.56, 3.125, 6.25, 12.5 and 25 μM) of CBD were pre-incubated with human isolated platelets for 5 minutes at 37ºC in an optical aggregometer. Then, the platelets were activated using 0.5 μg/ml CRP-XL (**A**), 1 μg/ml collagen (**B**), 5 μM U46619 (**C**) or 0.1 U/ml thrombin (**D**), and the level of aggregation was monitored for five minutes by optical aggregometry. The traces shown are representative of four separate experiments using platelets obtained from various donors. The bar diagrams show the cumulative data which were calculated by taking the level of aggregation obtained with the positive controls as 100%. Data represent mean ± SEM (n=4). The p values shown were as calculated by one-way ANOVA (*p<0.05, **p<0.01, ***p<0.001 & ****p<0.0001).

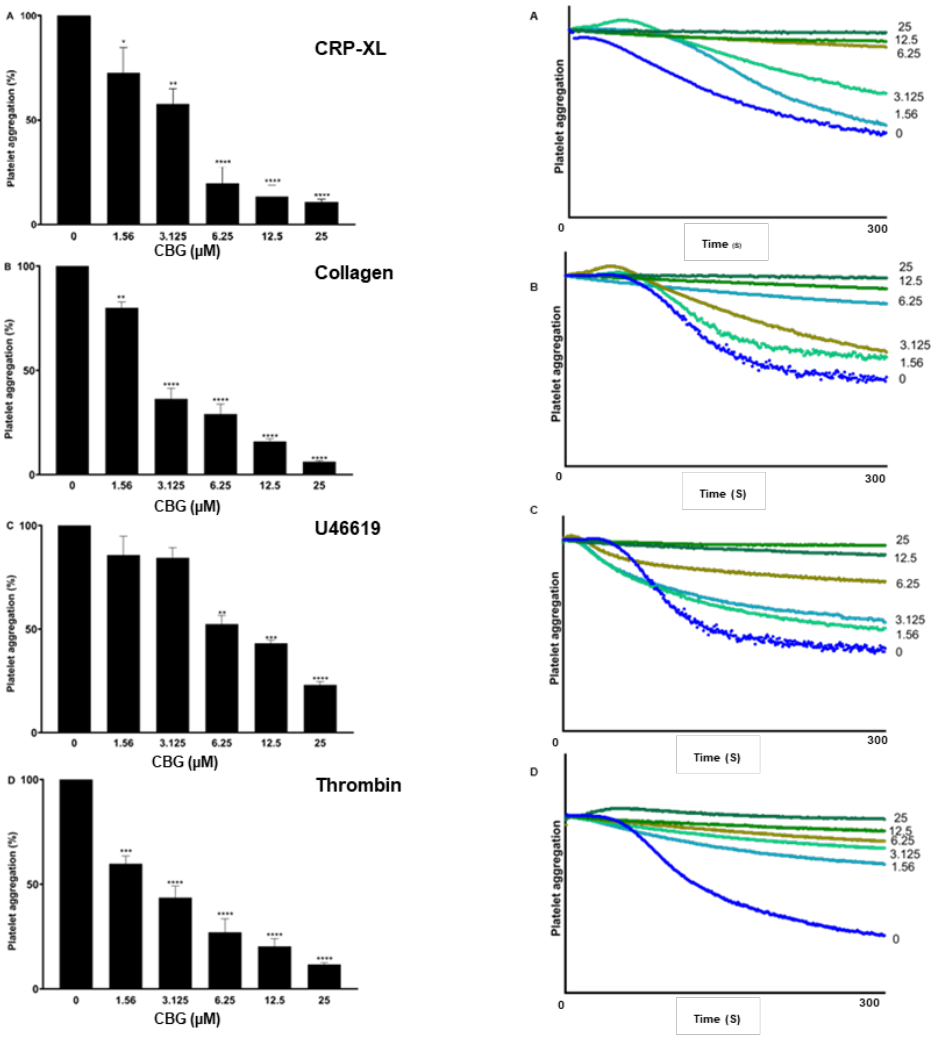
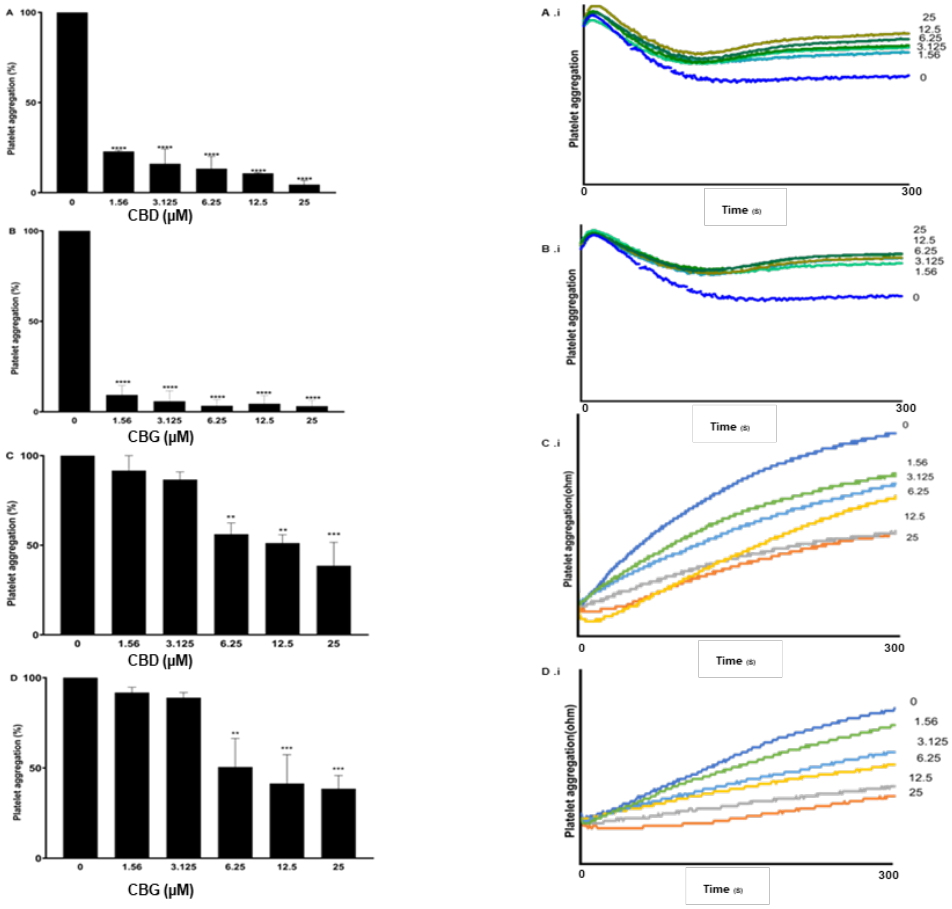


Figure 3.2: Effects of CBG on agonists-induced aggregation in human isolated platelets. Various concentrations (1.56, 3.125, 6.25, 12.5 and 25 μM) of CBG were pre-incubated with human isolated platelets for 5 minutes at 37ºC in an optical aggregometer. Then, the platelets were activated using 0.5 μg/ml CRP-XL (**A**), 1 μg/ml collagen (**B**), 5 μM U46619 (**C**) or 0.1 U/ml thrombin (**D**), and the level of aggregation was monitored for five minutes by optical aggregometry. The traces shown are representative of four separate experiments using platelets obtained from various donors. The bar diagrams show the cumulative data which were calculated by taking the level of aggregation obtained with the positive controls as 100%. Data represent mean ± SEM (n=4). The p values shown were as calculated by one-way ANOVA (*p<0.05, **p<0.01, ***p<0.001 & ****p<0.0001)

Following the investigation of the modulatory effects of CBD and CBG on isolated platelets, their effects were analysed in human PRP i.e., in the presence of plasma proteins as many plant-derived compounds were shown to demonstrate poor efficacy in PRP due to their high binding with plasma proteins. Moreover, the use of PRP represents a physiologically relevant condition (although not identical due to the absence of red and white cells) to determine platelet function. We used ADP (5 μM) (activates platelets through the $P2Y_{12}$ receptor) as an agonist to analyse the effects of CBD and CBG in human PRP. Both CBD (**Figure 3.3A**) and CBG (**Figure 3.3B**) inhibited ADP-induced platelet aggregation in a dose-dependent manner. Finally, human whole blood was treated with CBD and CBG and analysed their effects on platelets using impedance aggregometry. Collagen (5 μg/ml) was used to stimulate platelets that were pre-treated with various concentrations of CBD and CBG. Both CBD (**Figure 3.3C**) and CBG (**Figure 3.3D**) showed inhibitory effects on platelet activation in whole blood at higher concentrations (6.25 μM and more).

These data demonstrate that both CBD and CBG significantly inhibited the modulation of diverse agonists-induced platelet activation under various settings such as isolated platelets, PRP and whole blood. Therefore, they can be further investigated to explore their therapeutic potential as antiplatelet agents to control platelet reactivity under pathophysiological circumstances.



<u>Figure 3.3</u>: Effects of CBD and CBG on agonists-induced aggregation in human platelet-rich plasma (PRP) and whole blood. Various concentrations (1.56, 3.125, 6.25, 12.5 and 25 μM) of CBD (A) and CBG (B) were pre-induced with human PRP for 5 minutes at 37ºC in an optical aggregometer. Then, the platelets were activated using 5 μM ADP and the level of aggregation was monitored for five minutes by optical aggregometry. Similarly, the whole blood was incubated with CBD (C) or CBG (D) and the aggregation was stimulated using 5 μg/ml collagen in an impedance aggregometer. The traces shown are representative of four separate experiments using PRP or whole blood obtained from various donors. The bar diagrams show the cumulative data which were calculated by taking the level of aggregation obtained with the positive controls as 100%. Data represent mean ± SEM (n=4). The p values shown were as calculated by one-way ANOVA (**p<0.01, ***p<0.001 & ****p<0.0001).

3.2.2 CBD and CBG displayed no cytotoxic effects in platelets

To determine whether CBD and CBG exert any cytotoxic effects in platelets, and if this might have affected agonists-induced platelet aggregation, lactate dehydrogenase (LDH) cytotoxicity assay was performed. Different concentrations of CBD and CBG (1.56, 3.125, 6.25, 12.5, 25, and 100 μM) were treated with platelets (PRP) for 30 minutes, and the level of LDH released was measured using a spectrophotometer. As shown in (**Figure 3.4**), the results demonstrate that both CBD and CBG failed to exert any cytotoxic effects in human platelets. These results confirm that CBD and CBG are not toxic to platelets at least at the concentrations used in this study, and the effects observed in aggregation experiments were their actual pharmacological effects.

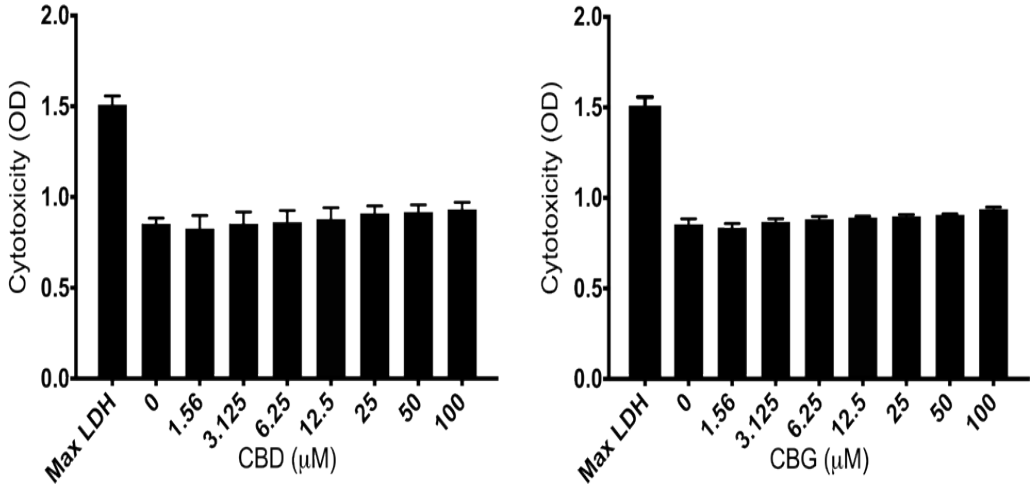


Figure 3.4: Cytotoxic effects of CBD and CBG on platelets. Human PRP was placed in a 96-well plate and treated with a vehicle control [0.01% (v/v) DMSO] or different concentrations of CBD or CBG (1.5, 3.125, 6.25, 12.5, 25, 50 and 100 μM) for 30 minutes at 37°C with 5% CO_2 prior to mixing with the LDH reaction mix and stop solution (provided with the kit) and measuring the absorbance at 680 nm and subtracted from the absorbance obtained at 490 nm to eliminate the background noise. A positive control was included to achieve the maximum level of LDH by treating platelets with a detergent solution provided in the kit. Data represent mean ± SEM (n=3).

3.2.3 CBD and CBG inhibit granule secretion in platelets

Platelets contain two different types of granules; α- and dense granules, and they release their contents upon activation (van der Meijden and Heemskerk, 2019b). These released molecules play critical roles in the regulation of secondary activation of platelets and the formation of thrombi. To assess the effects of CBD and CBG on platelet granule secretion, we used two different approaches. The first one was to measure the dense granule secretion by quantifying the amount of ATP released using a lumi-aggregometer. A luciferin-luciferase reagent was incubated with human-isolated platelets for two minutes before incubation with a vehicle control [0.01% (v/v) DMSO] or various concentrations (1.56, 3.125, 6.25, 12.5, and 25 μM) of either CBD or CBG for five minutes. Then the platelets were stimulated using 0.5 μg/ml CRP-XL and the level of granule secretion as well as aggregation was monitored for five more minutes. The results demonstrate that CBD at 25, 12.5, 6.25, and 3.125 μM were found to inhibit ATP release by 85%, 65%, 55%, and 45%, respectively (**Figure 3.5A**). Similarly, CBG has also significantly reduced ATP release at 25, 12.5, 6.25, and 3.125 with >60% inhibition (**Figure 3.5B**).

To determine the effects of CBD and CBG on α-granule secretion, the level of P- selectin exposure on the platelet surface was measured using PE-Cy5–conjugated P- selectin antibodies by flow cytometry in human PRP and whole blood. Both CBD and CBG at concentrations of 1.56, 3.125, 6.25, 12.5, and 25 μM significantly inhibited 0.5 μg/ml CRP-XL-induced P-selectin exposure in platelets (**Figure 3.5C**). Notably, the inhibitory effects of CBD and CBG were largely retained when human whole blood was used, suggesting that these compounds can affect platelet function in the presence of plasma proteins and other blood cells (**Figure 3.5D**). Together, these data suggest that CBD and CBG can affect platelet granule secretion with a concentration of as low as 3 μM, which will be easily achievable in physiological settings.

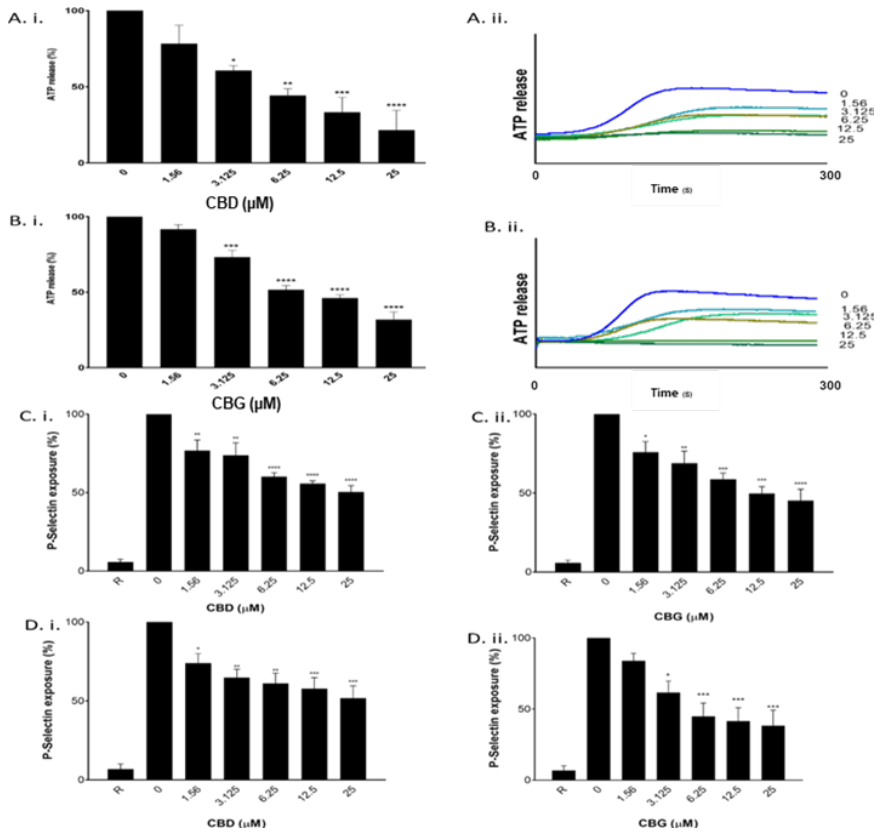


Figure 3.5: Effects of CBD and CBG on platelet granule secretion.

Human isolated platelets were mixed with a luciferin-luciferase reagent for two minutes followed by incubation with a vehicle control [0.01% (v/v) DMSO] or various concentrations of CBD (**A**) and CBG (**B**) for five minutes. Then, platelets were activated by 0.5 μg/mL CRP-XL and the level of ATP release was monitored for another five minutes by lumi- aggregometry. The traces shown are representative of four separate experiments. Human PRP was incubated with PE-Cy5 –conjugated P-selectin antibodies and a range of concentrations of CBD (**Ci**) and CBG (**Cii**) for 5 minutes before the addition of CRP-XL (0.5 μg/ml) and measuring the level of P-selectin exposure on the platelet surface. Similarly, citrated human whole blood was incubated with PE-Cy5 –conjugated P-selectin antibodies and various concentrations of CBD (**Di**) or CBG (**Dii**) for 5 minutes before the stimulation with CRP-XL (0.5 μg/ml). Then the level of P-selectin exposure on the platelet surface was measured by flow cytometry. Data represent median fluorescence intensity ± SEM (n=4) and were converted to percentages for easier comparison. The level of P- selectin exposure obtained with vehicle-treated samples was taken as 100%. The p values shown were as calculated by one-way ANOVA (*p<0.05, **p<0.01, ***p<0.001 & ****p<0.0001). R represents resting platelets.

3.2.4 CBD and CBG affect integrin αIIbβ3-mediated signalling in platelets

Since CBD and CBG affected platelet aggregation, it was hypothesised that they may affect inside-out signalling to integrin αIIbβ3, as this plays a critical role in the affinity modulation of this integrin and its subsequent binding to fibrinogen to facilitate platelet aggregation (Huang et al., 2019). To assess whether CBD and CBG influence the inside- out signalling to integrin αIIbβ3, the level of fibrinogen binding on the platelet surface was measured using flow cytometry in the presence or absence of different concentrations of CBD and CBG. Human PRP was incubated with various concentrations of CBD and CBG for 5 minutes before the addition of 0.5 µg/ml CRP-XL and further incubation of 20 minutes at room temperature. Then the cells were fixed, and the level of fluorescence was analysed by flow cytometry. The results demonstrated that both CBD (**Figure 3.6Ai**) and CBG (**Figure 3.6Aii**) inhibited CRP-XL-induced fibrinogen binding on platelets. Similarly, the level of fibrinogen binding was measured in human whole blood using flow cytometry. The whole blood pre-incubated with different concentrations of CBD and CBG for 5 minutes was stimulated with 1 µg/ml CRP-XL for 20 minutes at room temperature and the level of fluorescence was measured by flow cytometry. Both CBD (**Figure 3.6Bi**) and CBG (**Figure 3.6Bii**) displayed inhibitory effects on fibrinogen binding in a concentration-dependent manner. Similar to the aggregation data, these results further emphasise the potential of CBD and CBG to inhibit platelet activation.

Following the binding to fibrinogen, the integrin αIIbβ3 transduces signal (outside- in signalling) into the cell to trigger platelet spreading, which eventually promotes thrombus formation and clot retraction, eventually (Versteeg et al., 2013). The effects of CBD and CBG on integrin αIIbβ3-mediated outside-in signalling were assessed by measuring clot retraction. The clot formation was initiated by adding 1 U/ml thrombin to human PRP in the absence or presence of either CBD or CBG at these concentrations (6.25 and 12.5 µM). The rate of clot retraction was monitored over two hours by taking photographs every 30 minutes and measuring the remaining clot weights. Clot retraction was indeed reduced in the presence of CBD or CBG at 12.5 µM after two hours compared to the vehicle control-treated samples (**Figure 3.6C**). These data suggest that outside-in signalling mediated by integrin αIIbβ3 is modulated by CBD and CBG.

To further determine the effects of CBD and CBG on integrin αIIbβ3-mediated outside-in signalling, platelet spreading on fibrinogen was investigated in the presence and absence of various concentrations of these compounds (**Figure 3.7A**). CBD (at 6.25 and12.5 μM) and CBG (at 6.25 and 12.5 μM) significantly inhibited platelet adhesion on fibrinogen, with only a few platelets able to progress to fully formed lamellipodia and spread completely. Similarly, various concentrations of CBD and CBG inhibited platelet adhesion on immobilised collagen under static conditions (**Figure 3.7D**). Static platelet adhesion is driven through collagen receptors at the initial stage of platelet activation. Overall, these data demonstrate that CBD and CBG can affect integrin αIIbβ3-mediated outside-in signalling and collagen-mediated initial adhesion in platelets.

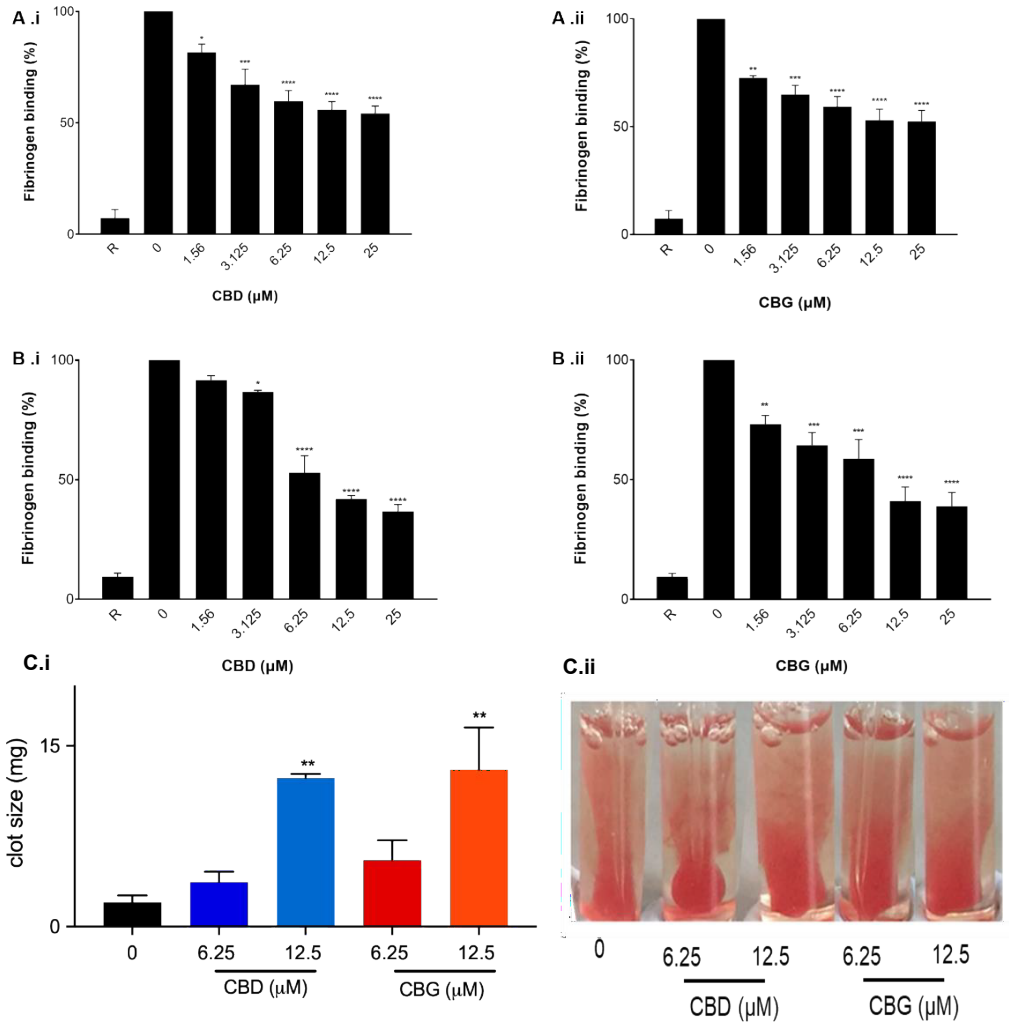


Figure 3.6: CBD and CBG affect integrin αIIbβ3-mediated signalling. Human PRP was treated with a range of concentrations of CBD (**Ai**) or CBG (**Aii**) for 5 minutes before the addition of CRP-XL (0.5 μg/ml) and measuring the level of fibrinogen binding using FITC-conjugated anti-fibrinogen antibodies by flow cytometry. Similarly, citrated human whole blood was treated with different concentrations of CBD (**Bi**) or CBG (**Bii**) for 5 minutes before stimulation with CRP-XL (0.5 μg/ml). The level of fibrinogen binding was quantified. Data represent the mean of median fluorescence intensity ± S.D. (n = 4). The effect of CBD and CBG (6.25 and 12.5 μM) on clot retraction was analysed *in vitro* (**Ci**). Data represents mean ± SEM (n=4) of clot weights measured at 2 hours. **Cii** shows a representative image of clot retraction at 90 minutes in the presence and absence of different concentrations of CBD and CBG. The p values shown were as calculated by one-way ANOVA (*p<0.05, **p<0.01, ***p<0.001 & ****p<0.0001). R represents resting platelets.

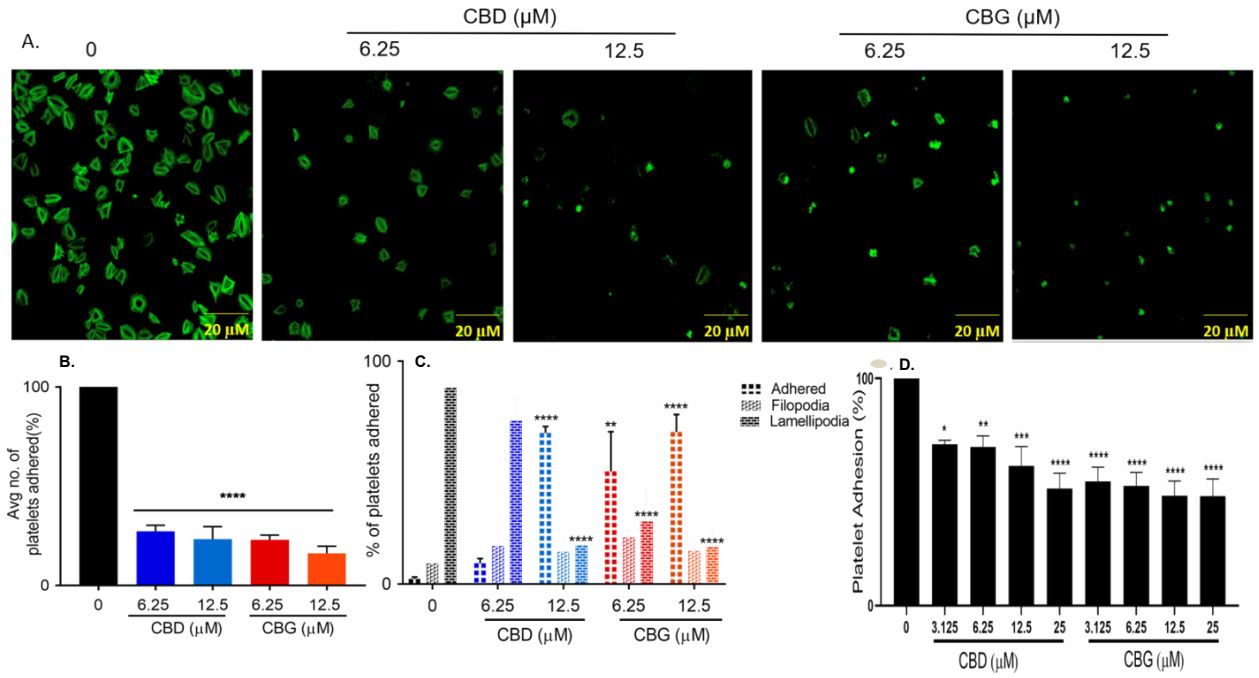
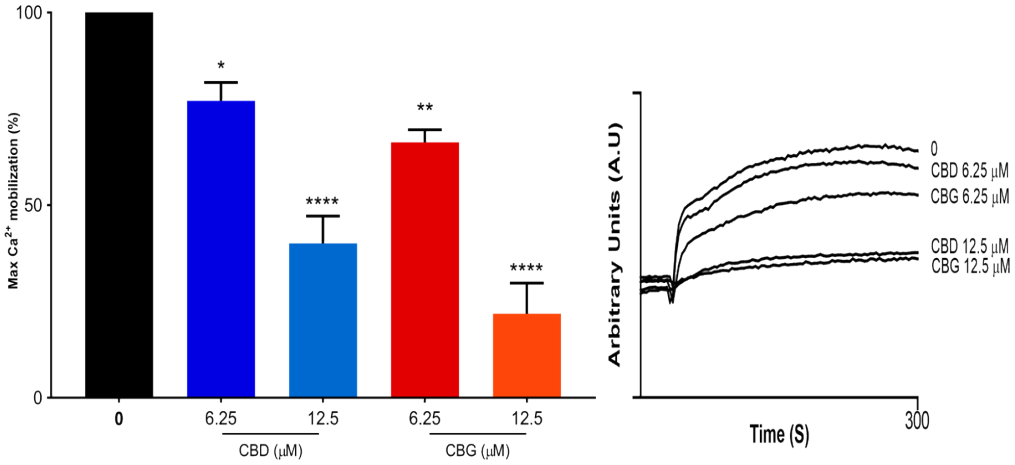


Figure 3.7: Impact of CBD and CBG on platelet spreading and adhesion. **A**, platelet adhesion and spreading on immobilised fibrinogen was analysed using human-isolated platelets in the presence and absence of CBD and CBG (6.25 and 12.5 μM) (platelets were stained with Alexa-Fluor 488 phalloidin and imaged using a 100X oil immersion lens on a Nikon A1-R confocal microscope. **B + C ,** represent the average number of adhered and percentage of platelet spreading at each stage was determined via analysing the images using ImageJ. Data represent mean ± SEM (n=3). **D**, Different concentrations of CBD and CBG were used to analyse their influence on human platelet adhesion on immobilised collagen under static conditions. Data represent mean ± SEM (n=5). The p values shown were as calculated by one-way ANOVA (*p<0.05, **p<0.01, ***p<0.001 & ****p<0.0).

3.2.5 Impact of CBD and CBG on calcium mobilisation in platelets

Upon platelet activation, intracellular calcium levels get increased to regulate platelet function including thrombus formation, cytoskeleton remodelling to promote their shape change, degranulation, and integrin αIIbβ3 affinity modulation (Shin et al., 2017). To assess if CBD and CBG affect intracellular calcium mobilisation in platelets, Fluo-4 AM dye-loaded human isolated platelets were incubated with a vehicle control [0.01% (v/v) DMSO] or CBD/CBG (6.25 and 12.5 μM) for five minutes before the addition of 1 μg/ml CRP-XL and measuring calcium levels using a spectrofluorometer. Both CBD and CBG inhibited cytosolic calcium levels upon stimulation with CRP-XL (**Figure 3.8**). In line with the above data, these results confirm that CBD and CBG can affect cytosolic calcium levels which in turn may affect other platelet functions.

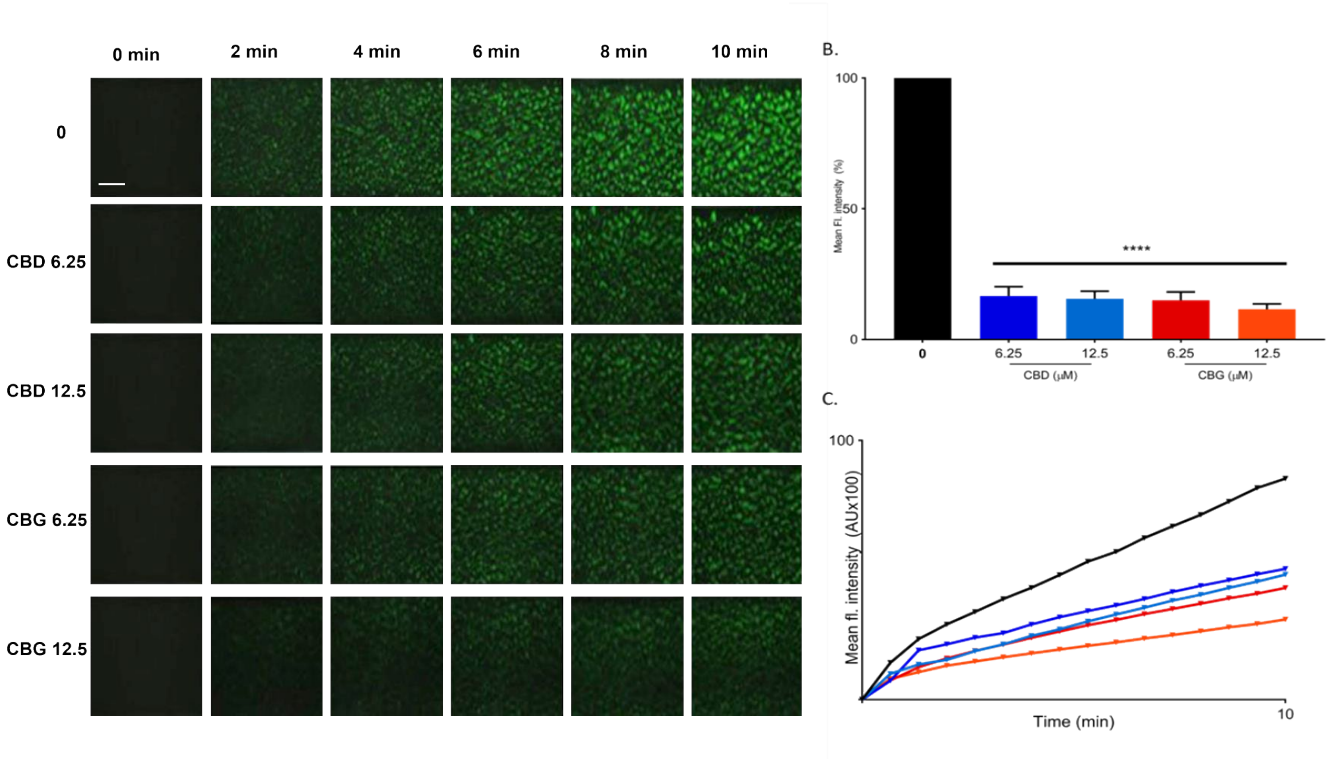


<u>Figure 3.8</u>: Effects of CBD and CBG on intracellular calcium mobilisation in platelets.

Fluo-4 AM dye-loaded human isolated platelets were incubated with vehicle control or CBD/CBG (at 6.25 and 12.5 μM) for 5 minutes at 37°C before stimulation with CRP-XL (1 μg/mL). The calcium levels were measured continuously for 5 minutes using a spectrofluorometer using an excitation wavelength of 340 to 380 nm and emission at 510 nm. Peak calcium level in the control was taken as the maximum value and the data were normalised accordingly for calcium levels in CBD and CBG-treated samples. Representative traces are shown on the right. Data represent mean ± SEM (n=3). The p values shown were calculated by one-way ANOVA (*p<0.05,**p<0.01, & ****p<0.0001).

3.2.6 CBD and CBG inhibit thrombus formation under arterial flow conditions

Since CBD and CBG inhibited platelet aggregation, granule secretion, integrin signalling, and calcium mobilisation, we assumed that they would ultimately affect thrombus formation. Therefore, ex *vivo* thrombus formation was analysed under arterial flow conditions using human whole blood in the presence and absence of CBD and CBG. The blood was labelled with DiOC6 fluorescent dye and then treated with vehicle control or different concentrations (6.25 and 12.5 μM) of CBD and CBG for five minutes. Then it was perfused through collagen-coated glass capillaries and the level of thrombus formation was monitored for 10 minutes by taking images every 30 seconds. In comparison to controls, CBD and CBG-treated samples displayed reduced thrombus volume and fluorescence intensity (**Figure 3.9**). These data suggest that CBD and CBG affect thrombus formation under arterial flow conditions, and therefore, they may have the potential to control thrombosis under pathological conditions.



<u>Figure 3.9</u>: CBD and CBG affect thrombus formation under arterial flow conditions.

DiOC6-labelled human whole blood was pre-incubated with CBD (6.25 and 25 μm) or CBG (6.25 and 12.5 μm) for 5 minutes before perfusion over collagen-coated (400 μg/ml) vena8 biochips. Images (**A**) shown are representative of three separate experiments. (10x magnification; scale bar - 10 μm). Data (**B**) represents mean ± SEM (n=3), and (**C**) represents the progression of thrombus formation over time. the p value shown was as calculated by one-way ANOVA (****p<0.0001).

3.2.7 CBD and CBG extended bleeding time in mice at higher concentrations.

Finally, to assess the effects of CBD and CBG on the regulation of haemostasis, a tail bleeding assay was performed in mice in the presence of a vehicle control [0.01% (v/v) DMSO] or CBD [at estimated (based on the calculated volume of blood using mouse weight) concentrations of 6.25 and 12.5 μM] or CBG (at an estimated concentration of 12.5 μM). Following five minutes after the administration of CBD/CBG, a 3 mm tail tip was dissected, and the volume of blood loss and bleeding time were monitored. Vehicle- treated mice bled between 309 and 460 seconds, whereas the administration of CBD extended the bleeding to between 328 and 688 seconds at 6.25 μM although this was not significantly different compared to the controls. However, at 12.5 μM, CBD increased the bleeding to 596 and 1200 seconds and this was significantly different from controls (**Figure 3.10**).

Similarly, CBG extended the bleeding to between 792 and 1200 seconds at 12.5 μM and it was significantly different from the controls. These data suggest that CBD and CBG can affect haemostasis in mice, although only at higher concentrations. At lower concentrations such as 6.25 μM, they might be safer to affect platelet activation without any adverse effects on haemostasis.

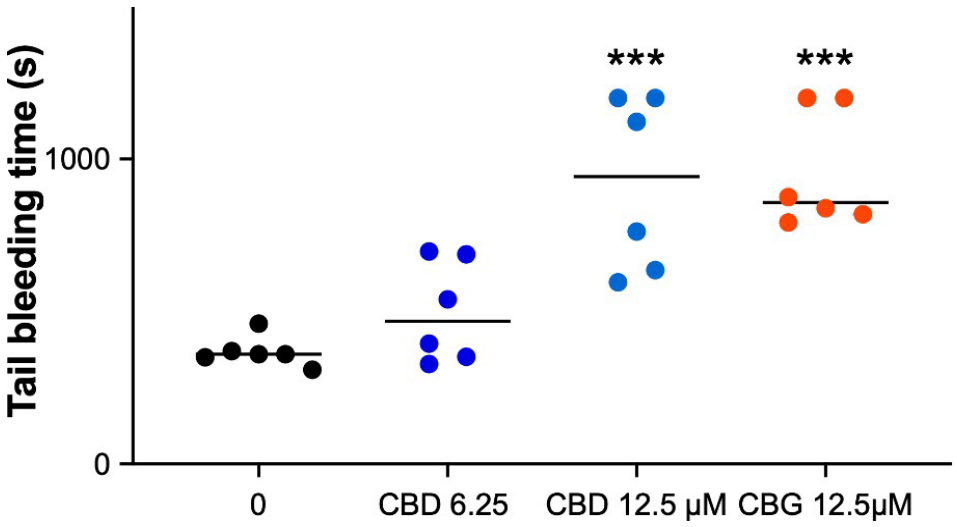


Figure 3.10: Effect of CBD and CBG on the modulation of haemostasis.

C57BL/6 mice (10-12 weeks old) were anesthetised 20 minutes before the infusion of CBD (6.25 and 12.5 µM) or CBG (12.5 µM) via the femoral artery. After 5 minutes, 3 mm tail tip was dissected, and the time to the cessation of bleeding was monitored. Data represent mean ± SEM (n=6 per group). The p values shown were as calculated by the Mann-Whitney non-parametric test (***p<0.001).

3.3 Discussion

Phytocannabinoids specifically non-psychoactive ones have been studied extensively in the last few decades as powerful therapeutic agents for various illnesses (McPartland and Russo, 2012; Cascio, Pertwee and Marini, 2017). Recently, the FDA has approved CBD for treating patients with seizures associated with Lennox- Gastaut syndrome as well as Dravet syndrome (Anon., 2020). Moreover, CBD and other non-psychoactive compounds are under clinical trials for various other diseases. The hyperactivity of platelets under diverse pathological conditions results in thrombotic conditions. Currently used anti-platelet drugs such as clopidogrel, aspirin and dipyridamole help control thrombotic risks, however, they are associated with increased risks in patients. Therefore, there is an urgent need to develop new anti-platelet agents for thrombotic diseases. Due to a range of beneficial effects and excellent pharmacokinetics, in this study, we investigated if CBD and CBG may possess therapeutic values to control thrombosis by modulating platelet function.

Formukong *et al. (*1989) demonstrated the effects of different cannabinoids on platelet aggregation using preliminary experiments. Here, they demonstrated the antiplatelet actions of CBD, CBG, THC, cannabinol (CBN) and olivetol on human and rabbit platelets (Formukong, Evans and Evans, 1989). However, they have not underpinned their impacts on various platelet functions under diverse experimental settings and the underlying molecular mechanisms. Another study by De Angelis *et al.* 2014(De Angelis et al., 2014) has elegantly reported that platelets obtained from individuals who regularly consumed cannabis have failed to aggregate upon stimulation with various agonists. Despite limited information, these studies have demonstrated the impact of cannabinoids and cannabis on the modulation of platelet reactivity and thus on thrombosis.

Our research has a unique approach in which CBD and CBG were investigated in parallel, to identify their efficacy that may lead to the development of dual therapeutic approaches. A variety of functional assays were employed to evaluate the effects of CBD and CBG under diverse settings such as isolated platelets, PRP – i.e., in the presence of plasma proteins and whole blood. Different agonists were used to underpin the effects of CBD and CBG on various stimulatory pathways in platelets. Our results demonstrate that CBD and CBG have strong inhibitory effects on isolated platelets and the effects were largely retained in PRP and whole blood.

When induced by a GPVI-selective agonist, CRP-XL, both compounds inhibited platelet aggregation even with a low concentration of 1.56 µM. CBG was displaying slightly higher inhibitory activities on platelets although they were not statistically significant when compared with CBD mediated effects. Furthermore, when the effects of CBD were compared with another agonist such as collagen, CBG displayed slightly higher inhibition although overall effects are the same and there is no statistically significant difference. Since collagen is involved only at the early stage of platelet activation, and the later stages are managed by other agonists such as ADP, thrombin and T_XA2, we sought to perform aggregation assays with these agonists to determine if CBD and CBG would exert similar effects. Indeed, they both exhibited similar inhibitory effects when these other agonists were used. These data demonstrate that their effects are maintained when different agonists are used, and therefore, they are not specific to any individual receptor-mediated signalling pathways. This might be beneficial in controlling thrombus formation through multiple pathways.

Several plant-derived compounds such as tangeretin, a flavonoid found in lemon peels (Oh et al., 2012) have previously been shown to bind plasma proteins. Therefore, the level of inhibitory effects was lower when PRP was used in comparison to isolated platelets. This is a significant issue in drug development although sometimes the plasma binding capacity is beneficial to carry the molecule in blood. To determine if CBD and CBG would display reduced inhibitory effects when PRP was used due to their binding to plasma proteins, aggregation assays were performed using PRP. Interestingly, CBD and CBG displayed higher levels of inhibitory effects in PRP similar to isolated platelets. Therefore, the binding of these compounds to plasma proteins might be minimal or it is not impacting their inhibitory effects on platelets. Similarly, when whole blood is used, the effects of many compounds will be largely reduced due to their binding to red and white blood cells (Abbate et al., 1986). However, our findings demonstrate that these compounds retained their inhibitory effects in whole blood. Overall, CBD and CBG significantly inhibited agonists-induced platelet aggregation in all experimental settings tested in contrast to a previous study which tested them only in isolated platelets. Grambow *et al.* 2016 (Grambow et al., 2016) reported that 0.1 - 10 µM of CBD did not affect human platelet activation when triggered with 10 µM TRAP6. Although this agonist was not used in this study, the concentration of agonist used in the previous study was very high, and therefore, the inhibitory effects would have been hidden or not sustained.

Calcium is an important factor in regulating cellular signalling pathways including in platelets (Nesbitt et al., 2003). Upon activation, the intracellular calcium levels increase, and this regulates various platelet functions (Gibbins, 2004). Moreover, calcium is a critical factor downstream of all agonists-induced activation pathways in platelets (Estevez and Du, 2017). To determine the effects of CBD and CBG on the modulation of intracellular calcium levels in platelets, the calcium levels were measured in the presence of these compounds. Indeed, the data demonstrate CBD and CBG affect calcium levels following stimulation with agonists such as CRP-XL. Due to a decrease in intracellular calcium mobilisation by the inhibitory effects of CBD and CBG, platelet function is highly likely to be altered downstream. To corroborate this further, platelet granule secretion was measured. Platelets consist of different granules and each type is associated with an established marker (Blair and Flaumenhaft, 2009a). Platelet aggregation leads to degranulation to promote secondary activation of platelets (Palta, Saroa and Palta, 2014). Our experiments demonstrated that CBD and CBG inhibited platelet granule secretion both for α- and dense granules. This action could be a consequence of calcium levels or due to the direct impacts of CBD and CBG on the granule secretion pathway that are largely regulated by cytoskeletal reorganisation. In a previous study (Grambow et al., 2016), CBD and CBG inhibited α-granule secretion as quantified by measuring P-selectin exposure in both isolated platelets and PRP similar to our study. They reported that platelets treated with CBD at a concentration range of 0.1 to 10 μM show a reduction in P-selectin exposure upon stimulation with TRAP6.

At the initial stage of platelet activation, an inside-out signal triggered from platelet cytosol to the integrin αIIbβ3 promotes the structural changes in this integrin to facilitate fibrinogen binding, which acts as a bridge to bind other platelets and promote aggregation (Gibbins, 2004). However, at a later stage, the fibrinogen-bound integrin αIIbβ3 triggers outside-in signalling to promote platelet spreading and clot retraction (Tucker, Sage and Gibbins, 2012). Hence, this integrin-mediated signalling is critical to maintain the initial activation of platelets and promote clot retraction (Zilberman-Rudenko et al., 2017) and subsequent wound healing. We found in our experiments that integrin αIIbβ3-mediated signalling was significantly inhibited by both CBD and CBG. The results showed that platelet adhesion and spreading on the fibrinogen-coated surfaces were reduced upon treatment with CBD and CBG. This was observed via the reduction in platelet spreading, lamellipodia formation and initial adhesion. It is unclear whether CBD and CBG have any direct effects on this integrin, or whether the effects were as a consequence of their impacts somewhere else in receptor-mediated activation pathways. A static platelet adhesion assay was performed to observe the effects of CBD and CBG on collagen, and this was

also reduced. The clot retraction mechanism of platelets combines their actions with the coagulation system, specifically thrombin which converts fibrinogen into fibrin during the coagulation cascades to aid in the development of a stable fibrin clot (Tucker, Sage, and Gibbins, 2012). The fibrin clot retraction process is largely mediated by integrin αIIbβ3 on platelets, which promotes the association between fibrinogen and the myosin-actin cytoskeleton (Li et al., 2010). The clot retraction test examines the retraction of a clot triggered by thrombin over time. CBD and CBG reduced clot retraction, and this suggests that they might have direct impacts on actin cytoskeletal arrangement and recruitment of cytoskeletal-associated proteins.

The ability of CBD and CBG to modulate thrombus formation was supported by their inhibition on numerous functions associated with platelet activation. As a result, it was hypothesised that they would inhibit thrombus formation in whole blood. *In vitro,* thrombus formation under arterial flow conditions was performed where whole blood samples treated with CBD and CBG were individually perfused at specific physiological shear rates over collagen-coated channels. CBD and CBG-treated samples were found to inhibit thrombus formation significantly compared to the controls. This is highly likely due to weakened platelet activity resulting in poor adherence to collagen and eventually leading to a decrease in thrombus formation. However, Grambow *et al.* 2016 reported that CBD was not significant in accelerating or decelerating thrombus formation *in vivo* (Grambow et al., 2016) which warrants further investigation to determine the effects of CBD and CBG under *in vivo* pathological settings.

The impact on thrombus formation may affect haemostasis in physiological settings (McRae, 2011). Therefore, a tail bleeding assay was performed using both CBD and CBG in mice. Both CBD and CBG demonstrated a slightly prolonged bleeding time in mice although at a lower concentration, it did not display any effects. The impact of these compounds on bleeding time may also occur as a result of their vasodilation effects. This was described in a previous study where CBD exhibited vasodilatory effects at a concentration of 10 μM on human-isolated pulmonary arteries and rat small mesenteric arteries (Baranowska-Kuczko et al., 2020). Overall, our results demonstrate the therapeutic benefits of CBD and CBG in preventing thrombotic events without interfering with physiological haemostasis which is essential for blood clotting upon vascular injury.

3.4 Conclusions

In conclusion, non-psychoactive phytocannabinoids derived from the *Cannabis sativa* plant have been widely utilised to cure a variety of pathological conditions in humans. Specifically, CBD is getting popular as an alternative therapy across the world for various conditions. Several studies have demonstrated their safety in the treatment of a variety of illnesses, including epilepsy, Parkinson's disease, and multiple sclerosis. This study has demonstrated the inhibitory effects of CBD and CBG on human platelet activation and thrombus formation with reduced effects on haemostasis under diverse experimental settings. As a result, these findings may be clinically relevant and potentially effective in reducing the risk of thrombotic diseases. Indeed, these data will form a basis for further *in vivo* and clinical studies to determine the therapeutic values of CBD and CBG to control thrombotic diseases.

CHAPTER FOUR

4. CBD and CBG Modulate Platelet-Mediated Inflammatory Responses

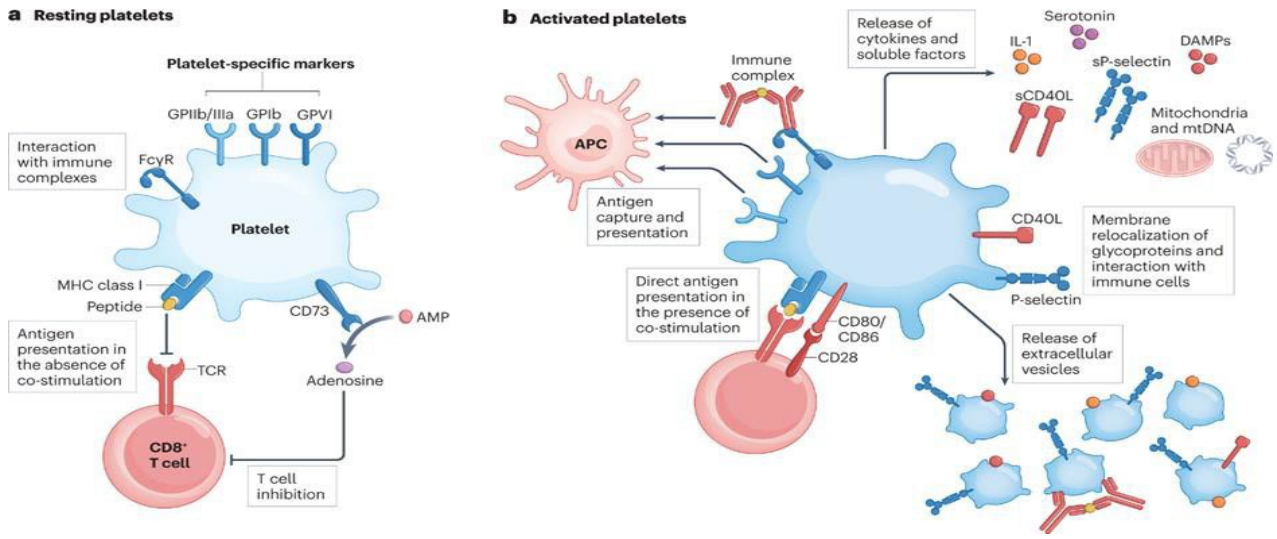
4.1 Introduction

Platelets possess a well-documented role in haemostasis as a crucial responder to vascular injury by binding to damaged endothelial cells at the site of injury to aggregate and form a stable platelet plug that prevents bleeding (Willerson and Ridker, 2004). Alongside this crucial role, they play an equally important role in inflammation and immunity, as shown in recent studies (Smyth et al., 2009; Eisinger, Patzelt and Langer, 2018). Indeed, crosstalk between platelets and other blood cells forms platelet–leukocyte aggregates (PLAs) which participate in the pathogenesis of thrombosis and inflammation (Golebiewska and Poole, 2015). In addition, they can directly interact with pathogens, control the functions of other immune cells, and mediate a wide range of physiological functions. Overall, platelets act as circulating sentinels because of their large number and ability to release a variety of immunomodulatory cytokines, chemokines, and other mediators under diverse pathological conditions (Panés, Perry and Granger, 1999).

Platelets interact with white blood cells and vascular endothelial cells both directly and indirectly via released immune mediators (Morrell et al., 2014). Platelet immunological responses are thus observed locally at platelet activation and thrombus formation sites as well as systemically at places far removed from platelet activation (**Figure 4.1**) (Stark and Massberg, 2021). Activated platelets bind to leukocytes and produce circulating PLAs, which are a reliable indicator of a prothrombotic state and are linked to a variety of cardiovascular diseases (Cerletti et al., 2012). Diseases that trigger inflammation can subsequently stimulate thrombosis such as deep vein thrombosis (DVT) (Branchford and Carpenter, 2018), stroke and atherosclerosis (Libby, Ridker and Maseri, 2002). As such during transient ischemic attacks and myocardial infarction, platelet-leukocyte aggerate is an important key diagnostic marker for thromboinflammatory processes (Rawish et al., 2020). Furthermore, many inflammatory molecules released by platelets under pathological circumstances have been linked to inflammatory disorders such as atherosclerosis and sepsis (Lipińska-Gediga, 2017). The quantities of cytokines generated in the blood are currently used to monitor inflammation levels in patients with cancer, infection, or autoimmune disorders. Clinical measurement of IL-6, for instance, is a powerful predictor of death in pancreatic and cardiovascular diseases (Gager et al., 2020).

Antiplatelet drugs disrupt platelet aggregation and thrombosis and target platelet immune-modulatory functions. Aspirin and $P2Y_{12}$ receptor antagonists such as clopidogrel, prasugrel, and ticagrelor moderate platelet-leukocyte contacts and inhibit leukocyte recruitment. As a result, antiplatelet medication influences a wide spectrum of pathologic disorders (Nylander and Schulz, 2016). Phytocannabinoids such as CBD and CBG have received a lot of attention for their significant effects on inflammation and the immune system, such as blocking pro-inflammatory cell activation and the generation of pro-inflammatory mediators or lowering intracellular and mitochondrial oxidative stress. Furthermore, CBD has been shown to have apoptotic effects in immune cell types, leading to cannabinoid-induced immunosuppression (Rieder et al., 2010). CBD has been shown to lower the levels of pro-inflammatory cytokines, suppress T cell proliferation, promote T cell death, and inhibit immune cell migration and adhesion in preclinical studies (Nagarkatti et al., 2009). In a mouse model of Alzheimer's disease, CBD was found to prevent β-amyloid-induced neurotoxicity, ROS production, and lipid peroxidation (Esposito et al., 2007). In colitis-induced mice, CBG pre-treatment reduced inflammation by lowering the expression of pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ (Gugliandolo et al., 2018).

Therefore, CBD and CBG have the potential to treat platelet-mediated inflammatory disorders as well as be effective in the treatment and prevention of thrombotic diseases. As a result, the effect of CBD and CBG on platelet-mediated inflammatory responses will be analysed in this chapter. The aim of this chapter is to focus on the potential of CBD and CBG to influence the production of several inflammatory cytokines from activated platelets as well as their impact on platelet- leukocyte interactions.



<u>Figure 4.1</u>: Interactions between platelets and immune cells play a crucial role in both innate and adaptive immune responses.

Platelets play a role in immune responses by both resting and activated states. Resting platelets scavenge immune complexes, inhibit CD8+ T cells, and produce anti-inflammatory adenosine. Activated platelets release molecules that promote interaction with immune cells, present antigens, and produce extracellular vesicles. **a)** Resting platelets have specific markers and receptors that allow them to scavenge immune complexes.These markers include glycoproteins GPIIb/IIIa, GPIb, GPVI, and Fcγ receptor (FcγR). CD73 on resting platelets contributes to their ability to inhibit CD8+ T cells. CD73 catalyzes the production of anti-inflammatory adenosine from adenosine monophosphate (AMP). **b)** Activated platelets releases cytokines and damage-associated molecular patterns. This promotes their interaction with immune cells. Activated platelets also present antigens to immune cells and produce extracellular vesicles containing various molecules. This figure was adapted from (Scherlinger, and Richez, 2023).

4.2 Results

4.2.1 CBD and CBG affect the production of reactive oxygen species in platelets

Reactive oxygen species (ROS) are highly reactive molecules that are produced in response to both endogenous and external stimuli. ROS includes both radical and non- radical oxygen-based compounds, including superoxide anion ($O2^{-}$), hydrogen peroxide (H2O2), and hydroxyl radical (OH) (Masselli et al., 2020). Oxidative stress is a common pathophysiological mechanism, and it has been linked to a wide range of pathological conditions, including cancer, diabetes, and cardiovascular disease, among others (Vona et al., 2021). Platelet responses are altered by ROS produced from both platelets and other vascular sources. CBD and CBG were tested to see whether they had any effect on platelet ROS generation in response to CRP-XL activation. A reagent called 2′,7′-Dichlorofluorescin diacetate (H_2DCFDA) was used to measure the intracellular ROS level in CRP-XL-stimulated platelets.

Human-isolated platelets ($4x10^8$/ml) were preincubated with 10 μM H_2DCFDA at 37°C for 30 minutes before being used in the experiment. Platelets loaded with H_2DCFDA were loaded to a 96-well plate with varying doses (6.25, 12.5, and 12.5 μM) of CBD and CBG and incubated for 5 minutes before being activated with CRP-XL (1 μg/ml). Then, diluted the samples ten-fold in modified tyrodes-HEPES buffer that included 0.1% BSA (BD Biosciences, UK). Analyses were carried out immediately using an Accuri C6 flow cytometer. By measuring the median fluorescence intensity and expressing it as a fold change, the amount of ROS produced was estimated. The amount of fluorescence obtained with H_2DCFDA-loaded resting platelets (without exposure to CRP-XL) was used as a reference to quantify the baseline level of ROS in platelets in this experiment. To determine the possible effects of CBD and CBG, the amount of fluorescence (ROS generation) achieved with CRP-XL-induced platelets, a positive control, was normalised to 100%.

Figure 4.2 shows a typical histogram produced from flow cytometric studies of platelet samples after stimulation with 1 μg/ml of CRP-XL in the presence or absence of various concentrations of CBD and CBG. Each sample includes both resting (unstimulated platelets) and positive (vehicle) controls. According to **(Figure 4.2A)**, CRP- XL stimulation increased baseline fluorescence significantly, but this did not occur in the vehicle-treated sample (resting platelets). The rise in ROS generation was greatly minimized by preincubating platelets with CBD and CBG before adding CRP-XL. At doses of 6.25 and 12.5

μM, CBD inhibited CRP-XL-induced ROS generation in platelets by 81% and 50%, respectively. CBG suppressed ROS generation to a comparable degree at 6.25 and 12.5 μM concentrations, at 82% and 42%, respectively.

In addition to the antiplatelet effects that were revealed in the previous chapter, these data show that CBD and CBG exert inhibitory effects on ROS production in platelets.

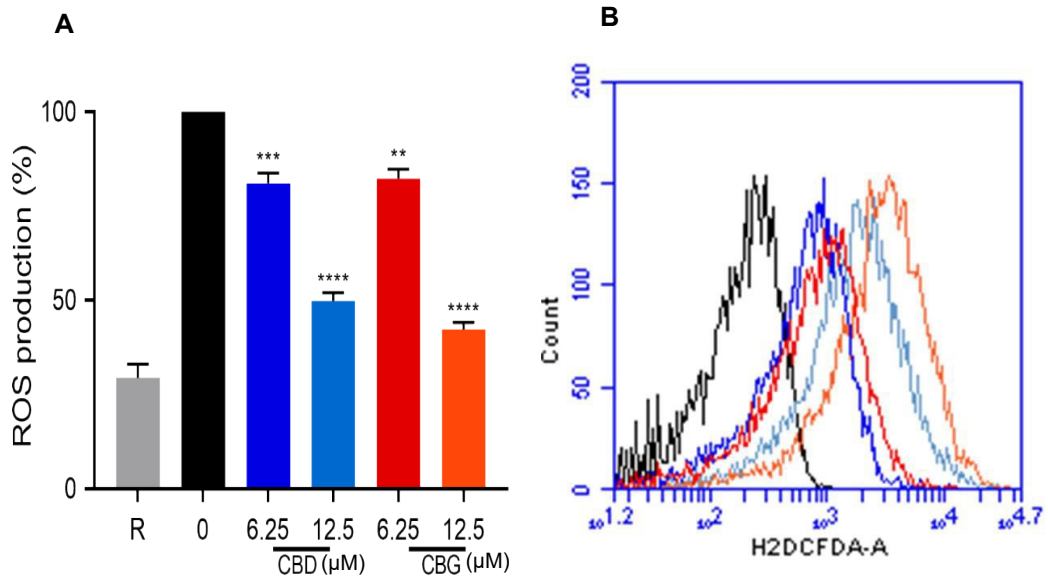


Figure 4.2: The effect of CBD and CBG on ROS generation by platelets.

H_2DCFDA, a fluorescent dye, was used to detect ROS formation in platelets. After 30 minutes of H_2DCFDA incubation at 37°C, the platelets were treated with different concentrations of CBD, CBG, or vehicle control [0.01% (v/v) DMSO] for 5 minutes before being stimulated with 1 µg/mL CRP- XL. Flow cytometry was used to quantify the generation of ROS in platelets. (**A**) The cumulative data and the significance of differences were assessed using One-way ANOVA for repeated measurements with Bonferroni's correction for multiple comparisons (n=4). (**p< 0.01, ***p<0.001, and ****p≤0.0001). (**B**) An example of a histogram displaying the ROS release. R represents resting platelets.

4.2.2 CBD and CBG affect the release of soluble P-selectin

The adhesion molecule P-selectin is one of the most prominent indicators of platelet activity (Blann, Nadar and Lip, 2003). The stimulation of platelets and endothelial cells leads to an increase in the surface expression of this molecule (Dixit and Simon, 2012). In addition, it has been shown that greater levels of the soluble P-selectin (sP- selectin) that is released into the circulation may be linked to an increased risk of developing cardiovascular disease and stroke (Burger and Wagner, 2003). The release of this adhesion molecule from active platelets or injured endothelial cells is reflected in its elevated plasma concentration (Amin et al., 2016). sP-selectin could be a potential disease biomarker in individuals with hypertension, hypercholesterolemia, and unstable angina. Human plasma has normal concentrations of 30–60 ng/mL sP-selectin, but under pathological conditions, this can increase to high concentrations that may range from 120 to 200 ng/L, with extreme values of up to 1000 ng/L (Woollard, 2005).

The effects of CBD and CBG on the release of sP-selectin during platelet activation were investigated in this research. Blood was drawn from healthy human donors, and the isolated platelets were collected in the same manner as previously reported. Pre- incubation of the platelets with two concentrations (6.25 and 12.5 μM) of CBD and CBG was done for five minutes before the addition of CRP-XL (1 μg/mL). Platelets were then centrifuged for 10 minutes at 1000 g at room temperature. To assess the amount of sP- selectin, the supernatant was collected and used in the experiment.

We used a human sP-selectin ELISA kit to test whether these phytocannabinoids influence platelet sP-selectin release in response to CRP-XL stimulation (Theromfisher, UK). With a mean concentration of 29.61 ng/mL in response to 1 mg/ml of CRP-XL, human-isolated platelets produced more sP-Selectin than resting platelets, which produced 1.47 ng/mL. CRP-XL-induced sP-Selectin production was dramatically reduced when platelets were incubated with CBD. A significant reduction in the level of sP-Selectin was observed at concentrations of 6.25 and 12.5 μM compared to the positive control (CRP-XL alone), with an inhibitory effect at a mean concentration of 15.31 ng/mL and 4.7 ng/mL, respectively, compared to sP-Selectin released from control platelets. CBG has also shown a considerable reduction of sP-Selectin secretion at concentrations of 6.25 and 12.5 μM, with a mean concentration of 14.13 and 3.45 ng/mL, respectively, at these concentrations (**Figure 4.3**). These findings

show that CBD and CBG may inhibit platelet sP- Selectin release in response to CRP-XL activation.

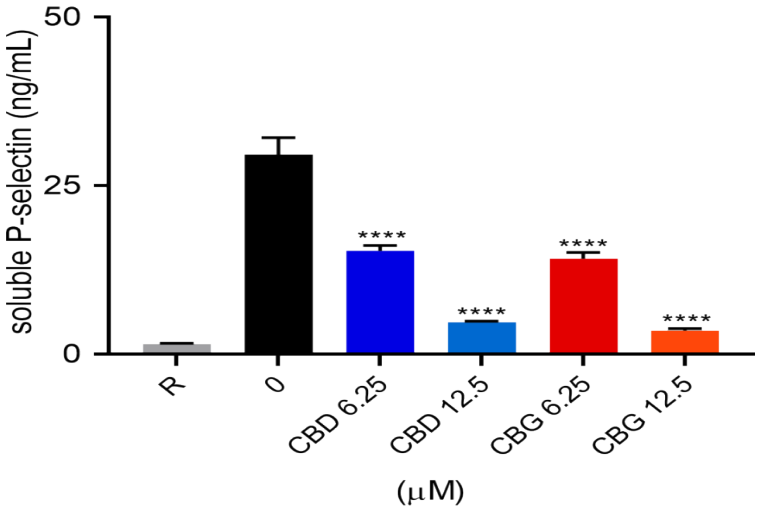


Figure 4.3: Effects of CBD and CBG on the release of sP-selectin from platelets.

Human isolated platelets were pre-treated for 5 minutes at 37°C with a vehicle control [0.01% (v/v) DMSO] or varying concentrations of CBD or CBG and then activated in an aggregometer for 5 minutes at 37ºC with CRP-XL (1 µg/ml) for 5 minutes at 37°C. The supernatant obtained after centrifuging the samples at 1000 g for10 minutes at room temperature was immediately used in this assay.A human sP-selectin ELISA kit according to the manufacturer's instructions was used to measure the concentration of sP-selectin. Cumulative data mean ± SEM (n=3). The p value shown (****$p<0.0001$) was calculated by one-way ANOVA followed by a Bonferroni's correction for multiple comparisons. R represents resting platelets.

4.2.3 CBD and CBG affect the release of Tumour Necrosis Factor-α (TNF-α) from platelets

TNF- α is a potent pro-inflammatory cytokine that is quickly produced upon trauma, infection, or exposure to bacterial-derived LPS and has been demonstrated to be one of the most abundant early mediators in inflamed tissue (Monaco et al., 2015). TNF-α has been considered a key factor in the activation and recruitment of inflammatory cells, as well as a key player in the development of many chronic inflammatory conditions (Davizon-Castillo et al., 2019). Platelet cytoplasm contains alpha granules and these granules store and release some pro-inflammatory cytokines upon activation (Blair and Flaumenhaft, 2009b). Chronic inflammation, characterised by elevated levels of TNF- α and IL-1β, plays a vital role in the development of cardiovascular disease (CVD) (Chen et al., 2018). Similarly, a condition with increased systemic TNF-α is associated with a higher prevalence of CVD and atherothrombotic events, suggesting that TNF-α plays a substantial role in the development of thrombosis (Davizon-Castillo et al., 2019). In some conditions, inhibiting TNF-α dependent cytokine cascades and leukocyte recruitment improves clinical and serological outcomes (Eggert-Kruse et al., 2007).

Here, we performed an ELISA test to investigate the effects of CBD and CBG on the release of TNF-α from platelets after they were activated. Platelets from human blood samples were isolated as previously described and then pre-incubated for five minutes with various concentrations of CBD and CBG before adding a vehicle or CRP-XL (1 µg/mL) and incubating for five minutes under stirring conditions to obtain resting or activated platelets, respectively. TNF-α levels in human platelets were measured using a commercially available human TNF-α ELISA kit according to the manufacturer's instructions. TNF-α level was significantly increased in platelet supernatants following stimulation with CRP-XL, reaching a mean concentration of 21.3 pg/mL, compared to a mean concentration of 0.9 pg/mL in unstimulated platelets (**Figure 4.4**). TNF-α levels in platelets treated with CBD and CBG were considerably lower than those in the positive control. For example, CBD treatment resulted in a substantial decrease in TNF-α concentrations at the tested concentrations. Its levels were lowered by a mean of 2.2 and 0.4 pg/mL at doses of 6.25 and 12.50 µM, respectively. CBG also demonstrated a significant decrease in TNF-α concentration at 6.25 and 12.50 µM, with TNF-α levels dropping by 2.5 and

0.5 pg/mL, respectively. This data shows that CBD and CBG could inhibit the release of TNF-α from human platelets in response to CRP-XL activation.

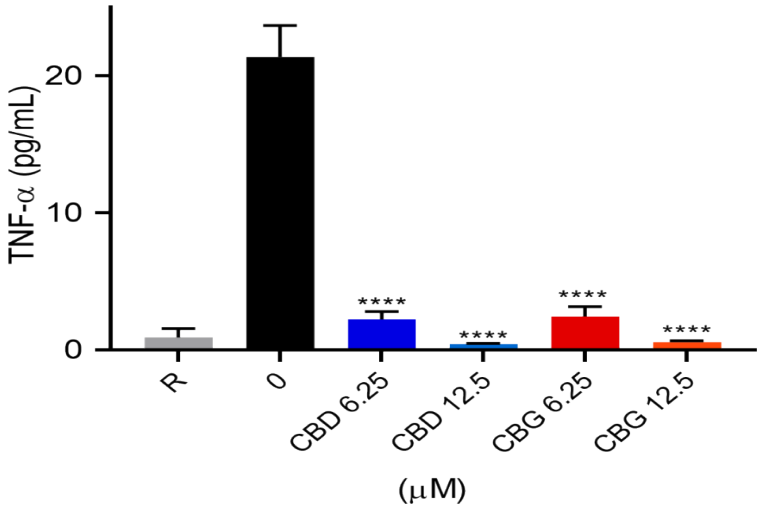


Figure 4.4: CBD and CBG suppress the release of TNF-α from human platelets.

Human isolated platelets were pre-treated with different doses of CBD, CBG, or vehicle control [0.01% (v/v) DMSO] for 5 minutes at 37°C, followed by 5 minutes of activation with 1 μg/ml CRP-XL under stirring conditions. To extract supernatants, the platelets were centrifuged at 1000 g for 10 minutes at room temperature. Following the manufacturer's instructions, a human TNF-α ELISA kit was used to measure TNF-α levels in platelet supernatants. Cumulative data represents mean ± SEM (n=3). p values (****p≤0.0001) shown were calculated by one-way ANOVA followed by a Bonferroni's correction for multiple comparisons. R represents resting platelets.

4.2.4 CBD and CBG reduce the secretion of RANTES from activated platelets

Numerous inflammatory molecules, including certain chemokines, are released upon platelet activation (Gear and Camerini, 2003). RANTES is a key molecule in the control of inflammation and pathological conditions including atherosclerosis. It is involved in regulating platelet activation and monocyte recruitment, which could accelerate atherosclerotic plaque development (Mause et al., 2005). As mentioned above, a large quantity of proinflammatory and immune-modulating substances are released by platelet α-granules. Therefore, platelet granules are a primary source of RANTES *in vivo*, which can further promote RANTES production in endothelial cells (Koper-Lenkiewicz et al., 2019). RANTES may inhibit monocytes moving on activated endothelium in flow and affect the adhesion of monocytes and T cells at the site of inflammation.

In thrombin-stimulated platelets, the effects of CBD and CBG on the release of RANTES were investigated. Before activation with 0.1 U/ml thrombin and stirring for 5 minutes, human-isolated platelets from different donors were prepared and incubated with various concentrations of CBD and CBG. The supernatants from the platelets were collected by centrifugation and used in the study. The quantity of RANTES produced from platelets after stimulation with 0.1 U/ml of thrombin was determined using a human RANTES ELISA kit (Theromfisher, UK).

CBD and CBG have been shown to suppress RANTES production from thrombin- stimulated platelets (**Figure 4.5**). In comparison to thrombin-activated cells, CBD and CBG at a concentration of 12.5 μM showed a comparable strong inhibitory effect, with around 80-90% inhibition. Moreover, low concentrations of CBD and CBG (6.25 μM) have decreased RANTES release to 88 ng/mL and 75 ng/mL, respectively. The above findings on RANTES and TNF-α reveal that CBD and CBG are capable of inhibiting platelet inflammatory molecules released in response to various agonists. This might be attributable to the fact that CBD and CBG, as previously discussed, suppress α-granule secretion that leads to suppress the inflammatory molecules release.

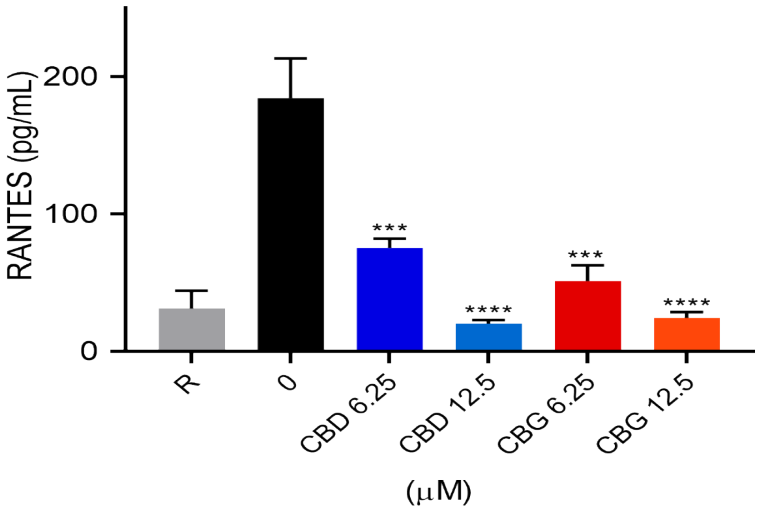


Figure 4.5: Effects of CBD and CBG on the release of RANTES from platelets.

Isolated platelets from human blood pre-treated with a vehicle or various concentrations of CBD or CBG were activated with 0.1 U/ml thrombin in an aggregometer for 5 minutes at 37°C. Platelets were then centrifuged at 1000g, for 10 minutes at room temperature, and resulting supernatants were collected and used in the assay. The concentrations of RANTES secreted from activated platelets were determined using a human RANTES ELISA kit according to the manufacturer's protocol. The bar chart represents concentrations of RANTES obtained in the presence of different concentrations of CBD and CBG. Cumulative data represent mean ± SEM (n=3). P-values (***$p<0.001$ and ****$p\leq0.0001$) shown were calculated by one-way ANOVA followed by a Bonferroni's correction for multiple comparisons. R represents resting platelets.

4.2.5 CBG and CBG affect the release of vascular endothelial growth factor (VEGF) from platelets

Platelet degranulation releases many soluble mediators, which is critical for wound healing, as well as the stimulation of angiogenesis (Golebiewska and Poole, 2015). The development of new blood vessels needs a combination of growth factors acting in a certain physiological ratio. VEGF, which regulates endothelial inflammation and integrity, is released by platelets and is a key regulator of angiogenesis (Niu and Chen, 2010). VEGF is stored in large amounts in platelet alpha granules, which are recognised as one of the primary storage locations, with roughly 80% of circulating VEGF stored in platelet granules (Salgado et al., 2001). Inhibitors of thrombin production, including fondaparinux (Xa inhibitor), reduce the release of VEGF from platelets by inhibiting the PAR1 activation process. Aspirin and other antiplatelet medications have been shown to impact the release of pro-angiogenic cytokines during platelet-tumour cell interactions (Tao et al., 2021). Based on the above findings, we hypothesised that CBD and CBG would have comparable effects on CRP-XL-induced VEGF release from platelets. To see whether these phytocannabinoids alter VEGF release from platelets, we used a human VEGF ELISA kit (Theromfisher, UK) to measure VEGF levels after stimulation of platelets with CRP-XL. Human platelets were isolated as stated in the methods section.

Platelets were incubated with varying concentrations of CBD, CBG, or a vehicle control. Following incubation, samples were stimulated with 1 µg/ml CRP-XL for 5 minutes in an aggregometer under constant stirring conditions. Following this, samples were centrifuged (1000 g for 10 minutes at room temperature), and supernatants were collected and used in the assay immediately, according to the manufacturer's procedure. The amount of VEGF produced from platelets activated with 1 µg/ml CRP-XL increased statistically significantly to 922.5 pg/mL on average, compared to 59.2 pg/mL in resting cells. However, after stimulation with CRP-XL, the amount of VEGF produced from platelets preincubated with various doses of CBD dramatically decreased, with mean concentrations of 116.8 pg/ml and 73.1 pg/mL at 6.25 and 12.5 µM, respectively. Similarly, CBG exhibited a considerable decrease with a mean of 88.6 and 70.70 pg/ml at 6.25 and 12.5 µM, respectively, when compared to the vehicle-treated platelets (**Figure 4.6**). This result shows that angiogenesis regulators like VEGF released by platelets may be affected by CBD and CBG.

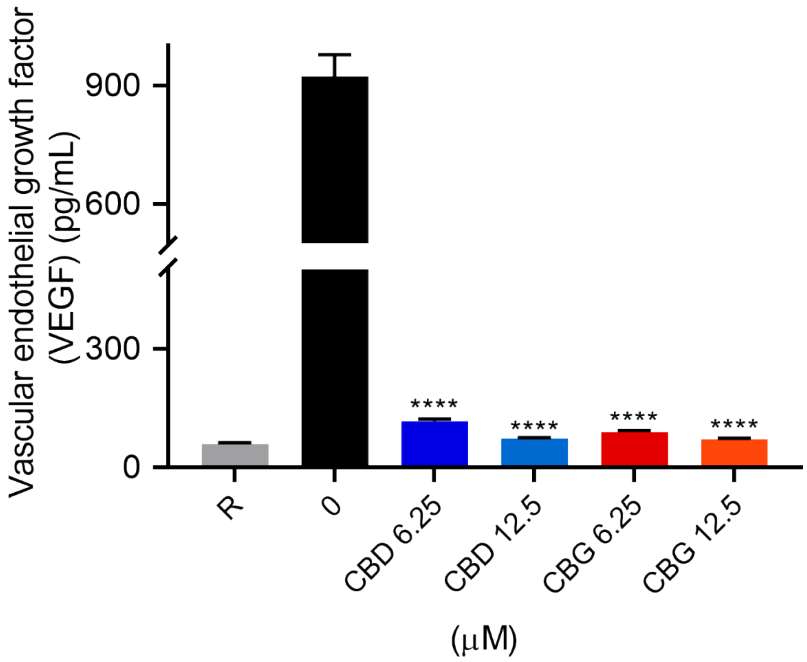


Figure 4.6: Effect of CBD and CBG on the release of VEGF from platelets.

Human isolated platelets were prepared and incubated with various concentrations of CBD and CBG or vehicle control [0.01% (v/v) DMSO] for 5 minutes at 37ºC, before stimulation with CRP-XL (1 µg/ml) under stirring conditions in an aggregometer for 5 minutes at 37ºC. Platelets were then centrifuged at 1000 g for 10 minutes at room temperature, and the resulting supernatants were used in the assay. The level of VEGF was measured using a human VEGF ELISA kit, according to the manufacturer's protocol. The graph depicts the mean ± SEM (n=3). P values (****p≤0.0001) shown were calculated using one ANOVA followed by Bonferroni's correction for multiple comparisons. R represents resting platelets.

4.2.6 CBD and CBG reduce the release of interleukin-1 beta (IL-1β) from platelets

Platelets are known to have a role in the early stages of atherosclerosis (Lopez- Castejon and Brough, 2011). For example, platelets release chemokines and inflammatory mediators that contribute to atherosclerosis (Libby, Ridker and Maseri, 2002). Although platelets lack nuclei, they contain a considerable quantity of messenger RNA (mRNA) that aids in the synthesis and release of several molecules including IL-1β which is a pro-inflammatory cytokine (Koupenova et al., 2018). Additionally, this cytokine has been demonstrated to have various roles in inflammation, CVD, endothelial dysfunction, and blood clotting disorders (Ait-Oufella et al., 2011). IL-1β may also activate immune cells by binding to the IL-1β receptor on their surface (Lopez-Castejon and Brough, 2011).

We examined the impact of CBD and CBG on the release of IL-1β from platelets when stimulated with an agonist. A human IL-1β ELISA kit was used to measure IL-1β levels from platelets. Different concentrations (6.25 and 12.5 μM) of CBD and CBG along with a vehicle control [0.01% (v/v) DMSO] were added to human platelets before they were activated with 0.1 U/ml thrombin for 5 minutes in an aggregometer. The cells were immediately centrifuged at 1000g for ten minutes at room temperature, and the supernatants were collected and used in the assay. The results demonstrate that the IL-1β released by non-stimulated platelets was 2.5 pg/mL. Thrombin (0.1 U/ml) significantly increased the release of IL-1β as shown in (**Figure 4.7**), with a mean concentration of 164 pg/mL. However, CBD decreased IL-1β release at concentrations of 6.25 and 12.5 μM, with inhibition levels at 13.67 and 4.39 pg/mL, respectively. Similarly, CBG at 6.25 and 12.5 μM dramatically reduced IL-1β release, with the levels at 14.57 and 5.29 pg/mL, respectively. These findings suggest that CBD and CBG appear to show a promising impact on platelet-mediated inflammatory responses.

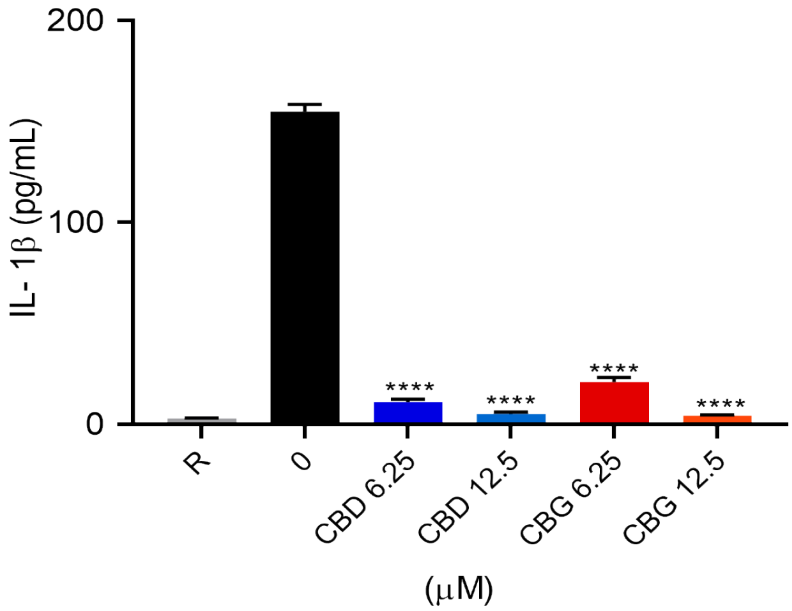


Figure 4.7: Effect of CBD and CBG on the release of IL-1β from platelets.

The levels of IL-1β released from platelets were measured using a human IL-1β ELISA kit. Human isolated platelets were incubated with vehicle control [0.01% (v/v) DMSO] or different concentrations of CBD and CBG for 5 minutes at 37°C, and then activated with thrombin (0.1U/ml) for 5 minutes at 37ºC. After the treatment, platelets were centrifuged at 1000g for 10 minutes at room temperature, and the resulting supernatants were used in the assay. The level of IL-1β was measured using a human IL-1β ELISA kit according to the manufacturer's protocols. The bar chart represents the levels of IL-1β obtained in the presence and absence of different concentrations of CBD or CBG. The graph depicts the mean ± SEM (n=3). p values (****p≤0.0001) were calculated using one ANOVA followed by Bonferroni's correction for multiple comparisons. R represents resting platelets.

4.2.7 CBD and CBG modulate platelet-leukocyte interactions

Endothelial activation due to damage or inflammation causes platelet rolling across intact endothelial cells (Jenne, Urrutia and Kubes, 2013). Endothelial stress is demonstrated during inflammation by an increase in the expression of E-selectin and a receptor (PSGL1) for platelet P-selectin. RANTES and HNP1 (human neutrophil peptide 1) proteins are secreted simultaneously by platelets and neutrophils during chronic inflammation (Nurden, 2011). In a model of myocardial infarction, these proteins form heterodimers that are responsible for monocyte adhesion and recruitment. Platelet-leukocyte (specifically with monocytes and neutrophils) complexes are also formed as part of the inflammatory responses (Totani and Evangelista, 2010).

Platelets may also influence the endothelium by raising their surface expression of CD40L and secretion of cytokines such as IL-1β, which is produced in response to inflammatory stimuli (Chen et al.,2020). Endothelial permeability, as well as leukocyte recruitment and adhesion to the endothelium, may be increased by released IL-1β. Interestingly, IL-1β improves platelet binding to collagen and fibrinogen and promotes aggregation in the presence of collagen (Lopez-Castejon and Brough, 2011). CBD and CBG both dramatically decrease the release of platelet-derived pro-inflammatory cytokines, as seen above. As a result, we examined the effects of CBD and CBG on platelet-leukocyte interactions to get a better understanding of their role in platelet- mediated inflammatory diseases.

Whole blood was obtained from healthy individuals and incubated for 10 minutes with various doses (6.25 and 12.5 μM) of CBD and CBG along with a vehicle control [0.01% (v/v) DMSO] before being stimulated with 10 μM of a thrombin receptor activation peptide (TRAP-6). Double-labelling of platelets and leukocytes was utilised to measure the degree of platelet-leukocyte interactions, which were then analysed using flow cytometry.

Before flow cytometry analysis, samples were doubly stained with a leukocyte pan marker, PE-labelled CD45, and a platelet marker, FITC-labelled CD42b. Leukocytes were detected in whole blood samples based on their size and CD45 expression. Leukocyte and platelet-positive cells were found inside the gated zone. When compared to resting cells, the data show that TRAP-6 considerably increased platelet-leukocyte interactions (**Figure 4.8**).

It was also shown that CBD and CBG at all doses reduced the TRAP-6- induced platelet-leukocyte interactions compared to the positive control.

There was a substantial decrease in the proportion of platelet-leukocyte interactions with CBD (6.25 and 12.5 μM) compared to the positive sample (78%). CBG (6.25 and 12.5 μM) was shown to be effective in reducing these interactions from 78% in TRAP-stimulated samples to 7.8% and 9.1%, respectively. Statistical analysis of these data suggests that CBD and CBG had substantial inhibitory effects on the platelet-leukocyte interactions caused by TRAP-6 (**Figure 4.8**).

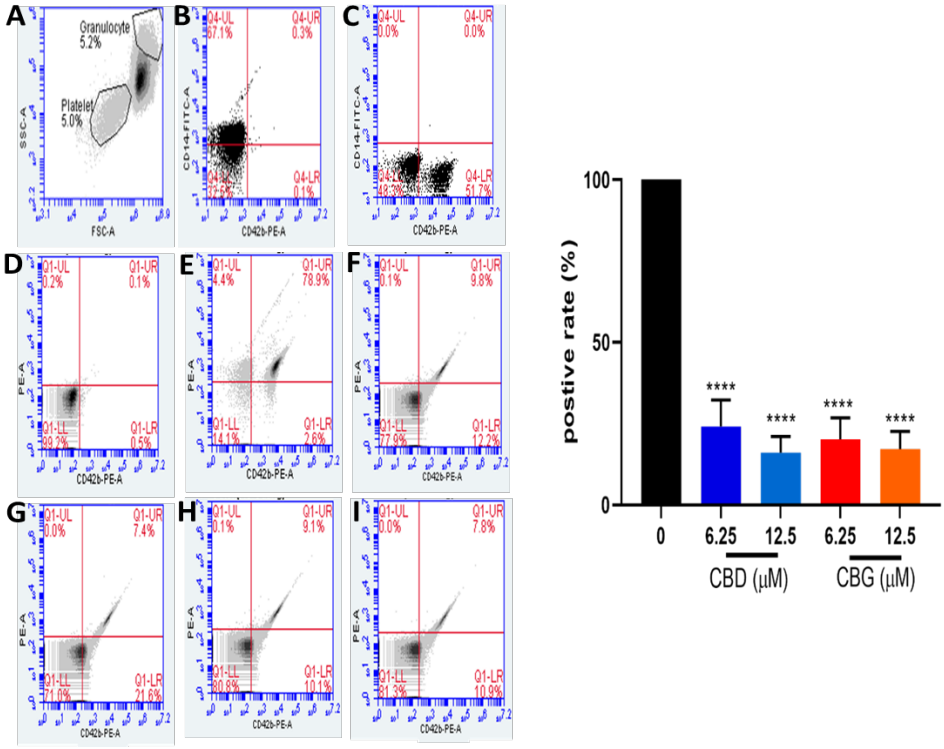


Figure 4.8: Effect of CBD and CBG on the formation of leukocyte-platelet interaction

Whole blood from human healthy donors was incubated with different concentrations (6.25 and 12.5 µM) of CBD and CBG for 10 minutes before stimulation with 10 µM TRAP-6. At first, the granulocyte population was identified in whole blood based on light scatter characteristics, size, and granularity (**A**). Leukocytes were identified in the blood sample by their size and expression of CD42b in flow cytometry, using a gated region for the quantification of platelet-leukocyte aggregates by flow cytometry. Stained with the leukocyte marker, PE-labelled anti-CD45 antibodies (**B**), and the platelet marker, FITC-labelled anti-CD42b antibodies (**C**), and double- stained cells in the presence of vehicle control [0.01% (v/v) DMSO] resting platelets (**D**) or activated by the presence of 10 µM TRAP (positive control) (**E**). CD42b positive events within the leukocyte gate were identified as platelet-leukocyte aggregates. Representative histograms show the level of CD42b as well as CD45 positive events in samples treated with different concentrations of CBD (**F-G**), and CBG (**H-I**). (**J**) Quantified data displays the percentage of inhibition by CBD and CBG- treated samples (vehicle-treated sample represents 100% platelet-leukocyte aggregates). Data represents mean ± SEM (n=4). The statistical significance was calculated by one-way ANOVA. P values shown (****p<0.0001) are calculated by one ANOVA followed by Bonferroni's correction for multiple comparisons.

4.3 Discussion

In addition to their haemostatic functions, platelets play a key role in establishing a host defence response and interacting with immune cells. As platelets reach the bloodstream, they invariably come into contact with pathogens. A large number of pro- inflammatory and pro-thrombotic molecules are released from platelets when they are activated by various molecules released during pathological conditions (Ed Rainger et al., 2015). These molecules play an important role in facilitating the interactions between activated platelets and immune cells in order to augment inflammatory responses (Chen et al., 2020). Hence, more platelets are activated when various molecules are released during pathological conditions (Libby, Ridker and Maseri, 2002). Platelets play an essential part in the progression of a wide variety of inflammatory disorders, including atherosclerosis and sepsis (Lipińska-Gediga, 2017). Platelets have been shown to have a role in adaptive immunological and inflammatory processes, since they may directly interact with bacteria, resulting in platelet activation and aggregation (Iba and Levy, 2018). Due to the consumption of circulating platelets by sequestration in lungs and microvasculature thrombi production, uncontrolled infection during sepsis causes widespread platelet activation, resulting in a reduced platelet count (thrombocytopenia)(Vadasz et al., 2015). Inflammation in the vascular endothelium is one of the primary causes of atherosclerosis, and this is linked to platelet activation (Van Ijzendoorn, Heemskerk and Reutelingsperger, 1995). Upon vascular damage, the circulating monocytes migrate to the affected region and transform into macrophages (Ghattas et al., 2013). When macrophages consume oxidised lipoproteins, they transform into foam cells and begin the formation of fatty streaks. Simultaneously, additional inflammatory cells, such as activated T cells and mast cells, adhere to the endothelium (Moore and Freeman, 2006).

Consequently, it is essential to understand the molecular processes underlying the regulation of platelet-mediated thrombo-inflammatory responses and to develop innovative therapeutic approaches for controlling these responses under pathological conditions (Chen et al., 2020). CBD has been shown to reduce inflammation and help in treating several neurodegenerative diseases that are linked to inflammation (Fernández- Ruiz et al., 2013). In hypoxic-ischaemic immature brains of newborn mice, CBD administration dramatically decreased the production of IL-6, TNF-α, and COX-2. Similarly, low-dose CBD therapy of mice treated with lipopolysaccharide lowered TNF-α production (Walsh et al., 2010).

CBD dramatically decreased both leukocyte migration into the lungs and levels of albumin, TNF-α, IL-6, and other chemokines in bronchoalveolar lavage fluid in a mouse model of acute lung damage (Ribeiro et al., 2012). CBD therapy also decreased inflammation and prevented a rise in brain IL-1β levels in neonatal pigs with brain hypoxic–ischemic (HI) damage. TNF-α, IFN-α, IL-6, IL-12, and IL-17 were likewise lowered considerably by CBD in this type of inflammation (Arruza et al., 2017). CBD and CBG have been proven to decrease apoptosis triggered by oxidative stress (Di Giacomo et al., 2020). CBG is known to be a potent regulator of oxidative stress, which is a leading cause of neurodegeneration (Gugliandolo et al., 2018). Both CBD and CBG prevent serotonin depletion, thus restoring its physiological levels (Di Giacomo et al., 2020). CBD also prevented the upregulation of 3-hydroxykynurenine (3-HK), an index of neurotoxicity, induced by an excitotoxic stimulus (Jîtcă et al., 2023).

Our findings demonstrate that CBD and CBG can control platelet-mediated inflammatory responses. For example, CRP-XL-induced ROS generation in platelets was reduced by CBD and CBG. ROS and other reactive species have the potential to harm various tissues including skin and accelerate its natural ageing process. However, Perez *et al.*'s 2022 study showed that CBG and CBD with an IC_{50} value of 0.003 nM could inhibit the generation of intracellular free radicals, making CBG and CBD 1800 times more potent than ascorbic acid (Perez et al., 2022). Additionally, Sun *et al*. 2017 showed that CBD has antioxidant properties as it reduced hippocampal oxidative damage following doxorubicin-induced cardiotoxicity (Sun et al., 2017). Based on our findings and those of the mentioned studies, CBD and CBG may control platelet-mediated antioxidant effects and, as a result, can influence platelet-mediated inflammatory responses. However, the methods by which CBD and CBG reduce ROS formation must be investigated further.

We examined the effects of CBD and CBG on the release of several inflammatory cytokines, including sP-Selectin, RANTES, VEGF, TNF-α, and IL-1β from agonist- stimulated platelets. Indeed, both CBD and CBG were shown to influence the release of these cytokines from activated platelets. These findings support that the effects of CBD and CBG on platelet function are the same as platelet-mediated inflammatory responses. Cytokines are secreted in response to an inflammatory response, and they play an important role in the pathogenesis of inflammatory diseases. Some of the pro- inflammatory chemicals generated by platelets following activation include circulating sP- selectin, RANTES, VEGF, TNF-α, and IL-1β(Kany, Vollrath and Relja, 2019). Some of these molecules are also considered platelet activation

markers (Woollard, 2005). In addition, it has been established that IL-1β is a crucial mediator in both the activation of platelets and the malfunctioning of endothelial cells (Lopez-Castejon and Brough, 2011). it has been proven that high blood levels of pro-inflammatory cytokines lead to vascular inflammation, atherosclerosis, and other pathological diseases (Kany, Vollrath and Relja, 2019). The pathogenic impact of platelet-derived inflammatory chemicals in the development of atherothrombosis has been demonstrated in several animal investigations (Chen et al., 2020). As a result, determining the effects of CBD and CBG on the release of various inflammatory molecules from platelets would provide additional insights into the effects on platelet-mediated inflammatory responses.

The findings of this study on the inhibitory effects of CBD and CBG are comparable to those of several previous studies which revealed their impact on the release of a variety of inflammatory mediators in other cell types. Myocardial dysfunction in diabetic cardiomyopathy was studied in C57BL/6 mice (Rajesh et al., 2010). Myocardial dysfunction in diabetic cardiomyopathy was characterised by an increased risk of heart attack and death, as well as an increased risk of oxidative stress; activation of NF-κB and mitogen-activated protein kinases (MAPK). However, these effects were suppressed by the administration of CBD.

CBD and CBG have already been shown to reduce platelet activation-induced P- selectin exposure and the subsequent release of RANTES which are necessary for the establishment of platelet-leukocyte interactions. The ability of TRAP-6 to promote the formation of platelet-leukocyte aggregates was validated by flow cytometric analysis. CBD and CBG significantly inhibited the formation of platelet-leukocyte aggregates in response to stimulation. The inhibition of RANTES release may potentially be related to the inhibition of platelet-leukocyte interactions (Totani and Evangelista, 2010). A decrease in platelet-leukocyte interactions following therapy with clopidogrel and prasugrel has been observed in other research, and our results are consistent with these findings (Pluta et al., 2022). Similar to the effects of CBD and CBG on platelet-leukocyte interactions, flavonoids such as flavonolignans demonstrated inhibitory effects on IL-1β-induced interactions between platelets and leukocytes (Bijak et al., 2017).

It is obvious from the results of this chapter that CBD and CBG have a significant anti-inflammatory impact, particularly on platelet-mediated inflammation. Further study is needed to investigate the effects of CBD and CBG on the regulation of platelet-mediated inflammatory processes *in vivo* and disease contexts and to elucidate the mechanisms underlying this suppression. However,

based on their influence on the management of thrombus formation, inflammation, and platelet activation, this may function as a potent antiplatelet medication to manage thrombotic and inflammatory diseases.

4.4 Conclusions

Platelets serve an important part in creating a host defence response and interacting with immune cells in addition to their haemostasis functions. Pro-inflammatory and pro-thrombotic molecules are released by activated platelets in pathological conditions. CBG and CBD effectively decrease the release of inflammatory cytokines such as sP-Selectin, RANTES, VEGF, TNF-α, and IL-1β. CBD and CBG also reduced the production of ROS in TRAP-6-induced platelets. Furthermore, in the flow cytometric analysis, CBD and CBG decreased the ability of TRAP-6 to form platelet-leukocyte aggregates. These findings suggest that CBD and CBG can be used to tackle thrombotic and inflammatory disorders.

CHAPTER FIVE

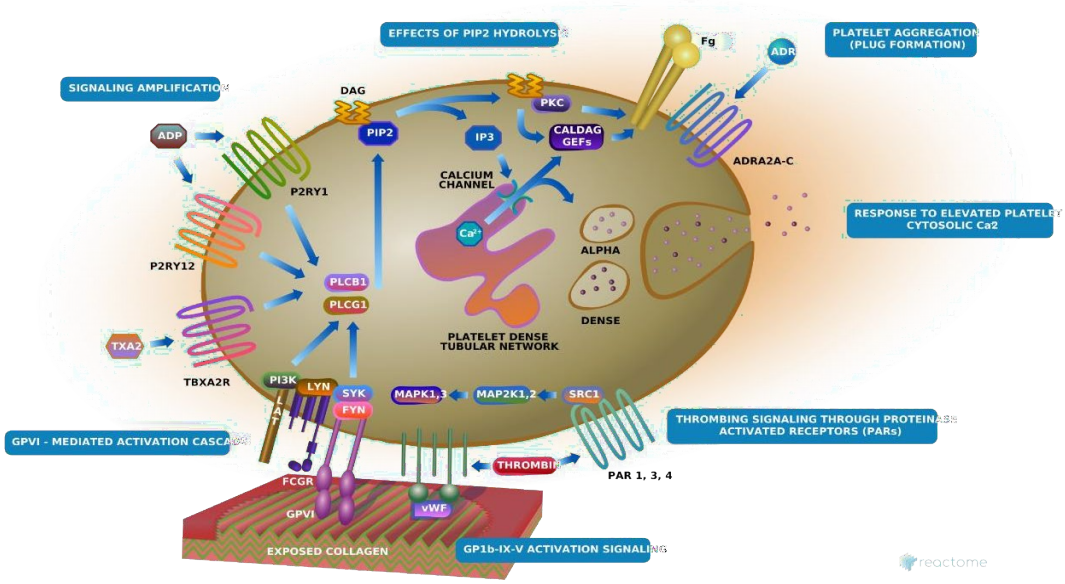
5. Mechanisms Through Which CBD and CBG Modulate Platelet Function

5.1 Introduction

CBD and CBG have demonstrated a significant impact on the modulation of platelet function, *in vitro* thrombus formation, and haemostasis under physiological conditions. In addition, they have been found to inhibit calcium mobilisation, collagen, thromboxane analogue, and thrombin-induced platelet aggregation. CBD and CBG have also been observed to prevent the release of dense and α-granules in platelets induced by CRP-XL and inhibit clot retraction and platelet spreading, indicating their influence on outside-in signalling via the integrin αIIbβ3 receptor. Overall, these results revealed that CBD and CBG decrease platelet activation, thrombosis, and platelet-mediated inflammatory responses. These results suggest that CBD and CBG might have potentially shared mechanisms to affect platelet activity.

Based on these findings, this chapter aimed to investigate how these compounds affect various signalling pathways in platelets. Specifically, the phosphorylation levels of key signalling proteins in the GPVI signalling pathway including the Src kinase family (**Figure 5.1**) were analysed. Moreover, their influence on the PI3K/Akt and MAPK/ERK pathways, which are critical for platelet activation and aggregation was analysed through immunoblotting to assess the phosphorylation levels of signalling proteins in these pathways, including Akt in platelets stimulated with collagen or CRP-XL in the presence or absence of CBD and CBG.

Furthermore, this study aimed to examine how CBD and CBG impact the cAMP signalling pathway, which is a crucial mechanism that provides negative feedback in platelets to prevent unnecessary activation while circulating in the bloodstream (Smolenski, 2012). Therefore, this chapter demonstrates potential mechanisms through which CBD and CBG modulate platelet function.



<u>Figure 5.1:</u> Some of the key signalling proteins in platelets along with their respective activators.

The phosphorylation of several intracellular proteins is crucial for the coordination of signal transduction downstream of each receptor. It is important to note that any changes in the phosphorylation of a single component can heavily impact the regulation of its associated effector molecules downstream. This figure was adapted from (de Bono, 2004).

5.2 Results

5.2.1 CBD and CBG affect PI3K/AKT signalling

AKT plays a key role in platelet activation since it is activated downstream of the GPVI receptor, GPCRs and the GPIb-IX-V complex (Bye., 2016). PI3K activation causes PIP3 synthesis, which in turn results in AKT and Rap1B activation leading to integrin αIIbβ3 activation (Hemmings and Restuccia, 2012). Several platelet surface receptors, cell motility, differentiation, and survival, are all dependent on PI3K as a potent second messenger (Liu et al., 2009). PI3K also plays a key role in the activation of platelets (Niu et al., 2012). AKT is the primary downstream signalling effector of PI3K. PDK1 activates and phosphorylates AKT upon recruitment to the plasma membrane via PIP3 binding (Manning and Toker, 2017).

Previous research has shown that CBD activates the AKT/PI3K pathways in different pathological conditions (Giacoppo et al., 2017) (Franco-Vadillo et al., 2021). However, the impact of CBG on cell signalling mechanisms was not studied in detail. Therefore, we sought to understand how CBD and CBG affect the phosphorylation of AKT in platelets upon agonist-induced activation. CRP-XL-stimulated platelets were examined for their effects on PI3K-mediated signalling via the phosphorylation of AKT (as a marker of PI3K signalling). Platelet lysates were used for the investigation, and immunoblot analysis was used to look at the phosphorylation of AKT at serine 473 (pS473) which is a critical phosphorylation site in this protein.

In the presence of various doses (6.25 and 12.5 μM) of CBD and CBG along with a vehicle control [0.01% (v/v) DMSO], human isolated platelets were activated with CRP- XL (0.5 μg/mL) before being lysed and immunoblotted to determine the phosphorylation of AKT (pS473). At 90 seconds, CRP-XL significantly increased the phosphorylation of AKT (**Figure 5.2**). However, CBG completely reduced its phosphorylation at both doses, whereas CBD inhibits AKT phosphorylation by 70% and 30% at 6.25 and 12.5 μM, respectively. These findings show that CBD and CBG may affect PI3K signalling pathways in platelets via AKT, which could have an impact on the modulation of platelet function.

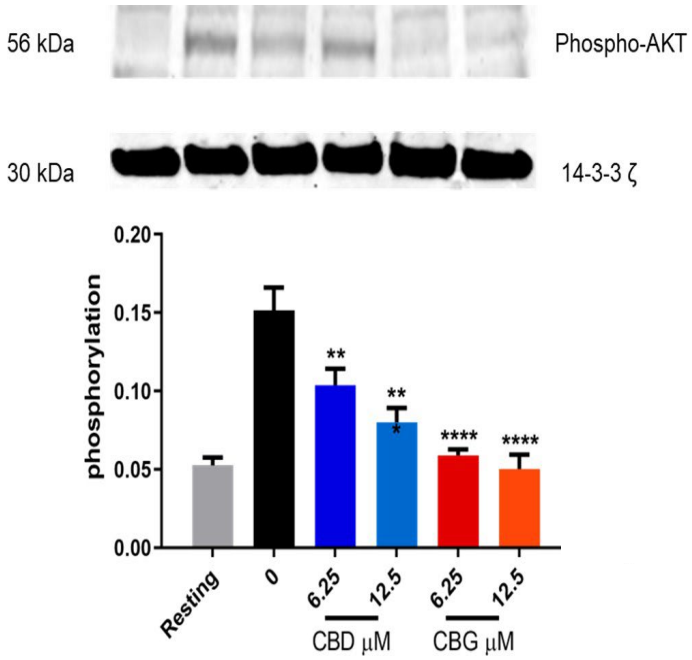


Figure 5.2: Effect of CBD and CBG on AKT phosphorylation.

Human isolated platelets were treated with a vehicle control [0.01% (v/v) DMSO] or various concentrations of CBD and CBG for 5 minutes before being stimulated with CRP-XL (0.5 μg/mL) for 5 minutes in an aggregometer at 37°C. The level of AKT phosphorylation at the S473 residue was detected using platelet lysates and phospho-specific antibodies against this site by immunoblot analysis. The level of 14-3-3ζ was detected as a loading control. The blots shown are representative of three separate experiments using platelets obtained from different individuals. The relative phosphorylation levels of AKT are represented as the mean ± SEM (n=3). p values shown (**p<0.001 and ****p<0.0001) are as analysed by one ANOVA followed by Bonferroni's correction. R represents resting platelets.

5.2.2 CBD and CBG affect cAMP levels and the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) in platelets

cAMP signalling in platelets is connected to the inhibition of platelet activation and it plays a vital role in the regulation of platelets in circulation (Nagy and Smolenski, 2018). cGMP and cAMP are elevated in the blood when healthy endothelial cells release nitric oxide (NO) and prostaglandin (PGI2), which suppress platelet activity (Smolenski, 2012). As a result, cAMP and cGMP levels are linked to the inhibitory signalling in platelets (Daniel, Ashby and Pulcinelli, 2002). Since the levels of cAMP in platelets are known to be affected by several small molecule inhibitors (Smolenski, 2012), we analysed the effects of CBD and CBG on the levels of cAMP in platelets upon activation by CRP- XL.

A cAMP ELISA kit was used to quantify the cAMP concentrations in platelets. Before activation with CRP-XL (0.5 µg/ml), human isolated platelets were incubated with a vehicle control [0.01% (v/v) DMSO] or two concentrations (6.25 and12.5 µM) of CBD and CBG. EGTA (1 mM) was used as a control for cAMP levels in unstimulated platelets. cAMP levels were then measured using the manufacturer's instructions after the platelets were lysed with 0.1 M HCl. CRP-XL-activated platelets had considerably lower cAMP levels than resting platelets (**Figure 5.3**). When compared to the vehicle control, pre-treatment with CBD at all tested doses (6.25 and 12.5 µM) significantly elevated cAMP levels in CRP-XL-activated platelets (**Figure 5.3**). However, CBG showed lower cAMP levels than CBD by around 20% and 18%, respectively, at tested concentrations of 6.25 and 12.5 µM.

Since the primary target of cAMP is protein kinase A (PKA), we then determined the phosphorylation levels of VASP (which is a substrate for PKA) at position S157, which can affect a variety of signalling components (Benz et al., 2009). To investigate the effect of CBD and CBG in this pathway, platelets were incubated with either CBD, CBG or a vehicle control [0.01% (v/v) DMSO] for 5 minutes before being stimulated with CRP-XL (0.5 µg/ml). Compared to the CRP-XL-activated platelets, the resting platelets had an increased level of phosphorylation of VASP S157 (**Figure 5.4**) due to the increased level of cAMP in resting conditions. The platelets that had been preincubated with CBD showed an increase in the phosphorylation level of VASP (S157), whereas the sample that had been treated with the vehicle control (stimulated platelets) showed no rise in the level of VASP phosphorylation (**Figure 5.4**). CBG, on the other hand, indicated less cAMP release. It became clear that CBD significantly increased the phosphorylation of VASP compared to the positive control sample. CBG had no significant

effect in comparison to CBD. These results suggest that CBD may have a significant impact on cAMP-mediated signalling in platelets, but CBG may have a different mechanism to modulate platelet activation in addition to cAMP-mediated signalling.

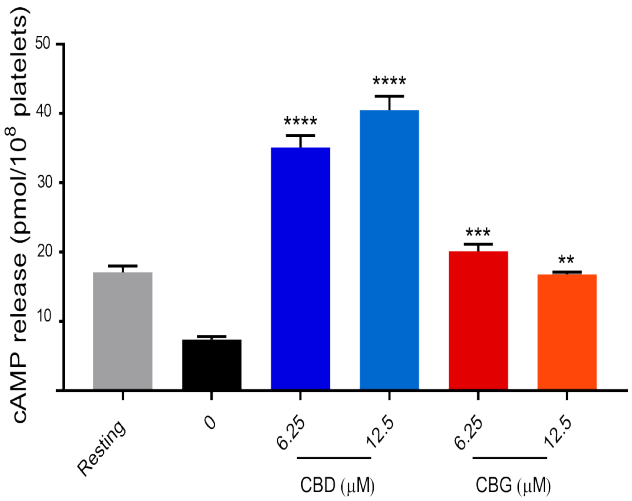


Figure 5.3: CBD and CBG affect the cAMP levels in platelets.

The total cAMP levels in platelets that were pre-treated with a vehicle control [0.01% (v/v) DMSO] or different concentrations (6.25–12.5 μM) of CBD and CBG before stimulation with CRP-XL (0.5 μg/mL) were determined using a cAMP ELISA kit. Cumulative data represent mean ± SEM (n=3). p values shown (**p<0.001, ***p<0.001 and ****p<0.0001) are as calculated by one-way ANOVA followed by Bonferroni's correction for multiple comparisons. R represents resting platelets.

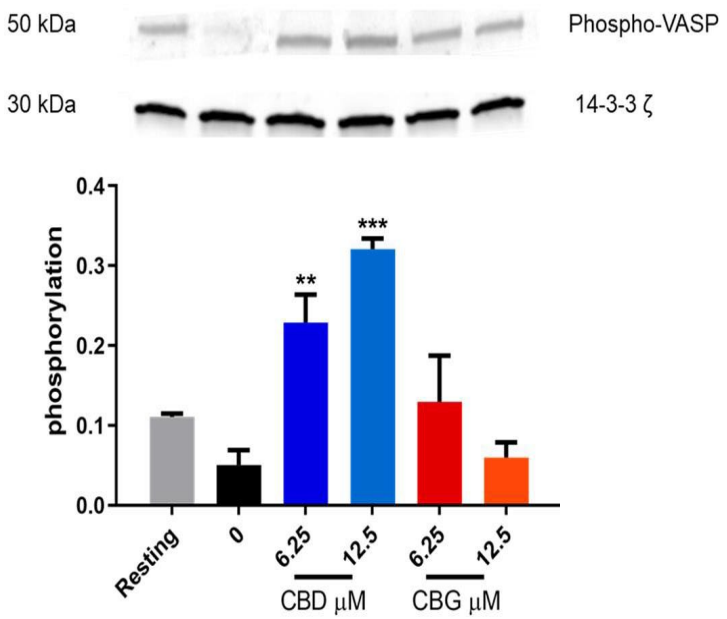


Figure 5.4: Effect of CBD and CBG on VASP phosphorylation.

Human isolated platelets were treated with either vehicle control [0.01% (v/v) DMSO] or various concentrations of CBD and CBG for 5 minutes before being stimulated with CRP- XL (0.5µg/mL) in an aggregometer at 37°C. The level of VASP phosphorylation in residue S157 was detected using platelet lysates by immunoblot analysis using phospho-specific antibodies for VASP S157. The level of 14-3-3ζ was detected as the loading control. The relative phosphorylation levels of VASP pS157 are represented as the mean ± SEM (n=3). P values shown (**p<0.01 and ***p<0.001) are analysed by one-way ANOVA followed by Bonferroni's correction for multiple comparisons. R represents resting platelets.

5.2.3 CBD and CBG affect Src family kinases

GPVI signalling is coordinated by the phosphorylation of several intracellular proteins. Changes in the phosphorylation of one component are likely to affect the regulation of downstream associated effector molecules. GPVI's function and downstream signalling depend on Src family kinases (SFKs)(Senis, Mazharian and Mori, 2014). The autophosphorylation of SFK is triggered by the collagen-mediated clustering of the GPVI receptors and the SFKs including Src, Fyn and Lyn play critical roles in this process (Roskoski, 2005). The autophosphorylation results in additional SFK-dependent phosphorylation of the FcR-chain complex-containing ITAM, followed by phosphorylation of SYK (Senis, Mazharian and Mori, 2014). Src has two types of phosphorylation sites: an activating phosphotyrosine site (pY 416), which is the result of autophosphorylation, and an inhibitory phosphotyrosine site (pY527), which is the result of phosphorylation by the C-terminal Src kinase and SH2 domains (Sotirellis et al., 1995). There is a correlation between the dephosphorylation of the inhibitory site (pY527) and an increase in Src kinase activity, as well as platelet activation. Src activity permits GPVI and integrin αIIbβ3 to activate platelets (Huang et al., 2019).

Human isolated platelets ($4x10^8$ cells/mL) were prepared and treated with CBD (6.25 and 12.5 μM), CBG (6.25 and 12.5 μM) or a vehicle control [0.01% (v/v) DMSO] for 5 minutes at 37°C to examine the effect of CBD and CBG on the modulation of Src phosphorylation in response to stimulation with a GPVI-specific agonist, CRP-XL. After incubation, 0.5 μg/mL CRP-XL was added for 5 minutes at 37°C. When CRP-XL was added, the phosphorylation of the inhibitory site (pY527) of Src was diminished (**Figure 5.5**). Notably, only CBG had a substantial effect by blocking the dephosphorylation of Src, whereas the dephosphorylation in the positive control sample remained at the same level as CBD. CBG increases the phosphorylation of the Src inhibitory site. After being quantified, normalised to the loading control of 14-3-3ζ and compared to the vehicle control [0.01% (v/v) DMSO] (0.5 μg/mL CRP-XL), this increase was substantial at all concentrations tested. Based on the findings presented here, it appears that CBG, but not CBD, can inhibit SFK, specifically Src, in platelets in response to stimulation with CRP-XL. Therefore, CBG may affect platelet function by targeting Src.

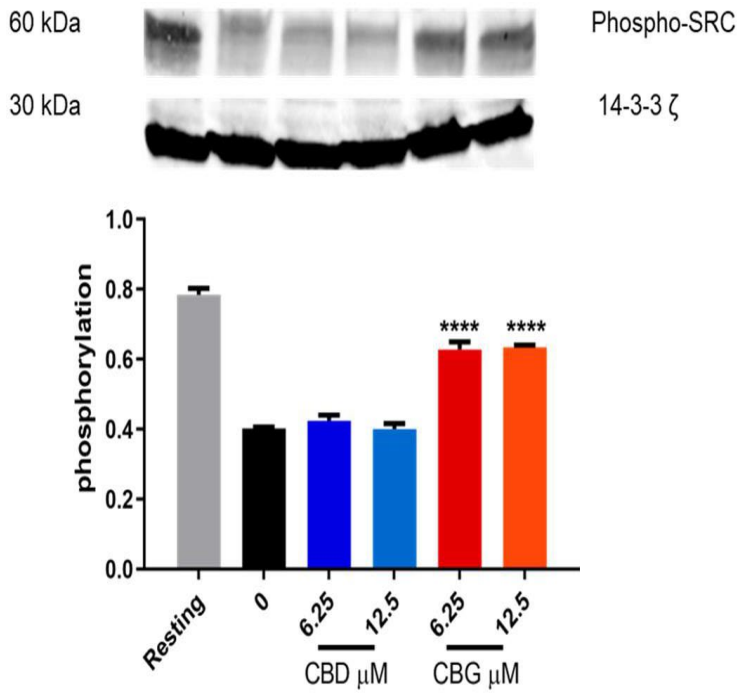


Figure 5.5: Impact of CBD and CBG on Src phosphorylation.

Human isolated platelets were treated with either vehicle control [0.01% (v/v) DMSO] or various concentrations of CBD or CBG for 5 minutes before being stimulated with CRP- XL (0.5 µg/mL) for 5 minutes in an aggregometer at 37°C. The level of Src phosphorylation at residue Y527 was detected using platelet lysates and phospho-specific antibodies against this site by immunoblot analysis. The level of 14-3-3ζ was detected as a loading control. The relative phosphorylation level of Src is represented as the mean ± SEM (n=3) after being normalised to the loading control. p-value (****p<0.0001) shown is calculated by one way-ANOVA followed by Bonferroni's correction for multiple comparisons. R represents resting platelets.

5.3 Discussion

In the vasculature, the unnecessary activation of platelets can lead to thrombosis, which is a major cause of death around the world. Improved therapeutic approaches to limit unnecessary platelet activation are vital in preventing and treating CVD, especially thrombotic disorders. Platelet activation, thrombus formation and platelet-mediated inflammatory responses are all significantly inhibited by CBD and CBG, as evidenced by the data reported in earlier chapters. To further understand how CBD and CBG affect platelet function, in this chapter, we aimed to identify the molecular pathways through which CBD and CBG affect platelet function. We investigated the influence of CBD and CBG on critical signalling pathways in platelets.

As a marker for PI3K signalling, we analysed the phosphorylation of AKT at Ser 473 in our study to assess the effect that CBD and CBG have on this signalling in platelets. CBG reduces CRP-XL-induced AKT phosphorylation. Moreover, CBD had a lesser effect on AKT phosphorylation than CBG. Indeed, CBD has been reported to suppresses the PI3K/AKT survival pathway in glioblastoma by lowering the phosphorylation of AKT1/2, according to prior studies (Marcu et al., 2010; Ivanov, Wu and Hei, 2017). However, a study by Giacoppo *et al.* 2017 revealed that CBD increased the phosphorylation of AKT in multiple sclerosis mice (Giacoppo et al., 2017). CBG, on the other hand, did not have much information to understand its effects on AKT phosphorylation or other signalling pathways.

We studied the effects of CBD and CBG on GPVI signalling proteins. GPVI is a significant collagen receptor on platelets because it is a potent stimulator of platelet activation and GPVI loss results in reduced thrombus formation at arterial shear rates (Nieswandt and Watson, 2003). Sequential activation of several signalling proteins, such as Src and PI3K, is required for GPVI signalling(Watson et al., 2005). We investigated the effect that CBD and CBG have on the phosphorylation of Src. Upon platelet activation, Src binds to the cytoskeleton and phosphorylates the critical substrates that control cytoskeleton remodelling(Liem, 2016). Additionally, Src is linked to integrin αIIb3-mediated signalling, most prominently via the subunits of this integrin (Huang et al., 2019). In platelets, CRP-XL induces Src dephosphorylation at the inhibitory site of pY529, which is inhibited solely by CBG but with no effect observed by CBD. Hence, CBG may alter platelet function by targeting Src. In platelets, the activation of integrin αIIb3 is mediated mostly by PI3K/AKT (Niu et al., 2012). Therefore, the inhibition of AKT further demonstrates the potent antiplatelet actions of CBG.

We hypothesised that CBD and CBG activate another signalling pathway, as they react differently to the GPVI signalling pathway. We explored whether well-known endogenous platelet inhibitory pathways (such as those regulated by NO and PGI2) may also be regulated by CBD and CBG. Indeed, cAMP-dependent PKA activation attenuates platelet shape changes more than cGMP-dependent PKG activation (Walter and Gambaryan,2004). PKA activity is therefore a primary factor responsible for mediating the cAMP-induced reduction of P-selectin exposure on the platelet surface (Fuentes and Palomo, 2013). Platelet aggregation is inhibited as a result of a decrease in integrin αIIb3 activation, which is connected to an increase in VASP phosphorylation, which is a marker of cAMP-mediated signalling(Daniel, Ashby and Pulcinelli, 2002). Also, the phosphorylation of VASP inhibits the formation of actin nuclei, which leads to a reduction in cytoskeletal remodelling(Benz et al., 2009). This process is essential for the transformation of platelet shape change (Cuenca-Zamora et al., 2019). Compared to the positive control, CBD increased VASP phosphorylation. This aligns with CBD-induced intracellular cAMP in platelets.

Based on these findings, it appears that CBD interacts with the cAMP signalling system, which ultimately leads to the suppression of platelet activation. CBG had only a slight impact on the phosphorylation of VASP, which is consistent with the levels of cAMP. Both CBD and CBG have been shown in these studies to be able to limit platelet function, but each has a unique way of targeting different molecules in platelets. This research shows the potential of phytocannabinoids, CBD and CBG in inhibiting platelet reactivity and could be used to develop new antithrombotic medicines.

5.4 Conclusions

CBD and CBG are phytocannabinoids derived from *cannabis* that have been shown to have anti-inflammatory properties as well as inhibitory effects on platelet activation. To conclude this chapter, CBG can block the phosphorylation of AKT induced by CRP-XL. CBD decreased phosphorylated AKT levels at higher concentrations. Src dephosphorylation at pY529 is blocked only by CBG, although CBD has no effect on platelets at this signalling pathway. To limit platelet activation, CBD reduces integrin αIIb3 activation as shown in chapter two, which is connected to an increase in VASP- phosphorylation, a marker for cAMP-mediated signalling. This leads to the suppression of platelet activation. CBG had a minimal effect on the phosphorylation of VASP, which is consistent with cAMP levels. CBD and CBG lower platelet reactivity, which may be employed to create novel antithrombotic therapeutics.

CHAPTER SIX

6. General Discussion

6.1 General Discussion

In this chapter, a brief overview of the project will be provided to the overall findings and address the potential implications that these findings may have in a more general clinical setting. This project's major aim was to examine the impact of CBD and CBG on platelet reactivity, thrombus formation, haemostasis, and leukocyte-platelet interactions.

Platelets are small discoid-shaped bodies that circulate in the blood, and they can adhere to each other to form a thrombus when there is vascular injury. This process is supported by certain adhesion proteins on the surface of platelets. Thrombotic diseases, such as stroke and heart attack, occur when platelets become inappropriately activated. Therefore, platelets are a crucial target for treating and preventing these diseases. However, current treatments like aspirin and clopidogrel have significant side effects, including prolonged bleeding. Hence, there is an urgent need to develop safer and more effective anti-platelet drugs to address thrombotic diseases.

To investigate the potential of CBD and CBG as antiplatelet agents, multiple functional characteristics of platelets such as calcium mobilisation, granule release, integrin activation and aggregation were evaluated in the presence of different concentrations of these compounds. In addition, their impact on haemostasis and cytotoxicity was analysed (chapter 2). Furthermore, a better understanding of the effects of CBD and CBG on inflammation will ultimately be beneficial for the development of new therapeutic medications in the treatment of thrombo-inflammatory conditions (chapter 3). Lastly, understanding the molecular mechanisms by which CBD and CBG were able to modulate the inhibitory effects on platelet activation was established (chapter 4).

The findings of this study show that the non-psychoactive phytocannabinoids, CBD and CBG have an inhibitory effect on platelet activation induced by multiple agonists. In human-isolated platelets, the presence of CBD and CBG caused a significant reduction in platelet activation in response to collagen and CRP-XL. This result indicates that CBD and CBG affect the GPVI signalling pathway. Additionally, it was shown that CBD and CBG suppressed thromboxane A2 analogue-induced platelet activation. This finding suggests their influence on the COX enzyme and TP receptor. In addition, when thrombin- activated platelets were treated with CBD and CBG, the PAR1 receptor was affected, inhibiting platelet activation. This inhibitory impact was also demonstrated in the presence of plasma proteins as a decrease in

aggregation was observed when ADP was used to activate platelets.

Flow cytometry-based tests further revealed that CBD and CBG have dose- dependent effects on CRP-XL-induced fibrinogen binding at different concentrations. The results of aggregation tests were in agreement with their effects on fibrinogen binding. CBD and CBG impact inside-out signalling to the integrin αIIb3. Furthermore, CBD and CBG influenced the outside-in signalling of integrin αIIb3 as evaluated by a clot retraction assay, as well as platelet adhesion and spreading on fibrinogen. The anticoagulant effects of CBD and CBG may be seen in clot retraction.

In addition, CBD and CBG inhibited agonist-induced platelet granule secretion which is an important event of positive feedback signalling that supports platelet activation and subsequent thrombus formation. This inhibition results in a reduction in platelet reactivity. CBD and CBG decreased CRP-XL-induced calcium mobilisation, which is essential for integrin activation and other platelet functions. We also observed no effects in tail bleeding *in vivo* in mice, which suggests that lower dosages may not have any effect on haemostasis. This is consistent with *in vitro* results showing the inhibitory effects of CBD and CBG on platelet aggregation, granule secretion, and integrin αIIb3 activation. CBD and CBG had a consistent effect on *in vitro* thrombus development under arterial flow conditions. This research, however, was unable to evaluate the impact of CBD and CBG on a laser/FeCl3-induced thrombosis model which can be considered in future work.

CVD has been linked to an increase in platelet-leukocyte interactions. These interactions have a key role in thrombosis and inflammation, which are associated with various pathological conditions including atherosclerosis and other inflammatory disorders. CBD and CBG effectively suppressed CRP-XL-induced ROS production in human platelets, according to our findings. This is consistent with their effect on granule release and aggregation.

RANTES stimulates leukocyte migration and adherence to the endothelium surface via binding to CD40 and CD40L which are suppressed by CBD and CBG. Additionally, they showed a significant decrease in the production of cytokines, such as sP-selectin, IL-1β, TNF-α, and VEGF. CBD and CBG significantly reduced the formation of platelet-leukocyte aggregation induced by TRAP-6. These findings provide further evidence for the antithrombotic activity of CBD and CBG. This activity can decrease platelet and leukocyte adhesion by lowering the formation of platelet-leukocyte interactions and cytokines

released by platelets. Overall, CBD and CBG appear to be able to reduce platelet-mediated inflammatory reactions, as shown by these findings. Platelet function has been inhibited by both CBD and CBG, but each has its distinct method for targeting mechanism. CBG inhibits the phosphorylation of AKT induced by CRP-XL, but CBD had a less significant effect on AKT phosphorylation. Dephosphorylation of Src at the pY529 inhibitory site of CRP-XL is blocked entirely by CBG, while CBD has no effect on this phosphorylation. Src is required for cytoskeleton remodelling during platelet activation. The cAMP signalling system appears to interact with CBD as also shown through VASP phosphorylation, resulting in a reduction of platelet activation. The phosphorylation of VASP by CBG was minimal, in line with the lower concentration of cAMP.

The initial results of the platelet functional assessment indicate that CBG has a greater impact than CBD. This difference can be attributed to the chemical structure of CBG and its role as a precursor to CBD. Specifically, CBG's structure may allow it to interact more effectively with certain biological targets involved in platelet function. This finding has significant implications for subsequent experiments. Given CBG's pronounced impact on platelet function, CBG also may exhibit stronger anti-inflammatory properties compared to CBD. This could be due to several factors such as receptor Interaction; CBG may bind more efficiently to cannabinoid receptors (such as CB1 and CB2) or other receptors involved in inflammation (like PPARs or TRP channels), leading to a more potent anti-inflammatory response. Also, the enzymatic Inhibition of CBG that might increases the efficacy of CBG compared to CBD. Cytokine modulation as CBG could have a stronger impact on the modulation of cytokines, which are signaling proteins that play a key role in inflammation. For instance, CBG might suppress the production of pro-inflammatory cytokines (e.g., TNF-α, IL-6).

Overall, these findings underscore the need for a more in-depth exploration of CBG’s pharmacological profile, especially in relation to its anti-inflammatory and platelet-modulating properties. Also, it demonstrates that phytocannabinoids CBD and CBG have the potential to inhibit platelet reactivity and could be employed in the development of novel drugs to treat thrombotic disorders.

6.2 future work

Phytocannabinoids are bioactive natural products found in flowers, seeds, and fruits. They can be beneficial for treating human diseases (such as multiple sclerosis, neurodegenerative diseases, epilepsy, and pain), the cellular metabolic process, and regulating biological function systems. Cannabigerol and cannabidiol have shown potential therapeutic benefits for a variety of human diseases and conditions. While the therapeutic potential is promising, it is important to note that more research is needed to fully understand the efficacy and safety of CBG and CBD for these conditions. Clinical trials and long-term studies are essential to establish standardized dosages, identify potential side effects, and understand how these compounds interact with other medications and treatments.

CBD a non-psychoactive compound from cannabis, is increasingly recognized for its therapeutic potential. Understanding its safety and toxicity is essential for its medical application. Generally, CBD is deemed safe and well-tolerated, with the World Health Organization (WHO) stating it has no potential for abuse or dependence. However, CBD can interact with medications metabolized by the liver's cytochrome P450 enzyme system. Optimal CBD dosages can vary; starting with a low dose and gradually increasing it while monitoring for adverse effects is recommended. Human studies have shown that doses up to 1,500 mg per day are generally well-tolerated, though high doses can sometimes cause liver abnormalities. Extremely high doses have been toxic in rodent studies, but these doses are far above typical human usage. There is limited data on the long-term safety of CBD, and ongoing research is examining its effects on liver function, reproductive health, and the immune system. While CBD is largely considered safe, it does carry risks, particularly regarding drug interactions, long-term use, and product quality.

CBG is another non-psychoactive cannabinoid found in the cannabis plant, similar to CBD. Initial studies suggest that CBG has a promising safety and toxicity profile. CBG is generally well-tolerated in both humans and animals, lacking the psychoactive effects of THC. Preliminary research indicates that high doses of CBG are well-tolerated, but more studies are needed to determine safe consumption limits. There is limited data on the long-term safety and cumulative effects of chronic CBG use. Long-term studies are necessary to evaluate its impacts on liver function, reproductive health, and the immune system. CBG may also interact with medications metabolized by the liver's cytochrome P450 enzyme system, potentially affecting the efficacy or toxicity of other drugs.

The primary objective of this PhD project was to evaluate the effect of CBD and CBG on platelet functions, platelet-leukocytes interactions, and the underlying mechanism of their effects. This study established that CBD and CBG have not impacted haemostasis. Indeed, these compounds have shown promising effects in reducing platelet aggregation, secretion, and thrombus formation, as well as modulating inflammatory responses. The findings suggest that CBD and CBG could be utilized in the development of novel drugs for treating thrombotic disorders by regulating platelet function and inflammation. Nonetheless, several questions remain unanswered which need to be investigated in future studies.

To gain a better understanding of the molecular mechanisms of action of CBD and CBG, as it is necessary to investigate the intracellular signalling pathways of several important receptor proteins such as GPR55, PPAR, and adenosine A2A receptor, which have previously been reported to be involved in CBD mechanism of action other cell types. Albeit localization of GPR55 on human platelet was proved by (Kargl et al., 2013), experiments of knockout GPR55 mice were planned to be done just before the break of Covid-19 which affected the lab work and were unable to be explored. This study might be a powerful tool to understand the mechanism of CBD and CBG on G-protein coupled receptor. Also, it was plan for feeding study to be conduct on C57BL/6 mice (12 weeks old) to test CBD and CBG effects on platelet functions, hemodynamic parameters, and mechanisms. However, this was terminated because of the outbreak and social distancing.

Since there was evidence of CB1 and CB2 expressed on human platelet (Catani et al., 2010), investigation can be performed in the future to characterise this unidentified pathway that CBD and CBG might involve the endocannabinoid system to mediate their effects to inhibit platelet activation which will be beneficial in many neurovascular disorders. Also, understand the crosstalk of these two receptors in the innovation of anti- platelet drugs as it has been recognised that the activation and consequent signalling cascades of CB1 and GPR55 receptors are linked (Waldeck-Welermair et al., 2008). It would be interesting to test CBD and CBG on mouse model for risk assessment in cardiovascular diseases such as thrombosis, ischemic stroke, bacterial/viral infection, and tumours.

To empower this research and to have a great insight about the impact of both CBD and CBG, a mathematical modeling of platelet activation, aggregation assays to understand platelet behavior under various conditions and replicate the biological processes of platelet adhesion, activation, and

aggregation.

7. References

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