The Investigation of the anti-platelet properties of Cucurbitacins.

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The Investigation of the anti-platelet properties of Cucurbitacins.

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1 Abstract

Background: Cucurbitacin's are dietary compounds that have been shown to cause anti-tumour, anti-inflammatory and anti-atherosclerotic activities. Mechanisms of action include dysregulation of the actin cytoskeleton and disruption of integrin function. Integrin outside-in signalling and cytoskeletal rearrangements are important for stable thrombus formation and clot retraction after platelet adhesion at the site of vessel damage.

Aims: To identify the effect of cucurbitacin's B, E and I on platelet function and thrombus formation. Methods: Human washed platelets, platelet rich plasma and whole blood *in vitro* platelet function assays were used including real time and end point platelet aggregation, platelet adhesion assay with DiOC6 and phalloidin staining, thrombus formation, flow cytometry and western blots to determine actin polymerisation activity and integrin function.

Results: Anti-platelet and anti-thrombotic effects following treatment with cucurbitacin's B, E and I were observed. Treatment of platelets with cucurbitacin B, E or I lead to reduction of platelet aggregation and fibrinogen binding following stimulation with ADP and collagen. Cucurbitacin B, E and were found to inhibit other integrin mediated events, including adhesion and spreading on adhesive surfaces such as fibrinogen. Additional investigation of cytoskeletal dynamics found treatment with cucurbitacin's B, E and I increased actin polymerisation and myosin light chain phosphorylation which has been shown to disrupt integrin activation and platelet spreading. Treatment with cucurbitacins was also found to disrupt stable thrombus formation, with an increase in the formation of unstable thrombi and a reduction in thrombus density compared to control.

Conclusions: Cucurbitacins have anti-platelet and anti-thrombotic activity, which appear to be linked to dysregulation of actin cytoskeletal dynamics and inhibition of integrin activity.

2 Introduction

Cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality in the world (Clark, 2013). Types of conditions covered by the term CVD include coronary artery disease, stroke, atherosclerosis, congenital heart disease, peripheral arterial disease, myocardial infarction (MI), deep venous thrombosis, pulmonary embolism and many other conditions linking to the cardiovascular system. The activation of blood platelets and aggregation is linked to the development of atherosclerosis, formation of blood clots and blockage of arteries. Platelet activation is essential for haemostasis, which is the binding of platelets to damaged blood vessels followed by aggregation and thrombus formation, preventing excess bleeding. However, platelet activation is also one of the main pathophysiology's of atherosclerotic plaque formation, promoting the formation of a thrombus at site of a plaque rupture consequently leading to a myocardial infarction. (Thomas and Storey, 2015).

There are a number of anti-platelet drugs used for the treatment of CVD, including aspirin and clopidogrel. However, these drugs are associated with several side effects in patients, some of which are potentially life threatening such as excessive bleeding (Guthrie, 2011). Additionally, certain patients show resistance to anti-platelet drugs (Algra, 2013). For this reason, safer more effacious anti-platelet drugs that target different mechanisms are needed for the prevention and treatment of CVD. It's been found that increased level of inflammation in the blood is what accelerates many diseases including CVD. Oxidative stress triggers elevated levels of inflammation, certain antioxidants found in fruits, vegetables and dietary supplements have been linked to the slowing of the progression of CVD (O'Kennedy et al., 2017). Cucurbitacins are dietary compounds which are found in the plants of family known as Cucurbitaceae. They have been known to show anti-inflammatory (Yuan et al., 2006) and antitumor activity (Alghasham, 2013) as well as anti-atherosclerotic activity (Bernard and Olayinka, 2010). Therefore, it is important to investigate the anti-platelet properties of cucurbitacins due to the increasing incidence of cardiovascular disease, the negative effects that current antiplatelet therapies produce and the potential for cucurbitacins to elicit additional positive properties.

2.1 Platelets

Platelets are anuclear cells with a diameter of approximately 3-4 µm and an average life span of 8-10 days. Platelets originate from bone-marrow megakaryocytes and circulate within the vascular system (Hou et al., 2015). They are discoid in shape and are encased by a plasma membrane which contain glycoproteins (GP) such as GPIa, GPIb and GPIIb/IIIa (Minors, 2007). Platelets contain messenger RNA (mRNA) so can synthesise a small number of proteins, but do not contain a nucleus which means they do not contain DNA (Italiano Jr and Shivdasani, 2003). The schematic drawing of a platelet in figure 1 shows the contents of platelets, containing mitochondria, a canalicular system and specialised secretory granules. These include alpha, dense and lysosomal granules with each containing many pre-formed megakaryocyte-derived molecules. Granule secretion supports platelet activation and thrombus formation.

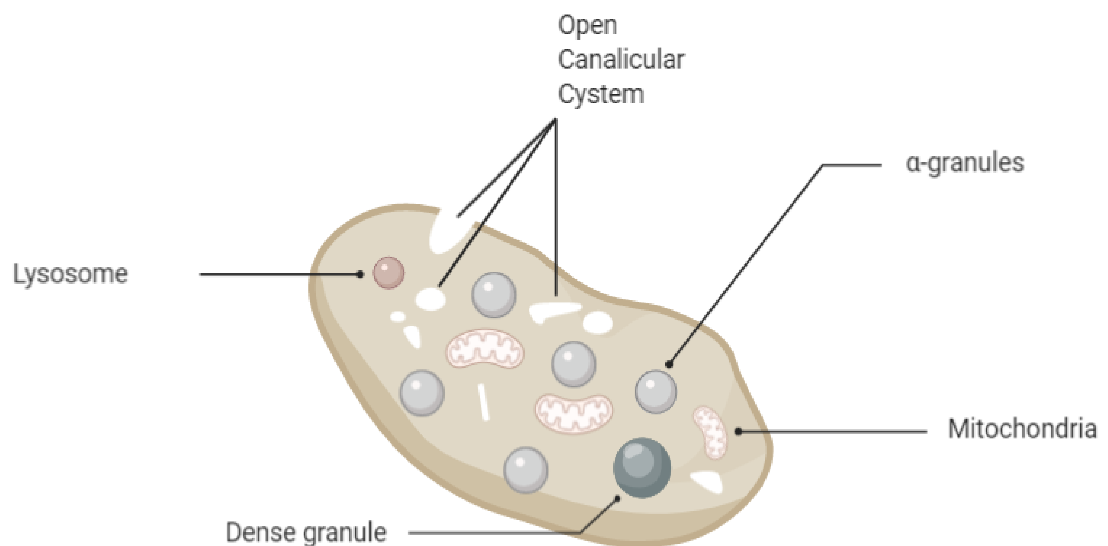

Figure 1: Schematic drawing of a platelet *Figure adapted from (Fitch-Tewfik and Flaumenhaft, 2013). Detailing contents of a platelet, with the small size each platelet contains surface receptors, a canalicular system, lysosomes, α-granules, dense granules and mitochondria.*

α- Granules contain different mediators including chemokines such as CXCL4 (PF4), coagulant/anticoagulant factors such as factor V, factor IX which are important for secondary haemostasis but also anti-thrombin which cleaves clotting factors in both intrinsic and extrinsic pathways (pathways of coagulation) (Blair and Flaumenhaft, 2009). They contain adhesion proteins like fibrinogen, fibronectin, von Willebrand

factor (vWF) which main roles include contribution to platelet-endothelial and platelet-platelet adhesion through the formation of cross-bridges, P-selectin – a glycoprotein also found in the granules are translocated from the granules to the platelet surface following platelet activation which allows for the platelets to interact with endothelial cells, monocytes, neutrophils and lymphocytes. They also mediate platelet and neutrophil rolling on activated endothelial cells (Fitch-Tewfik and Flaumenhaft, 2013) and growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelium growth factor (VEGF) and others. They also contain approximately one-half to two-thirds of αIIbβ3 in a resting α-granule membranes although they are also found on platelet plasma membrane (Blair and Flaumenhaft, 2009). Dense granules contain cations such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), nucleotides such as adenosine diphosphate (ADP) – an important agonist for platelet aggregation and ATP, serotonin and histamine (Sonmez and Sonmez, 2017). Finally, lysosomal granules include proteases such as cathepsin and glycohydrolase which are the lysosomes cargo and the lysosomes express CD63 - markers for platelet activation (Polasek, 2005).

Platelets circulate in the vascular system in a quiescent state, inactivated by soluble mediators released by the endothelium including prostacyclin and nitric oxide (NO). These inhibit the activation and aggregation of platelets (Thomas and Storey, 2015).

2.1.1 Platelet adhesion

Following injury to vessel wall, platelets attach to subendothelial matrix proteins such as collagens, which are exposed to the blood. Plasma vWF is released from platelets, endothelial cells and megakaryocytes and binds to collagen forming a bridge between collagen and platelets. In high shear stress conditions GPIbα-IX-V- the VWF receptor found on platelets, initiates the platelet adhesion to the injured site via binding of vWF. This GPIbα-VWF interaction is important for the tethering of circulating platelets to the vessel wall and slowing down the platelets so that GPVI can bind to collagen (Induruwa et al., 2018). This interaction and the integrin α2β1 binding to collagen initiates platelet signalling events which elicit platelet activation. In addition to GPIbα, the binding of other platelet integrin's to their ligands on the extracellular vessel matrix consequently mediates stable adhesion. These include integrin αIIbβ3 to fibrinogen/fibrin and fibronectin (Ni and Freedman, 2003). In regions of low shear

stress such as veins, these interactions can directly trigger platelet adhesion (Bledzka et al., 2013).

2.1.2 Platelet activation

The interaction and binding of platelet surface receptors, to their ligands (e.g. collagen, fibronectin) can induce platelet activation (Berndt et al., 2014). Activation of agonists leads to an increase in cytosolic calcium, which is essential for platelet activation. This occurs through the activation of phospholipase C (PLC) which hydrolyses Phosphatidylinositol 4,5-bisphosphate (PIP2) to Inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 and DAG are secondary messengers which result in intracellular calcium release and activation of protein kinase c (PKC) respectively, which are important mediators of several platelet processes including granule secretion (Stalker et al., 2012). The release of platelet granules amplifies the activation process to support additional platelets to be activated from a further distance. In addition to granule release, thromboxane (Tx) is generated and released and both ADP and Tx are secondary mediators of platelet activation. Secreted ADP activates further platelets surrounding the site, making it possible for platelets to activate without contact with the vessel wall. (Johnston-Cox et al., 2011). ADP activates G-protein coupled receptors $P2Y_1$ and $P2Y_{12}$ to amplify platelet activation (Storey et al., 2000). Furthermore, the increase in calcium aids the shape change of platelets into activated form of platelets. TxA2 role in platelet activation consists of converting arachidonic acid to short lived agent TxA2 through actions by cyclooxygenase-1 (COX1). TxA2 attaches to its receptor the GPCR thromboxane A2 receptors (TP) and further triggers platelet activation (de Groot et al., 2012). In addition to platelet activation, the coagulation system is also activated following vascular injury (Triggers Mackman, 2008). This produces the platelet activating factor, thrombin (Sambrano et al., 2001). Thrombin generation is driven by the exposure of phosphatidylserine (PS) on the platelet and endothelial cell membrane surface enabling further platelet activation. (Berndt et al., 2014). At concentrations as low as 0.1 nM , thrombin is able to activate platelets (Stalker et al., 2012). Thrombin stimulates two G_q coupled receptors, protease-activated receptor (PAR); PAR1 and PAR4 which are expressed on human platelets. Together, thrombin generation and these receptors activate platelets (Figure 2).

2.1.3 Platelet aggregation

Following platelet activation, integrin αIIbβ3 binds its major ligands, including fibrinogen. Integrins are transmembrane receptors involved in cell signalling which have cytoplasmic tails of α- and β-subunits. The integrin αIIbβ3 is usually kept in a low affinity conformation "resting" state but after platelet activation transitions to a high affinity ligand binding state and this is known as inside-out signalling. For this to occur, intracellular activators known as talin and kindlins bind to the β3 integrin tail (Shattil et al., 2010). Additionally, a process known as outside-in signalling occurs whereby on extracellular ligand binding to the activated integrin and initiates cell signalling events. The extracellular ligand fibrinogen attaches to the αIIbβ3 integrin and this mediates platelet aggregation by acting as a bridge to other platelets, thus resulting in the aggregation of platelets (Ley et al., 2016).

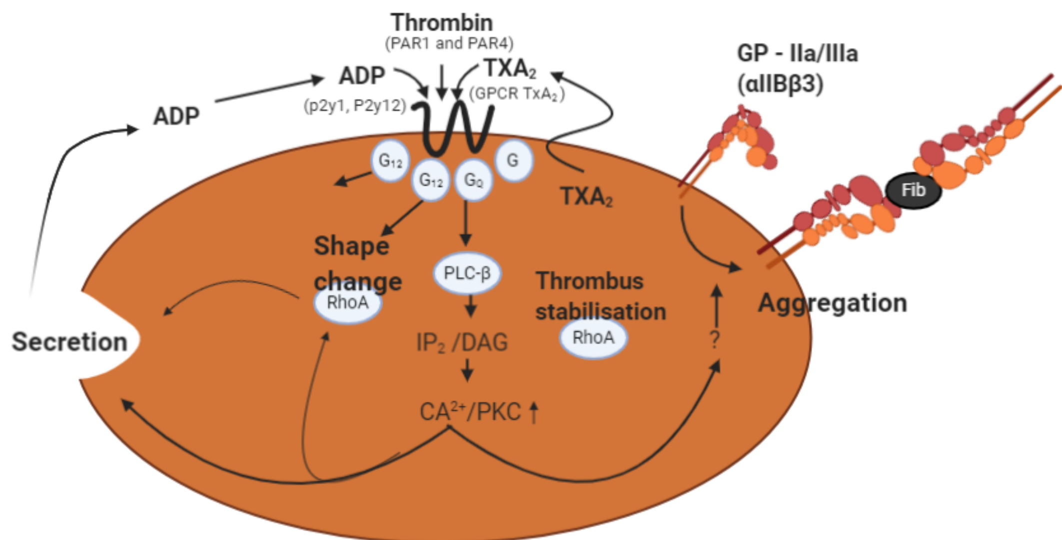

Figure 2: Process of platelet activation. *Figure adapted from (Periayah et al., 2017). Detailed process of platelet activation. Including the activation of protein kinase c (PKC) important for secretion of granules and their attachment to their respective receptors, increase in calcium for platelet activation.*

2.2 Platelet Shape Change

The platelet cytoskeleton has an important role in maintaining the function of platelets in all platelet processes (Fox, 1993). Throughout the platelets function, the cytoskeleton rearranges to in each process to regulate platelet function. Resting platelets are discoid in shape and the matrix of the interior of platelets is the sol-gel

Zone and this is where actin microfilaments, microtubules and intermediary filaments- cytoskeletal proteins are stored. The microtubules maintain the resting/discoid platelet by forming a closed circular bundle known as marginal band. These microtubules of the marginal band are crossed linked and form a bundle allowing the platelets to maintain the discoid shape. Additionally, the platelets contain cross-linked actin fibres and spectrin that form a lining in the inner surface of the plasma membrane (Mathur et al., 2018).

It is believed that the rearrangement of actin is crucial for platelet shape change (Antonipillai et al., 2018), following platelet activation actin polymerisation increases and the length of the actin increases too. Actin monomers (G-actin) form filamentous actin (F-actin) and during activation, the formation of these F actin increases. These filaments form four structures, - filopodia, lamellipodia, stress-like fibres and a contractile ring.

Following platelet activation, the platelet cytoskeleton dismantles and reorganises. Platelets change from a spherical discoid shape, by extending pseudopods which allows for the platelets to adhere to the surface. This is then followed by an extension of the platelet membrane alongside filament arrangement and the formation of filopodia and lamellipodia which increases the surface area of the platelets, and is known as platelet spreading. (Sandmann and Köster, 2016).

2.3 Platelet Agonists

There are several different platelet agonists that stimulate platelets. The most common agonists include ADP, collagen, thrombin, fibrinogen and arachidonic acid.

2.3.1 Collagen

Collagen induces platelet activation and has a central role in platelet adhesion and initiation of platelet activation. The most important signalling receptor for collagen in platelet activation is GPVI. Following interactions between collagen and the receptor, platelets trigger strong activation which leads to the release of α- and dense granules (Yun et al., 2016). Furthermore, integrin α2β1 is a second receptor for collagen and is an important mediator for platelet adhesion (Estevez and Du, 2017). The binding of collagen to this integrin triggers outside-in signalling which reinforces platelet activation. (Rivera et al., 2009) This makes collagen an important agonist to stimulate and activate the platelets.

2.3.2 ADP

ADP activates platelets through the $P2Y_1$- Gq coupled receptors and $P2Y_{12}$- Gi coupled receptors which are essential for the platelet activation, aggregation, procoagulant activity and thrombus formation under flow. $P2Y_1$ receptor is important for ADP-mediated platelet shape change and rapidly reversible wave of platelet aggregation. $P2Y_{12}$ has shown to be not as important in causing ADP-mediated shape change as the $P2Y_1$ but it does have a role in potentiating TxA2 mediated dense granule secretion (Murugappa and Kunapuli, 2006). This makes ADP a potent agonist for platelet aggregation to amplify secondary mediator signalling and platelet activation. The receptors for the ADP have shown great importance in the development of anti-platelet drugs such as ADP receptor antagonists like clopidogrel. Which are used frequently in the clinic (Wijeyeratne and Heptinstall, 2011).

2.3.3 Fibrinogen

Fibrinogen is a protein involved in platelet aggregation as it binds to integrin αIIbβ3 and this forms cross-links between platelets allowing them to aggregate and form a thrombus. αIIbβ3 is the most abundant cell surface receptors (French and Seligsohn, 2000). Following the inside-out signalling of the integrin αIIbβ3, outside-in signalling of the integrin is initiated by the binding of soluble fibrinogen to the activated integrin (Huang et al., 2019) . This facilitates for the cytoskeleton of the platelet to further rearrange, spreading of platelets and adhesion. Immobilised fibrinogen affects the kinetics of filipodia by extending them and development of lamellipodia in αIIbβ3-mediated platelet spreading (Jiroušková et al., 2007).

2.4 Platelet Dysfunction in Cardiovascular disease

In normal vascular function, platelets circulate around the endothelial wall in the vessel without attaching to it (Ruggeri, 1997). However, platelets respond quickly to changes in endothelial cells under pathological conditions, including atherosclerotic plaque rupture and fatty streak formation. The platelets attach to the site of lesion and form thrombi through dysfunctional platelet aggregation and this is associated with cardiovascular ischaemic events such as MI and stroke. (Body, 1996). Following a plaque rupture, platelets bind to the site of rupture, activate and form a thrombi in an intact blood vessel which can result in a MI. Arterial thrombosis is the main cause of MI and stroke, these thrombi are rich in platelets and form at sites of high shear

stress. Healthy endothelial cells regulate platelet function by limiting platelet activation through a release of NO. However, in atherosclerosis, endothelial cells lose this ability to limit thrombosis due to decreased secretion of NO (Koupenova et al., 2017). One of the most common modifications in platelet function in diseased conditions is platelet hyperaggregation. This is seen more commonly in patients with diabetes mellitus (DM) type 1 and type 2, with DM patients showing an increased risk of CVD related events (Ghoshal and Bhattacharyya, 2014).

2.5 Anti-platelet treatment

Antiplatelet therapy is vital for the prevention and treatment of an arterial thrombosis (Hamilos et al., 2018). Antiplatelet drugs target the activation and aggregation of the platelets in order to prevent further thrombus formation. The most commonly used anti platelet drugs are aspirin, a cyclooxygenase (COX) inhibitor, and clopidogrel a thienopyridine and is a P2Y12 receptor blockers. (Triggers Mackman, 2008).

Aspirin prevents platelet aggregation by the irreversible inhibition of COX1, which is important for the production of TXA2; a vital agonist for the activation of platelets, amplification of the platelet response and induces platelet granule release (Behan and Storey, 2004). Aspirin is particularly effective as primary prevention strategy, significantly reducing the risk of an MI in men that have a possibility of developing CVD (Berger et al., 2006). Furthermore, it is also successful when used as secondary prevention, reducing the risk of a second MI in patients (Hennekens et al., 2006). However, despite these benefits, aspirin is associated with excessive bleeding events, with long-term aspirin therapy linked to a substantial increase in the occurrence of gastrointestinal haemorrhage (Gregg and Goldschmidt-Clermont, 2003).

The thienopyridine class of antiplatelet drugs work by blocking ADP from binding to one of its receptors; $P2Y_{12}$, reducing platelet activation (Gachet and Hechler, 2005). The most commonly used drug in this class is clopidogrel, which is used for the treatment of Acute Coronary Syndrome (ACS) (Cannon et al., 2007). Other thienopyridines such as ticlopidine, have had limited use due to the side effects associated with treatment the most serious being neutropenia accounting for 2.1% of ticlopidine-treated patients which sometimes results in fatalities (Quinn and Fitzgerald, 1999) (Valgimigli et al., 2018). Another recently developed $P2Y_{12}$ antagonist prasugrel is similar to clopidogrel in that it involves hepatic metabolism to make an

active metabolite, which performs as an irreversibly binding $P2Y_{12}$ antagonist (Hamilos et al., 2018). A study showed that prasugrel had decreased the amount of ischaemic events but increased the risk of bleeding compared to clopidogrel suggesting that prasugrel is more potent (Triggers Mackman, 2008).

A combination of anti-platelet therapies has been recognised as being significantly better at reducing and treating cardiovascular ischaemic events compared to single drug use. As aspirin and clopidogrel work by inhibiting different platelet activation pathways, dual antiplatelet therapy is now used. In clinical trials, patients with ACS without ST-segment elevation received clopidogrel in addition to aspirin as opposed to another group who received a placebo drug in addition to the aspirin. Results found a benefit of taking clopidogrel in the first 30 days, which continued during the 12 months of the trial. However, an increase in severe bleeding events in the group with clopidogrel compared to the placebo group (aspirin alone) was observed (Yusuf, 2001). Moreover, a randomised trials suggests that taking both aspirin and clopidogrel together or any $P2Y_{12}$ inhibitor together with aspirin offers no increased protection and in fact decreases efficacy of the $P2Y_{12}$ compared to group who took a lower dose of Aspirin that had a reduction in cardiovascular event (Warner et al., 2011). This highlights the disadvantages and risks of current anti platelet therapy and this gives rise to the need to identify novel compounds to develop as an anti-platelet therapy.

2.6 Diet and CVD

There is substantial evidence that associates healthy diets and a decrease in cardiovascular risk and an improvement of cardiovascular health (Casas et al., 2018). Unhealthy dietary patterns are associated with an increased risk of CVD such as intake of processed foods, increased sodium, unhealthy fats and a decreased intake of fruit and vegetables, fish, grains and nuts (Anand et al., 2015). For this reason, research into how a healthy diet can improve cardiovascular health is a priority. For instance, research has shown that a Western diet, generally a meat -based diet has shown higher levels of proinflammatory markers and decreased anti-inflammatory markers whilst "healthy" diets like the Mediterranean diet have shown the opposite with anti-inflammatory and cardioprotective effects. (Barbaresko et al., 2013). Furthermore, the Mediterranean diet has shown to decrease and modulate pro-atherothrombotic genes such as cyclooxygenase-2 (COX-2) and monocyte chemoattractant protein

(MCP-1) important for leukocyte recruitment (Esposito et al., 2006). This is central for research into atherosclerosis as a decrease in these genes reduces plaque stability. In atherosclerosis, platelets adhere to the endothelial cells contributing to recruitment of leukocytes thus contributing to inflammation (Nishijima et al., 2004).

In addition to their role in the formation of a thrombus, following a plaque rupture resulting in life threatening ischaemic events, platelets also have a role in intensifying inflammation through use of pro-atherogenic actions at all stages of atherosclerosis. They do this by helping the interaction between immune and endothelial cells by expressing molecules such as p-selectin (cell surface adhesion molecule found in α granules within platelets) (Fuentes Q et al., 2013). With the evidence of a positive link between healthy diet and atherosclerosis and platelets role in CVD, research into diet and dietary compounds as an anti-platelet agent is important as a primary prevention.

2.7 Anti-platelet properties of Dietary compounds

Research into natural dietary compounds have identified several dietary derived compounds with anti-platelet properties, which supports the link that a healthy diet decreases CVD.

2.7.1 Adenosine

A research study isolated and studied the antiplatelet effects of bioactive compound of ripe tomato (Solanum *lycopersicum*). Reversed-phase high-performance liquid chromatography (HPLC) was used to establish the concentration of adenosine in the tomato fruit (pulp and skin extracts) and the by-products (paste and pomace). Adenosine is an endogenous anti-aggregating substance which influences the activity of platelets by increasing the levels of 3′,5′-cyclic adenosine monophosphate (cAMP) and 3′,5′-cyclic guanosine monophosphate (cGMP) which inhibit platelet activity (Anfossi et al., 2002). A higher concentration of adenosine was found in aqueous extracts which resulted in inhibition towards platelet aggregation, adhesion and secretion as well as platelet thrombus formation (Fuentes et al., 2012).

2.7.2 Polyphenols

Inhibition of platelet function was observed following treatment with dietary polyphenols. Polyphenols are naturally occurring bioactive compounds that are characterised by their structure, this includes the amount of phenol rings they contain. Due to the difference in structure, the polyphenols are classified into

different groups and can vary in size depending on the phenolic structure (Ludovici et al., 2018). They act as secondary metabolites for plants and are generally found in vegetables, fruits, drinks and cereals (Beckman, 2000).

Several studies have shown that polyphenols elicit inhibition towards platelet function. One study used olive leaf extracts which are rich in the polyphenol oleuropein. The study found that oleuropein inhibited platelet aggregation in whole blood in a dose dependant manner. The study suggested that this was possibly due to their reactive oxygen species (ROS) - hydrogen peroxide (H_2O_2) scavenging properties and although the data shows that there is an inhibition of platelet function, the mechanism by which they elicit this activity has not yet been discovered (Singh et al., 2008). Furthermore, previous literature has found that oleuropein is not the only polyphenol found in olive leaves and another study (Talhaoui et al.) found various other polyphenols in olive leaf but that there was a larger concentration of oleuropein. Other polyphenols isolated included hydroxytyrosol, caffeic acid, luteolin, rutin and catechin. These polyphenols are also recognised to have antioxidant and H_2O_2 scavenging properties. The study suggested that the polyphenols are working together to inhibit platelet function as the polyphenols have the ability to show synergistic behaviour, however further work is required to quantify the polyphenols in olive leaf extract and determine the mechanism by which they inhibit platelet function.

In addition, another study investigating chlorogenic acid a polyphenol found abundantly in different foods including apples, kiwis, eggplants and coffee. The study found that chlorogenic acid inhibited ADP induced P-selectin expression and GPIIb/IIIa activation (Fuentes et al., 2014). P-selectin is also an inflammatory mediator. Chlorogenic acid was also shown to reduce ADP and collagen induced platelet aggregation. The study observed attenuation of platelet adhesion and aggregation formation on collagen under flow conditions following treatment of whole blood with chlorogenic acid. The study wanted to see whether the inhibition of platelet aggregation was due to an increase in cAMP levels (secondary messenger which inhibits platelet function modulating platelet function when activated), cAMP levels had increased with chlorogenic acid present and they had associated the inhibition of platelet aggregation with this increase. Moreover, Protein Kinase A (PKA)

– a potent antagonist of platelet activation (cAMP dependent) that maintains circulating platelets in a resting state was found to be increased with chlorogenic acid (Fuentes et al., 2014).

2.7.3 Flavonoids

Various studies have identified that flavonoids; a class of polyphenols that are found widely in fruits and vegetables and their secondary metabolites also have inhibitory effects on platelet function.

Hesperetin is a flavonoid found in grapefruits and oranges which showed inhibition of agonist induced platelet aggregation following stimulation by collagen and arachidonic acid through the inhibition of PLCγ2 phosphorylation and COX1 (Jin et al., 2007). The study demonstrated that hesperetin inhibited arachidonic acid mediated platelet aggregation, preventing production of TxA2 and prostaglandin D2 (PGD2) both produced by the COX1 pathway in platelets thus inhibiting platelet aggregation. In addition, hesperetin inhibited collagen mediated platelet aggregation. As collagen induces platelet activation through PLCγ2 resulting in increased calcium, which regulates platelet shape change and granule release, hesperetin was found to inhibit cytosolic calcium mobilisation and platelet function.

Another flavonoid that shows strong anti-platelet properties is tangeretin, a polymethoxylated flavone found abundantly in the peel of citrus fruits. The study found that tangeretin inhibited collagen-induced platelet aggregation in a concentration dependant manner. However, thrombin-induced platelet aggregation was inhibited at a lower concentration than collagen. Platelet aggregation is modulated by the integrin αIIbβ3 by inside-out signalling to increase fibrinogens ability to bind the integrin, treatment with tangeretin was found to inhibit the integrin αIIbβ3 inside-out signalling determined by measuring the fibrinogen binding. Moreover, tangeretin inhibited α-granule secretion, measured by P-selectin exposure. In addition to this, tangeretin also inhibited calcium mobilisation and tangeretin treatment resulted in reduced thrombus formation. These inhibitory effects were found to be due to an increase in the levels of cGMP, a key inhibitory mediator of platelet inhibition. This study went further, identifying that tangeritin showed no alteration of phosphodiesterase activity, indicating tangeritin does not increase cGMP by increasing hydrolysis of cGMP. The authors conclude that this

indicates that tangeretin increases the production of cGMP via regulation of guanylyl cyclase activity. (Vaiyapuri et al., 2013).

Structurally similar to tangeretin, nobiletin is a flavonoid found abundant in the peel of citrus fruits. The study reported that nobiletin inhibited platelet activation following stimulation with collagen and collagen-related peptide (CRP-XL) which is a GPVI specific agonist. Nobiletin was also able to inhibit aggregation through the inhibition of the integrin αIIbβ3. Similar to tangeretin, nobiletin inhibited platelet granule secretion and calcium mobilisation. Additionally, treatment with nobiletin affected the outside-in signalling of integrin αIIbβ3 by inhibiting clot formation with nobiletin. Of all the concentrations of nobiletin used, there was a clear reduction of thrombus formation compared to the vehicle. Nobiliten also increased cGMP levels like tangeretin and is not dependant on stimulation by agonist or platelet activation. The study further investigated and found that vasodilator-stimulated phosphoprotein (VASP) phosphorylation was also increased, which is phosphorylated by PKG – cGMP dependant protein kinase, indicating as with tangeritin, nobiletin regulates guanylyl cyclase activity (Vaiyapuri et al., 2015).

2.8 Cucurbitacins

Cucurbitacins are a large family of triterpenoid compounds found in a plant family known as cucurbitacea (figure 3). Cucurbitacins are characterised by tetracyclic cucurbitane nucleus skeleton called 19-(10→9-β)-abeo-5 alpha- lanostane (also known as 9-β-methyl-19-nor-lanosta-5-ene) (Alghasham, 2013). Chemically, cucurbitacins have different characteristics and are subdivided into 12 groups, most found in curcurbit group (Figure 3) (Chen et al., 2005). Originally, cucurbitacins were isolated from cucurbitacae but were later found in other plants of families including *Scrophulariaceae, rassicaceae, Thymelaeaceae, Datiscaceae, Begoniaceae, Elaeocarpaceae, Polemoniaceae, Desfontainiaceae, Primulaceae, Rubiaceae, Sterculiaceae, Rosaceae* (Chen et al., 2005). Cucurbitacins are found in edible vegetables and fruits, for instance cucumbers, squash, melons and watermelons but in low concentrations (Gry, 2006). Traditionally, used as folk remedies they have been shown to possess a wide range of pharmacological activities. Including their cytotoxic properties which gives some vegetables a bitter taste to deter animals in consuming the plant in excess. (Kaushik et al., 2015).

Several cucurbitacins have been shown to have several anticancer and anti-inflammatory activities. Cucurbitacins elicit cytotoxic activity on breast cancer cell lines such as SKBR-3 and MCF-7. (Dakeng et al., 2012). In vitro proliferation activity of cucurbitacin B was found to significantly inhibit the growth of colon (HCT-116), breast (MCF-7), lung (NCI-H460) and central nervous system (SF-268) cancer cell lines. (Jayaprakasam et al., 2003).

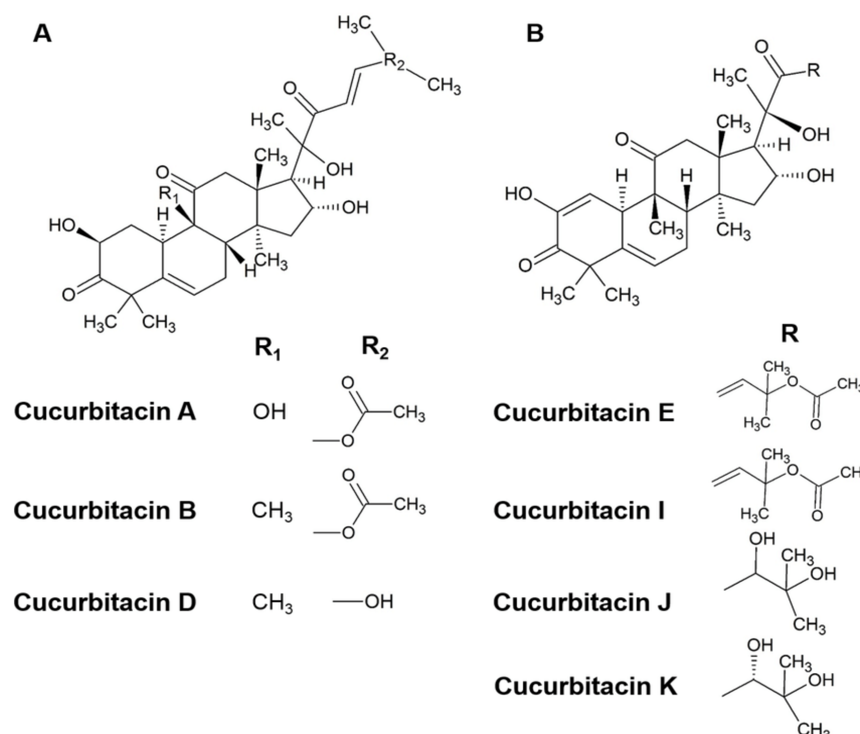

Figure 3: Structure of cucurbitacins. *A) The structure of cucurbitacin A, B and D. B) Structure of E, I, J and K*

Cucurbitacin D has also shown cytotoxicity towards many human cancer cell lines, such as lung cancer, brain cancer, breast and some human colon cancer cell lines (Jayaprakasam et al., 2003). Cucurbitacin E inhibits cell adhesion by disrupting the cytoskeleton of JY and HeLa cell lines (Musza et al., 1994) and has shown antitumor activity on ovarian sarcomas and prostate carcinomas (K. L. Duncan et al., 1996). Similarly, cucurbitacin I displays cytotoxicity towards KB human cell cultures (Chen et al., 2005) and anti-tumour activities in other cancers such as colon, breast, lung and brain. In addition to their cytotoxic anti-cancer properties, Cucurbitacins B, D, E and I have been reported to show inhibition of COX-2 enzymes, important for promoting inflammation but not COX-1 (responsible for thromboxane A2 synthesis) (Jayaprakasam et al., 2003). Reports have shown cucurbitacin B and E to elicit

inhibitory effects on products of lipid oxidation as lipid oxidation causes damage to cells through the degradation of lipids on the lipid membrane by free radicals. (Tannin-Spitz et al., 2007). This highlights the cucurbitacins potential as a preventative treatment for atherosclerosis as lipid oxidation plays a role in plaque formation. Taken together these reports identify cucurbitacins as future potential anti-atherosclerotic, anti-cancer and anti-inflammatory therapies. However, the antiplatelet activity of cucurbitacins is unknown. Therefore, this study will investigate the anti-platelet activity of cucurbitacins by monitoring the effect of cucurbitacin treatment on platelet function including measurement of platelet aggregation, platelet adhesion and thrombus formation.

2.9 Aims

The aim of this study is to identify the effect of the dietary compounds cucurbitacins on platelet function and thrombus formation.

2.10 Objectives

This project sets out to establish the effect of cucurbitacin compounds on the underlying cellular events of platelet function and thrombosis. We will do this by achieving the following objectives:

1. Investigate the anti-platelet properties of cucurbitacins ex vivo.
2. Investigate the anti-thrombotic properties of cucurbitacins using in vitro ex vivo assays.
3. Investigate the role for cucurbitacins in the regulation of platelet signalling components.

2.11 Hypothesis

We hypothesise that cucurbitacins B, E and I will have an effect on platelet function and elicit anti-platelet properties.

2.11.1 Null Hypothesis

We hypothesise that cucurbitacin B, E and I will show no anti-platelet properties and thus show no affect towards platelet function.

3 Materials and Methods

3.1 Blood collection

Informed consent was obtained, and blood taken from healthy, aspirin-free human volunteers into 4% sodium citrate according to ethical standards approved by Manchester Metropolitan University Ethics committee, ETHOS: 14849 (Appendix 1)

3.2 Platelet preparation

3.2.1 Platelet rich plasma

For platelet rich plasma (PRP), citrated whole blood was centrifuged at 100 xg for 20 minutes at room temperature and the supernatant PRP collected into falcon tubes.

3.2.2 Washed platelets

For washed platelet preparation, following protocol to prepare PRP. Add 150µLof Acid citrate dextrose ACD (29.9 mM trisodium citrate, 113.8 mM glucose and 2.9 mM citric acid [pH 6.4]) per ml of PRP and then centrifuge at 350 xg for 20 minutes. Remove the supernatant and re-suspend the platelet pellet in Tyrodes- HEPES buffer (134mM NaCl, 2.9mM KCl, 0.34mM Na2HPO4.12H2O, 12mM NaHCO3, 20mM HEPES and 1mM MgCl2 [pH 7.3]) containing glucose. Platelets were counted using Sysmex Blood analyser to guarantee at least a concentration of $2x10^8$ cells/ml.

3.3 Aggregation Assay

3.3.1 End-point – 96 well plate-based aggregation assay

Platelet aggregation was determined using optical aggregometry. For the end-point 96 well plate-based aggregation assay, agonists were prepared at 10 x final concentration in Tyrodes buffer as shown in table 6. A stock concentration of 10µg/ml of collagen (LabMedics - 300-0385) (-0.03-30µg/mL) and ADP (Sigma Aldrich – A5285) (0.01-10 µM) solution was used to make a collagen and ADP serial dilution series. The cucurbitacins (Cambridge Biosciences, Cucurbitacin B - CAY14820-1 mg, Cucurbitacin E - CAY14821-1 mg , Cucurbitacin I - CAY14747-1 mg) were also prepared at 10x final volume (0. 0.1, 0.3, 1, 3, 10 µM). A 10% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich 10213810) -tyrodes mix was prepared for the cucurbitacin serial dilution (final concentration of DMSO 0.1%). Using a multichannel pipette, 4.5µL of cucurbitacins was added to half area, flat-bottom, clear 96-well microtiter plate. The PRP or washed platelets were transferred to a reservoir and using a multichannel pipette 40.5 µL of

platelets were transferred into each cucurbitacin/vehicle containing well of the half area plate using inverse pipetting to avoid creating bubbles. This was incubated at room temperature for 10 minutes. A plate shaker was pre heated to 37°C, and following 10 minutes of cucurbitacin incubation, platelet activation was stimulated by adding 5µL of agonist (collagen or ADP) into the corresponding the wells, and the 96 well plate was then shaken for 5 minutes at 1,200 rpm at 37°C. Light absorbance was measured at 450 nm using a Synergy HT microplate reader. Light absorbance is a measure of platelet aggregation as is based on principle that light passes easier through a clear solution than when turbid. When platelet aggregates form, less light is absorbed, resulting in reduced absorbance and increased light transmission which is proportional to the amount of aggregation.

Table 1: Table showing dilutions of ADP, Collagen and Cucurbitacins in 96-well full area plate.

	ADP (µM)	Collagen (µg/mL)	Cucurbitacin (µM)
A	10	10	10
B	3	3	3
C	1	1	1
D	0.3	0.	0.3
E	0.	0.1	0.1
F	0.03	0.03	0 (Vehicle)
G	0.01	0.01	//////////////
H	0 (Vehicle)	0 (Vehicle)	///////////////

3.3.2 Analysis of platelet aggregation

Light absorbance is converted to %light transmission (%LT) using the equation:

$\%LT = 10^{-ABS} \times 100$

To convert %LT to % Aggregation, the following equation is used:

%Aggregation = 100/ (Resting – Blank) x %LT – Blank.

A dose response curve for each agonist and cucurbitacin was then plotted using GraphPad prism using a dose-response –stimulation curve analysis.

3.3.3 Real Time Aggregation

Real time aggregation is the "gold standard" turbidimetric platelet aggregation assay which uses optical aggregometry and measures light transmission over time using an aggregometer. A stock concentration of 100µM of ADP and collagen (100 µg/mL) was prepared in tyrodes buffer. Different concentrations of cucurbitacin were prepared at a 100x final concentration (final concentrations; 10, 1, 0.1 µm) that were used and 10% DMSO containing tyrodes buffer used as a diluent and vehicle control. In separate cuvettes, 2.5 µl (1/100 volume) of the different concentrations of cucurbitacins and vehicle was incubated with 225 µl of washed platelets. The cuvettes were placed into CHRONO-LOG® Model 700 Whole Blood/Optical Lumi-Aggregometers and baseline set in comparison to 250 µL of tyrodes buffer solution. The solution was stirred at 1200 rpm at 37 ^{o}C. After one minute, to establish baseline, the platelets were stimulated with 25 µL (1/10th volume) of the agonists ADP (10 µM final) and collagen (1 µg/mL final). Aggregation was measured for 5 minutes.

3.4 Platelet Adhesion and Spreading

Wells of a full area flat-bottomed 96-well plate were coated with 50 µL of fibrinogen (Sigma-Aldrich - F3879-100MG) (100 µg/ml) or 1% Bovine serum albumin (BSA) (Sigma-Aldrich - A7030-10G) (5 µg/mL) either overnight at 4 ^{o}C or for 1 hour for 37 ^{o}C. The wells were then washed with tyrodes buffer and then blocked with 50 µL of 1% BSA (5 µg/mL) and left for 1 hour at room temperature. After blocking the BSA is washed off three times with tyrodes buffer. Cucurbitacins were prepared at 10x final concentration (final concentrations; 0. 0.1, 0.3, 1, 3, 10 µM). A 10% DMSO-tyrodes mix was used as a diluent (final concentration 0.1% DMSO). 5µL (1/10th volume) of each cucurbitacin concentration was added to 45 µL of washed platelets at $2x10^{7}$ cell/mL and allowed to incubate for 10 minutes. 50 µL of cucurbitacin or vehicle control treated platelets was added to the fibrinogen and BSA coated wells and left to adhere at room temperature for 1 hour. Unbound platelets were aspirated off and discarded and wells washed with tyrodes buffer. Care was taken to not dislodge any adhered platelets. Adhered platelets were then fixed with addition of 50 µL of 4% Paraformaldehyde (PFA) (Sigma-Aldrich P6148) for 10 minutes, then washed again

with PBS (137 mM NaCl, 10 mM Na2HPO4, 2.7 Mm KCl, 2 mM KH2PO4 [pH 7.4]). To enable visualisation, adhered platelet was stained with 3,3′-Dihexyloxacarbocyanine iodide (DiOC6) (Sigma-Aldrich 318426-250mg) or Alexa-rhodamine conjugated phalloidin (Life Technologies- ThermoFisher Scientific A12379). 50 µL of DiOC6 (10 µM)/phalloidin in tyrodes buffer was added to the wells and incubated for 1 hour then removed and wells washed with tyrodes. The plate was stored in the dark at 4^oC until imaging.

3.4.1 Imaging and analysis

Fluorescent images of the adherent platelets were captured using CELENA S Digital Imaging system with the 20x objective lens. One image was captured at the centre of each of the well and analysed, atleast 100 platelets in the control sample was counted. Only one well per concentration was captured and analysed. Image J software was used to quantify the number of platelets that adhered to the plate (DiOC6) and total surface area covered by platelets that adhered and the average size of each platelet (phalloidin). The average platelet area was estimated by dividing the total surface area coverage by the number of platelets adhered on fibrinogen once corrected for non-specific BSA binding. To correct for non-specific binding, number of BSA adhered platelets were subtracted from the fibrinogen adhered platelets. Whilst attempts were made to set the ImageJ threshold (see following section) so that overlapping platelets were counted individually, manual checks revealed this was not always the case, therefore average platelet size can only be considered an ‘estimate’.

When imaging the wells, one image was taken in both pseudocolor for presentation of representative images and monochrome as seen in figure 4 and figure 5 For image J analysis, the monochrome image was used, and a ‘threshold’ was created to identify each single platelet by its fluorescent intensity.

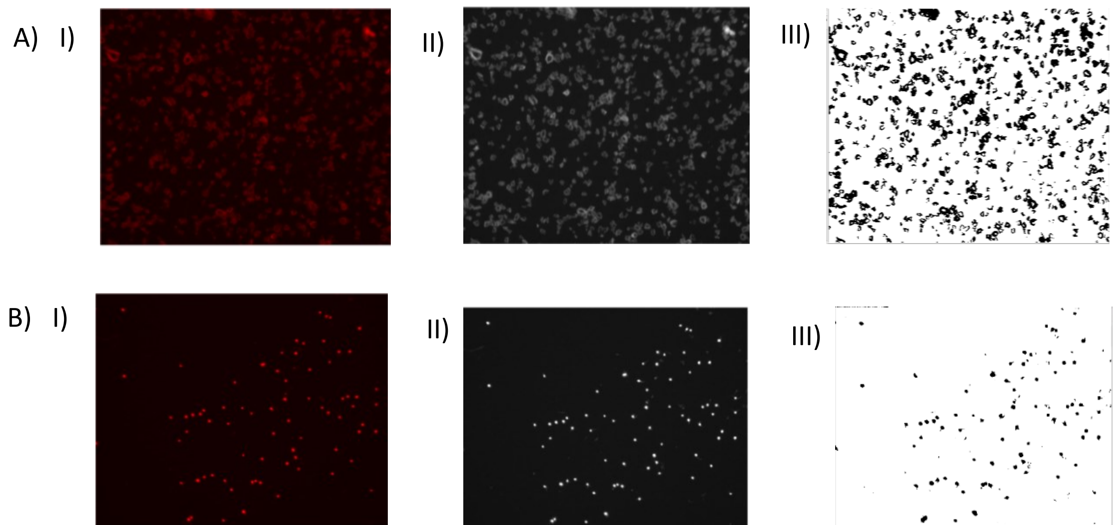

Figure 4: Imaging and Analysis of DIOC6 staining of Cucurbitacin B. *One fibrinogen image captured at 20x objective per well and analysed. A) 0 µL of cucurbitacin B. B) 10 µL of Cucurbitacin B. I) pseudo colour image, II) monochrome image and III) Threshold image created in image J for analysis.*

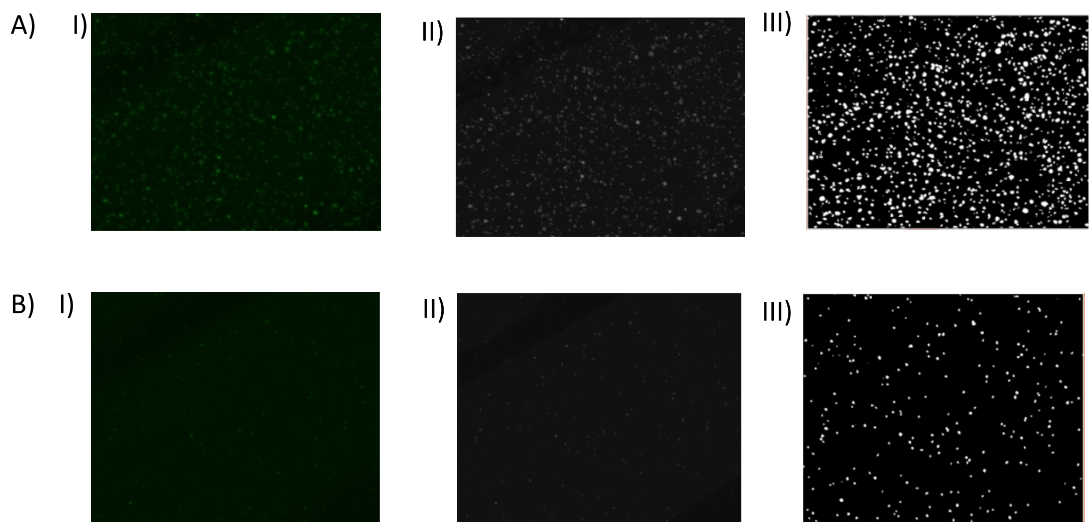

Figure 5: Imagine and Analysis of Phalloidin Staining of Cucurbitacin B. *One fibrinogen image captured at 20x objective per well and analysed A) 0 µL of cucurbitacin B. B) 10 µL of Cucurbitacin B. I) pseudo colour image, II) monochrome image and III) Threshold image created in image J for analysis.*

3.4.2 Platelet adhesion and spreading analysis in Image J

For platelet adhesion and spreading data analysis, images were opened in image J and a 'threshold' was created and background adjusted to detect adhered platelets (Figure 6-E). Number of platelets per image was quantified by using the 'analysis' and 'outlines' option within Image j which can be adjusted for size or circularity. The data is expressed as the total number of platelets, the total area of the platelets and average size of the platelets (Figure 6H). A step by step guide of the image J analysis protocols can be found in Appendix 8.1.

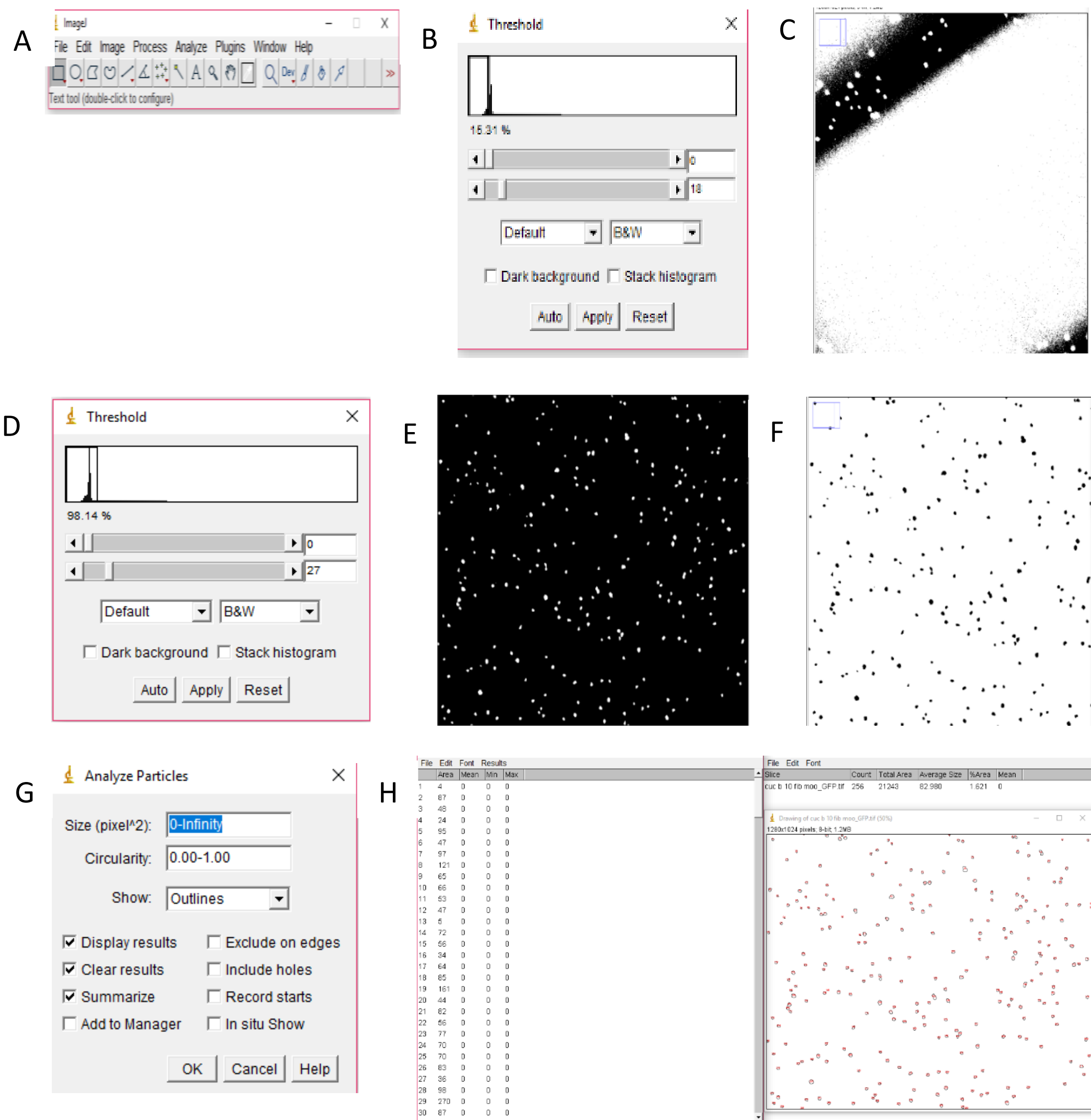

Figure 6: Image J analysis of platelet adhesion and spreading. *Step by step guide to analyse an image to count how many particles (platelets) and the average surface area of each particle in image J. A) Task bar for image J with all the options required to analyse. B) Threshold extension tab. C) The image created once a threshold is created. D) The intensity of fluorescence that shows in the image that shows each single platelet clear. E) The image created with the threshold. F) Image it is converted to be able to be analysed. G) Analysis extension tab to analyse the image with options of changing size of particles to be recognised and analysed. H) Summary of results, results of each platelet (area and mean) and image showing where each platelet is.*

3.5 Thrombus formation under flow

10µM DiOC6 (stains the platelet membrane) labelled human whole blood was treated with 10 µM of Cucurbitacin B, E or I or vehicle (0.1% DMSO) for 10 minutes. Channels of Vena8Fluor+ Biochips (Cellix), were coated overnight at 4° with collagen (100 µg/mL), then blocked with 1% BSA for one hour at room temperature and washed and replaced with PBS. Cucurbitacin pre-treated whole blood was perfused over the collagen coated Vena8fluor chips at an arterial shear rate 20 dyn/cm^2 ($1000s^{-1}$) for 10 minutes. A 20-x magnification lens on a Nikon A1-R confocal microscope was used to visualise platelet adherence and thrombus formation. Image J was used to analyse and determine surface area coverage, fluorescence intensity and the thrombus instability index (Δ Sd/ Δ T (%)) – change in surface distribution relative to change in time (Pugh et al., 2017), This is a measurement of objects in the video that haven't adhered and move from frame to frame , indicating instability these are determined as follows:

3.5.1 Δ Sd/ Δ T (%) analysis in Image J

Nikon ND2 plugin for image J was used to open the ND2 video file (Figure 7A), and the converted into an image sequence (Figure 7B). Frames at 10 second intervals were analysed (Figure 7C) to enable the distinction of movement (instability index) of the platelets. Like the previous image J platelet adhesion analysis, a threshold was created to recognise the platelets movement per image. The images were analysed for % surface area coverage of the thrombus. Furthermore, the images were analyses to find Δ Sd/ Δ T (%) which is a record of the change in surface distribution with time. The images were duplicated and one slice from the start of one duplicate was deleted and the end image of the second duplicate (Figure 7 E). Once deleted, the image sequence was subtracted from one another (Figure 7 F). This visualised the thrombi/platelets that appear to be moving/unstable and anything stable was eliminated. This was further analysed, and the data was presented as Δ Sd/ Δ T (%) which is the δ in surface distribution relative to change in time. A step by step guide of the analysis in image J can be found in Appendix 8.2.

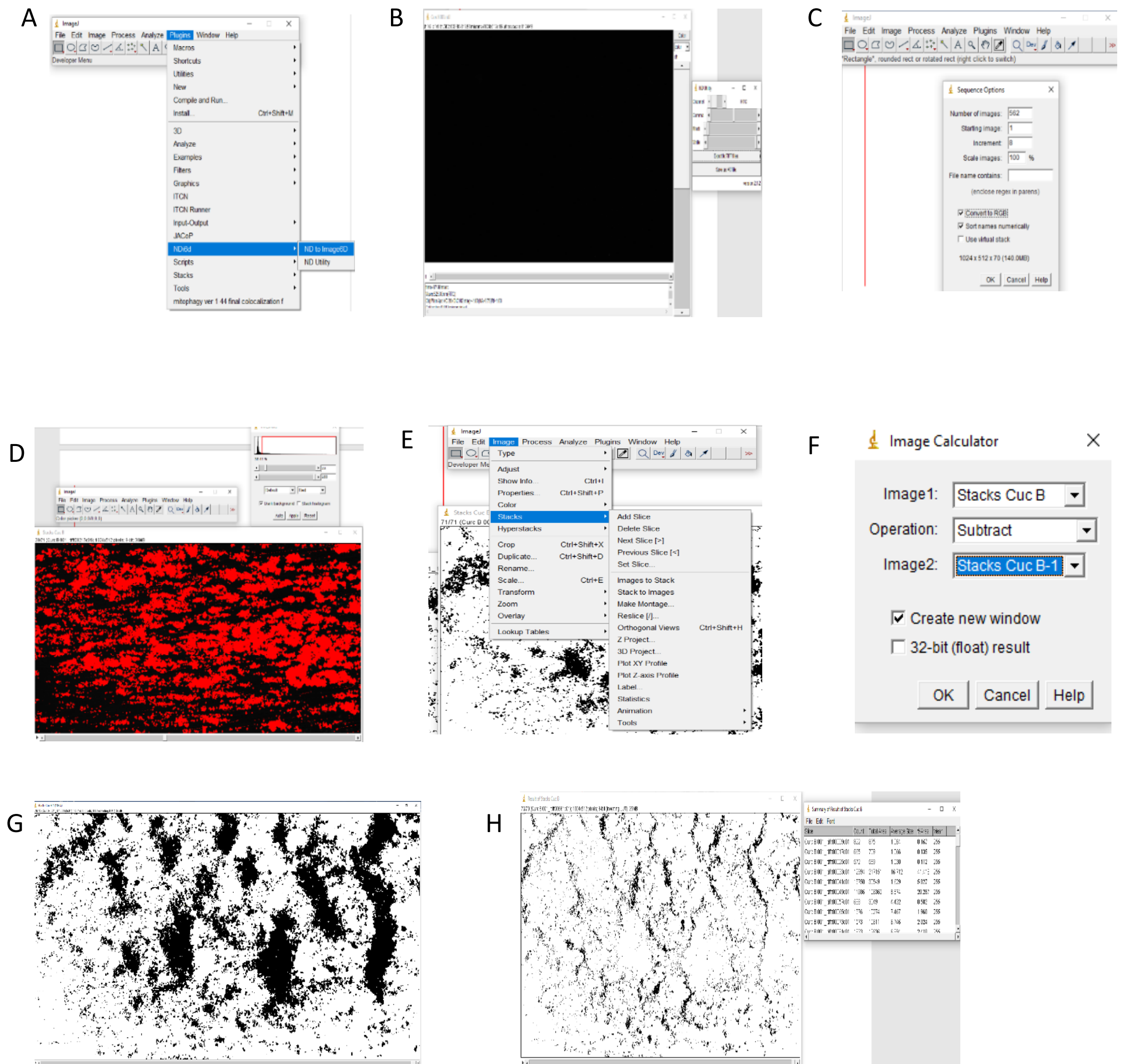

Figure 7: Image J analysis of thrombus formation video. *Step-by-step guide on how to analyse the thrombus formation videos to attain the % surface area coverage of each frame and Δ Sd/ Δ T (%)– a record of the change in surface distribution with time. A) ND2 plugin file for the video. B) The ND2 video opened in image J and option to convert to a video sequence. C) Video sequence with a frame every 8th image. D) 8 bit video with a threshold created for analysis. E) Second part of the analysis process with option to duplicate the video and delete a slice from the beginning of one video and end of the other video. F) The image calculator option to subtract both videos. G) The end of non-subtracted video to analyse surface area of coverage. H) The end of subtracted video to analyse Δ Sd/ Δ T (%)*

3.6 Flow Cytometry

3.6.1 Fibrinogen Binding – integrin GPIIb/IIIa activation

Platelet rich plasma was diluted 1/10 in tyrodes buffer and treated with increasing concentrations of cucurbitacins (0.1, 1, 10 µM) or vehicle control (0.1% DMSO/Tyrode's). An unstimulated (resting) sample and unstained sample were also prepared as negative controls and for gating. Cucurbitacin treated samples were stimulated with ADP (10 µM) and left to incubate with an anti-fibrinogen (Polyclonal Rabbit Anti-Human Fibrinogen/FITC Agilent Dako F0111) antibody for 30 minutes in the dark at room temperature. The samples were then fixed with 2% paraformaldehyde for 10 minutes. Data for 10,000 events in the platelet gate were collected for each sample using a MACSQuant (Miltenyi Biotec MACSQuant 16) flow cytometer and analysed using FlowJo software to determine median fluorescence intensity. Samples were prepared as follows (table 7):

Table 2: Table showing the samples prepared for Fibrinogen binding

Sample	Agonist	PRP + antibody mix	2% PFA
Cucurbitacin B – 10 µM	ADP	Antibody	PFA
Cucurbitacin B – 1 µM	ADP	Antibody	PFA
Cucurbitacin B – 0.1 µM	ADP	Antibody	PFA
Vehicle	ADP	Antibody	PFA
Cucurbitacin E – 10 µM	ADP	Antibody	PFA
Cucurbitacin E – 1 µM	ADP	Antibody	PFA
Cucurbitacin E – 0.1 µM	ADP	Antibody	PFA
Vehicle	ADP	Antibody	PFA
Cucurbitacin I – 10 µM	ADP	Antibody	PFA
Cucurbitacin I – 1 µM	ADP	Antibody	PFA
Cucurbitacin I – 0.1 µM	ADP	Antibody	PFA
Vehicle	ADP	Antibody	PFA
Unstimulated	-	Antibody	PFA
Unstained	-	No antibody	PFA

3.6.2 Actin Polymerisation

Human washed platelets prepared at $8x10^8$/mL in tyrodes HEPEs buffer were treated with increasing concentrations of cucurbitacins (0.1, 1, 10 µM) and 0.1% DMSO/tyrodes as vehicle control which was left to incubate for 10 minutes. ADP (10 µM) stimulated platelets with no cucurbitacin was added as positive control and ADP was only added to this one sample and not in the cucurbitacin treated platelets. An unstained sample (negative control) was also prepared with platelets and tyrodes to enable gating on flow cytometer. All samples were fixed with 2% paraformaldehyde and centrifuged for 10 minutes at 1000 xg at 4°C. The supernatants are discarded and resuspended in tyrodes buffer then centrifuged again with the same centrifuge settings as stated above. The pellet was then resuspended with BD Phosflow Perm Buffer III (BD Biosciences) and incubated on ice for 30 minutes. Once incubation is over, the samples are centrifuged again and resuspended in Tyrode's to wash and centrifuged once more. The samples except for the unstained samples were stained with Tyrode's containing conjugated phalloidin (Alexa-488) to detect F-actin for 30 minutes in the dark. Data for 10,000 events per sample were collected in the platelet gate using a MACSQuant (Miltenyi Biotec MACSQuant 16) flow cytometer and analysed using FlowJo software to determine median fluorescence intensity.

3.7 Western Blotting

3.7.1 Preparation of platelet lysates.

Human washed platelets were resuspended to 4×10^8 /ml in modified Tyrodes HEPEs buffer and increasing concentrations of cucurbitacin B, E and I prepared at a 10x final concentration (final concentrations; 10, 1, 0.1 µM) and 10% DMSO containing tyrodes buffer used as a diluent and vehicle control (final concentration 0.1%). Platelets were incubated with the increasing concentrations of cucurbitacins or vehicle control 10 minutes at room temperature. Platelets were also stimulated with ADP (10µM) for 10 minutes as a positive control. Following incubation, lysates were prepared by addition of an equal volume of laemlli buffer (1M Tris-HCl , 8% Sodium Dodecyl Sulfate (SDS), 40% glycerol, β-mercaptoethanol, 0.5 M Ethylenediaminetetraacetic acid (EDTA), bromophenol Blue [pH 6.8]) and heated to 90°C for 5 minutes, before storage at -20C for future use.

3.7.2 SDS-PAGE and Blotting

Samples were separated by SDS PAGE using pre-cast gradient 4-20% Tris-Bis gels and 15µL samples loaded as per table 8. The SDS PAGE gel was run with Tris-glycine Running biffer (192 mM Glycine, 25 Mm Tris, 1% SDS [pH 8.3]) at 90 volts for 10 minutes then 130 volts at 50 minutes until bands have reached black line of gel. Separated samples were then transferred onto Polyvinylidene difluoride (PVDF) membrane with 1% Transfer buffer (Tris Glycine 10x transfer buffer- 192 mM Glycine, 25 Mm Tris, 20% Methanol) at 90v for 90 minutes at 4°C. Following transfer, the PDVF membrane was blocked with 5% BSA (50ml TBST and 2.5 mg BSA) for 1 hour at room temperature.

Table 3: Order of lysates gel. Veh = vehicle control (0.1% DMSO)

Well	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Ladder	X													
Cucurbitacin		B	B	B		E	E	E		I	I	I		
Control					Veh				Veh				Veh	ADP
µM		10	1	0.1	0	10	1	0.1	0	10	1	0.1	0	10

Primary antibodies were prepared in BSA (5%) anti-Actin (AC-15 Anti-beta Actin - ab6276, ABCAM) antibody was prepared at 1:5000, (2 µL of actin and 10ml of BSA), and anti-phospho-myosin light chain 2 (Ser19) (pS19 Myosin Light chain 2 – 3671, Cell signalling technologies) was prepared at 1:1000 (5 µL in 5ml of BSA) and incubated at 4°C overnight. Blots were then washed 3 times for 10 minutes with Tris buffered saline Tween-20 (TBS-T) (10Mm Tris, 150 mM NaCl, 0.2% Tween- 20) with agitation. Secondary antibodies were prepared**.** Goat anti-Rabbit IgG Secondary Antibody (Li-cor - 926-32211) and Goat anti-Mouse IgG Secondary Antibody (Li-cor - 926-32210) at 1;10000 (1 µL of antibody in 10ml of BSA) and membranes incubated in light sensitive containers on a rocker for 1 hour at room temperature. The membranes were washed again 3 times for 10 minutes with TBST. Membranes were imaged using the Li-cor Odyssey Fc, for fluorescence intensity. ImageJ was used to quantify band density and fluorescence intensity.

3.8 Statistics

All experiments were performed at least in triplicate. The data obtained from plate-based aggregation, real time aggregation and *in vitro* thrombus formation, DIOC6 stained adhesion 96-well were analysed using two-way Anova. Phalloidin stained adhesion 96-well plate were analysed using one-way Anova. Median fluorescence intensity value as obtained in fibrinogen binding and actin polymerisation assays were analysed using Kruskall-wallis (parametric and non-parametric) test. All tests were performed on GraphPad Prism (version 8.01) from graphpad Software Inc. P≤0.05 was considered statistically significant. Values were all expressed as mean ±SEM.

4 Results

4.1 Cucurbitacins inhibits platelet aggregation.

Previous studies have shown that cucurbitacins can disrupt integrin signalling in breast cancer cell lines, specifically ITGA6 and ITGB4 (integrin α6 & integrin β4) which are overexpressed in breast cancer (Gupta and Srivastava, 2014). Integrin αIIbβ3 mediates platelet aggregation and is the dominant integrin in platelet function and essential for platelet aggregation. Therefore end-point aggregometry was used to analyse the effects of increasing concentrations of cucurbitacins B, E and I on integrin function and platelet aggregation. Both human platelet rich plasma (figure 8 and figure 10) and washed platelets (figure 9 and figure 11), were treated with increasing concentrations of cucurbitacin B, E or I (0, 0.1, 0.3, 1, 3, 10 µM), and aggregation to collagen (0.03-30 µg/mL) (Figure 8 and Figure 9) and ADP (0.01-10 µM) (Figure 10 and Figure 11) was determined after 5 minutes by monitoring light transmission. Treatment with either of the different cucurbitacin derivatives caused a dose dependent attenuation in platelet aggregation following stimulation with agonists collagen (Figures 8 and 9) and ADP (Figures 10 and 11). Following treatment of platelets with the highest concentration of all cucurbitacins (10 µM), aggregation was compared across increasing agonist concentrations, and a reduction in the amount of aggregation was observed. Despite an overall inhibition of aggregation, treatment with the cucurbitacins did not seem to affect the sensitivity of the aggregation to collagen or ADP, as the EC50 for either agonist was unchanged following treatment with 10uM Cucurbitacin B, E or I (Figure 8, 9, 10 and 11 A). The inhibition caused by cucurbitacins was not overcome by the increasing concentrations of collagen and

ADP. The IC50 for all cucurbitacins are all similar at all concentrations of ADP and collagen tested (Figure 8B, 9B, 10B and 11 B). Although inhibition is observed in both PRP and WP, it is elevated in WP (figure 9 and figure 11) compared to PRP (figure 8 and figure 10). For example, whilst aggregation to ADP was not significantly altered following treatment with 10µM cucurbitacin I in PRP compared to the vehicle (Figure 10A II), a significant inhibition of aggregation was observed compared to the vehicle in washed platelets (Figure 11A II). Similar results were observed with cucurbitacin B and E. This is likely due to cucurbitacin binding to plasma proteins in PRP, therefore decreasing their ability to inhibit aggregation. In conclusion we observed, cucurbitacin mediated inhibition of platelet aggregation in a dose dependant manner following stimulation with ADP and collagen. The concentration of agonist did not affect inhibition of platelets caused by cucurbitacin and the highest concentration of each cucurbitacin caused a decrease in overall amount of aggregation. Although no significance difference was observed across any of the data, there is a visual shift in the graphs.

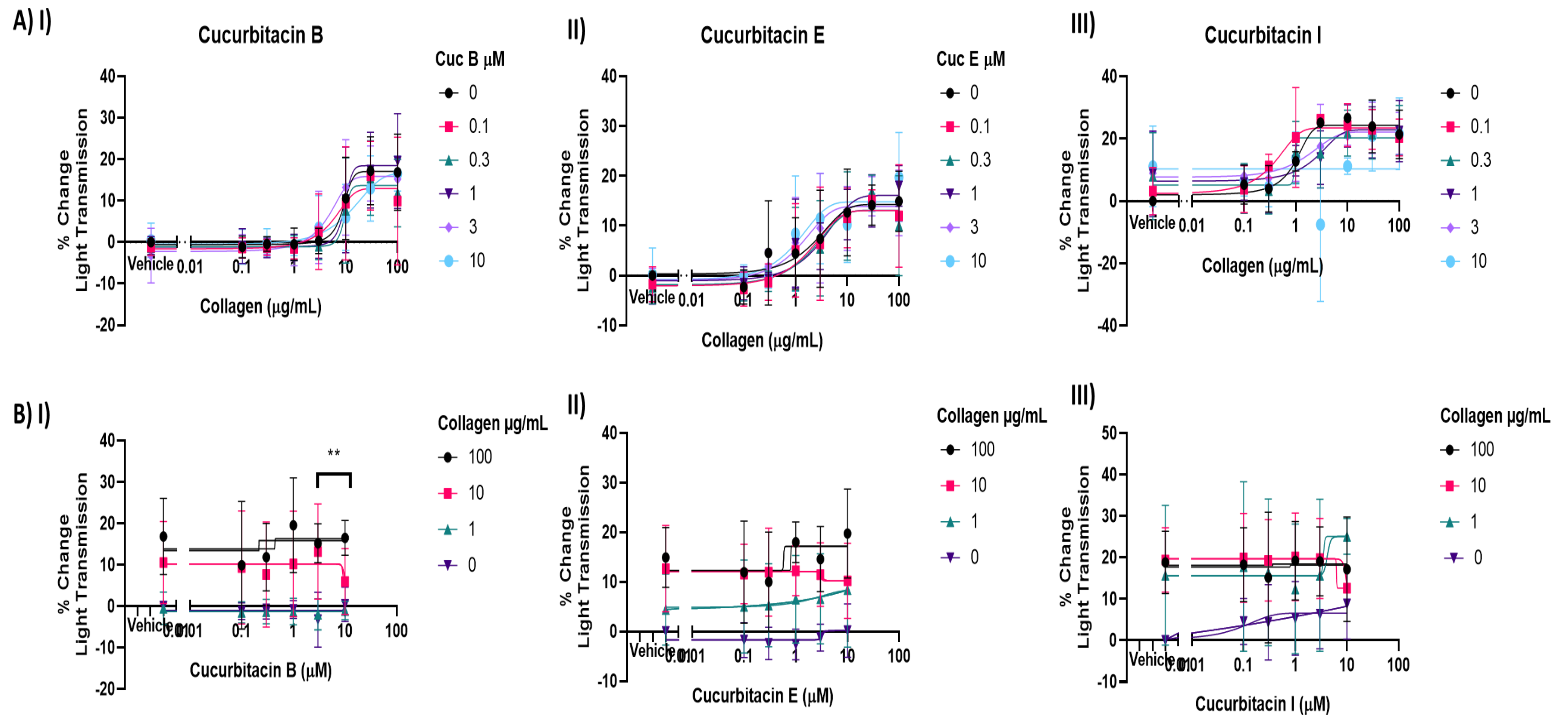

Figure 8: Cucurbitacins inhibit platelet aggregation with agonist collagen in PRP. *Human platelet rich plasma (PRP) in 96 well plates were treated with increasing concentrations of cucurbitacin i) B, ii) E and iii) I (0. 0.1, 0.3, 1, 3, 10 µM) and incubated at room temperature for 10 minutes. Plates were shaken at 1200 rpm for 5 minutes at a temperature of 37º C following addition of increasing concentrations of agonist Collagen (0. 0.1, 0.3, 1, 3, 10 µg/mL). Light absorbance measured at 450 nm and converted to % light transmission and A) Corrected change light transmission curve in PRP. B) Inhibition curve of cucurbitacins in PRP plotted using GraphPad Prism software. Error bars in graphs represent variability of the data collected to indicate any errors in experiments including variability in participants and potency of each blood sample to cucurbitacin and agonist.. Data expressed as mean + S.E.M for N≤5. * indicates p<0.05 in comparison to vehicle controls, where normalised data shown, statistics were performed prior to normalisation. Data was analysed using two-way Anova.*

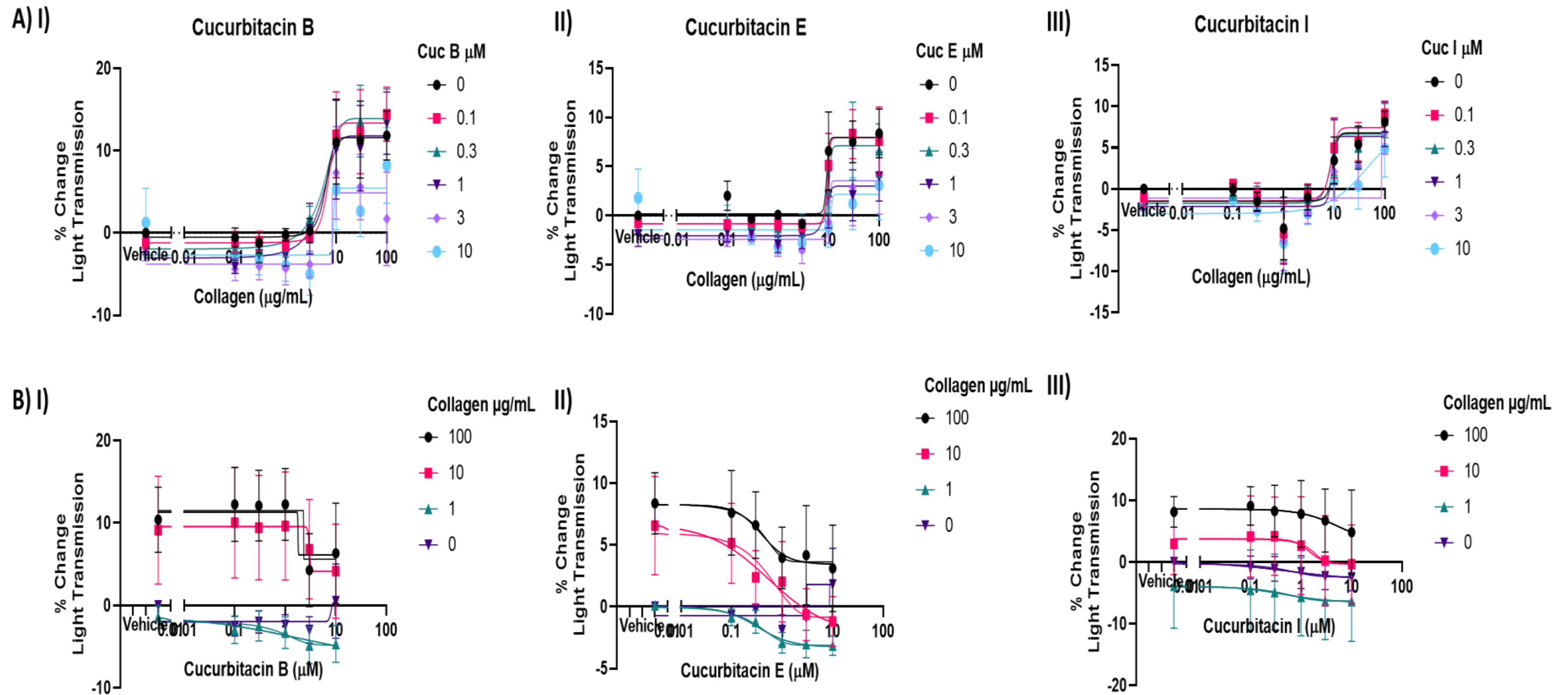

Figure 9: Cucurbitacin inhibits platelet aggregation with agonist collagen in WP. *Human washed platelet (WP) in 96 well plates were treated with increasing concentrations of cucurbitacin i) B, ii) E and iii) I (0. 0.1, 0.3, 1, 3, 10 µM) and incubated at room temperature for 10 minutes. Plates were shaken at 1200 rpm for 5 minutes at a temperature of 37°C following addition of increasing concentrations of agonist Collagen (0. 0.1, 0.3, 1, 3, 10 µg/mL). Light absorbance measured at 450 nm and converted to %light transmission. A) Corrected change light transmission curve in WP. B) Inhibition curve of cucurbitacins in WP. Error bars in graphs represent variability of data collected to indicate any errors in experiments including variability in participants and potency of each blood sample to cucurbitacin and agonist. Concentration curves plotted using GraphPad prism. Data expressed as mean + S.E.M for N≤5. * indicates $p<0.05$ in comparison to vehicle controls, where normalised data shown, statistics were performed prior to normalisation. Data was analysed using two-way Anova.*

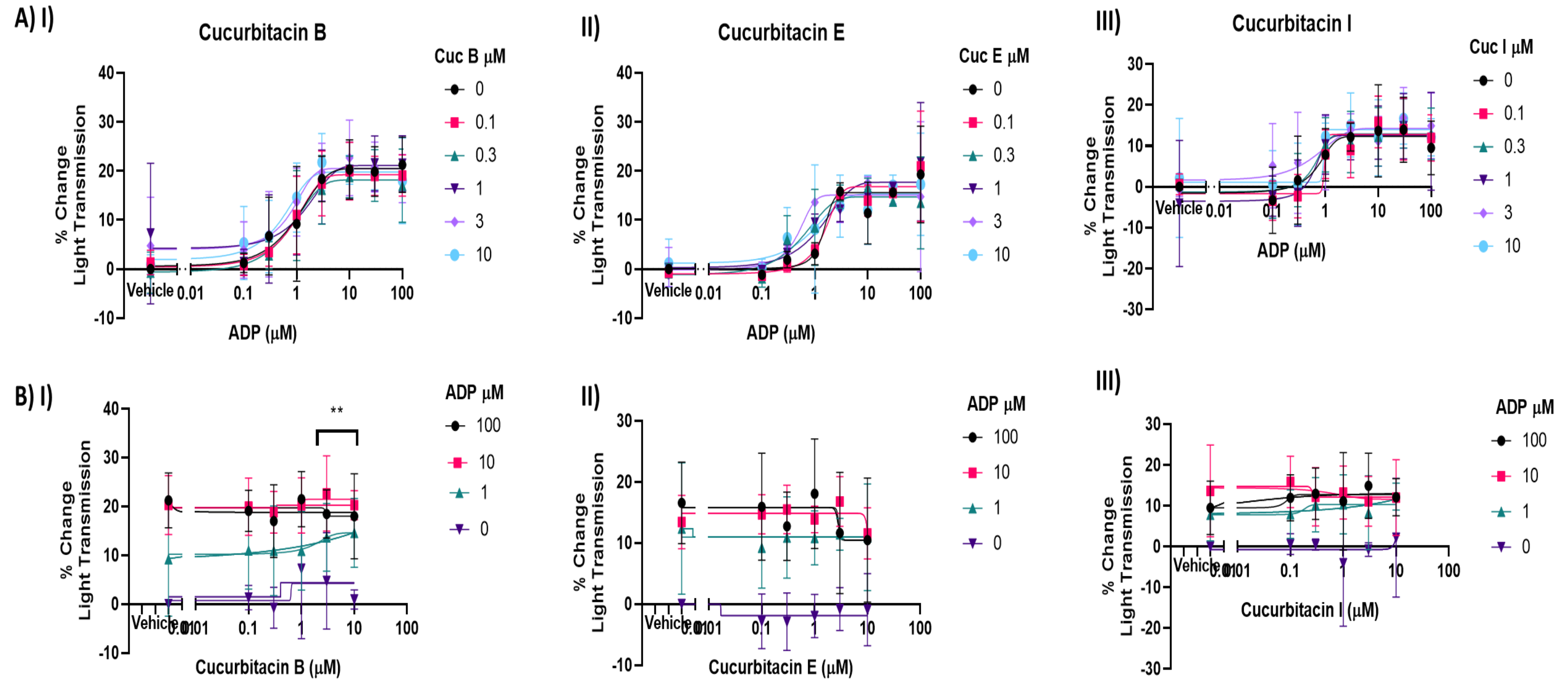

Figure 10: Cucurbitacin inhibits platelet aggregation with agonist ADP in PRP. *Human platelet rich plasma (PRP) in 96 well plates were treated with increasing concentrations of cucurbitacin i) B, ii) E and iii) I (0. 0.1, 0.3, 1, 3, 10 µM) and incubated at room temperature for 10 minutes. Plates were shaken at 1200 rpm for 5 minutes at a temperature of 37°C following addition of increasing concentrations of agonist ADP (0. 0.1, 0.3, 1, 3, 10 µM). Light absorbance measured at 450 nm which was converted to %light transmission. A) Corrected change light transmission curve in PRP. B) Inhibition curve of cucurbitacins in PRP. Error bars in graphs represent variability of data collected to indicate any errors in experiments including variability in participants and potency of each blood sample to cucurbitacin and agonist. Concentration curves plotted using GraphPad prism. Data expressed as mean + S.E.M for N≤5. * indicates $p<0.05$ in comparison to vehicle controls, where normalised data shown, statistics were performed prior to normalisation. Data was analysed using two-way Anova.*

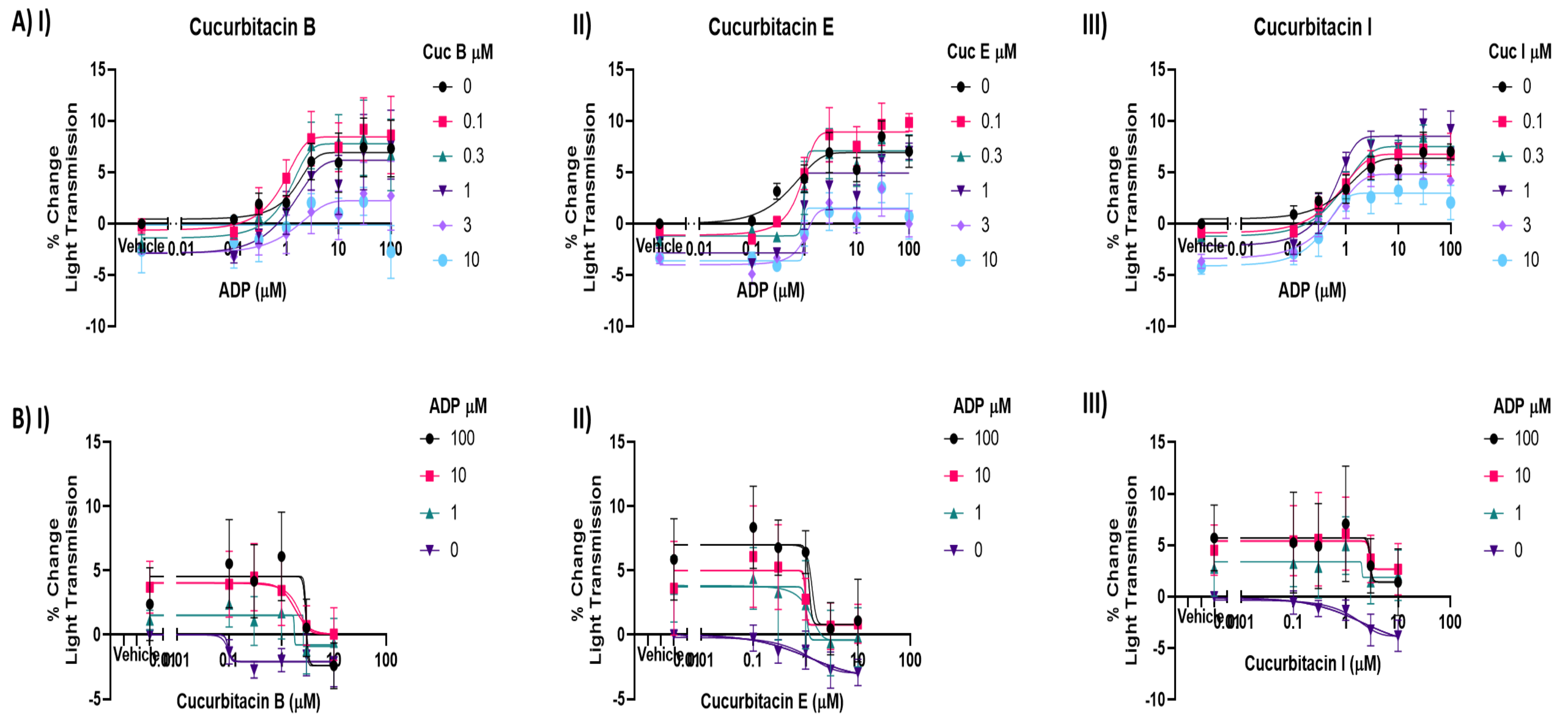

Figure 11: Cucurbitacin inhibits platelet aggregation with agonist ADP in WP. *Human Washed platelet (WP) in 96 well plates were treated with increasing concentrations of cucurbitacin i) B, ii) E and iii) I (0. 0.1, 0.3, 1, 3, 10 μM) and incubated at room temperature for 10 minutes. Plates were shaken at 1200 rpm for 5 minutes at a temperature of 37°C following addition of increasing concentrations of agonist ADP (0. 0.1, 0.3, 1, 3, 10 μM)). Light absorbance measured at 450 nm and converted to % light transmission. A) Corrected change light transmission curve in WP. B) Inhibition curve of cucurbitacins in WP. Error bars in graphs represent variability of data collected to indicate any errors in experiments including variability in participants and potency of each blood sample to cucurbitacin and agonist. Concentration curves plotted using GraphPad prism. Data expressed as mean + S.E.M for N≤5. * indicates $p<0.05$ in comparison to vehicle controls, where normalised data shown, statistics were performed prior to normalisation. Data was analysed using two-way Anova.*

4.2 Cucurbitacins inhibit real-time aggregation.

Real time aggregation was used to analyse the effect of cucurbitacin B, E and I on the kinetics of platelet aggregation in response to the different platelet agonists; ADP (10 µM) and collagen (1µg/mL), to determine whether the cucurbitacins altered the early time points of aggregation in addition to overall levels of aggregation after 5 minutes following stimulation. As with end point aggregation, in washed platelets, cucurbitacin B, E and I were found to cause a concentration dependent decrease in platelet aggregation following stimulation with ADP (Figure 12) and collagen (Figure 13) at 5 minutes. Interestingly aggregation was also found to be inhibited at earlier time points. Maximum inhibition in cucurbitacin B was attained with 10 µM at 5 minutes (figure 12A) with stimulation with ADP 10 µM. Interestingly, aggregation was also inhibited at earlier time points by the cucurbitacins indicating that cucurbitacins delay aggregation. For example, at 2 minutes, cucurbitacin B (10µM) caused ~30% aggregation compared to vehicle ~35% which carried on at each time point. Cucurbitacin E showed similar results with 10 µM causing a ~36% aggregation compared to vehicle ~40%. Cucurbitacin I 1 µM showed a reduction in aggregation, for example at 2 minutes there was a ~37.5% aggregation compared to vehicle ~40.5% and was seen across each time. Similarly, stimulation with agonist collagen showed similar results. Cucurbitacin B (10µM) caused ~42% aggregation compared to vehicle ~90% which carried on at each time point. Additionally, Cucurbitacin E 10µM showed ~62% aggregation compared to vehicle ~90% at 2 minutes and cucurbitacin I 10µM showed aggregation to be ~35% in comparison to vehicle ~90% at 2 minutes (Figure 13). This demonstrates that cucurbitacins delay platelet aggregation when stimulated with ADP and collagen.

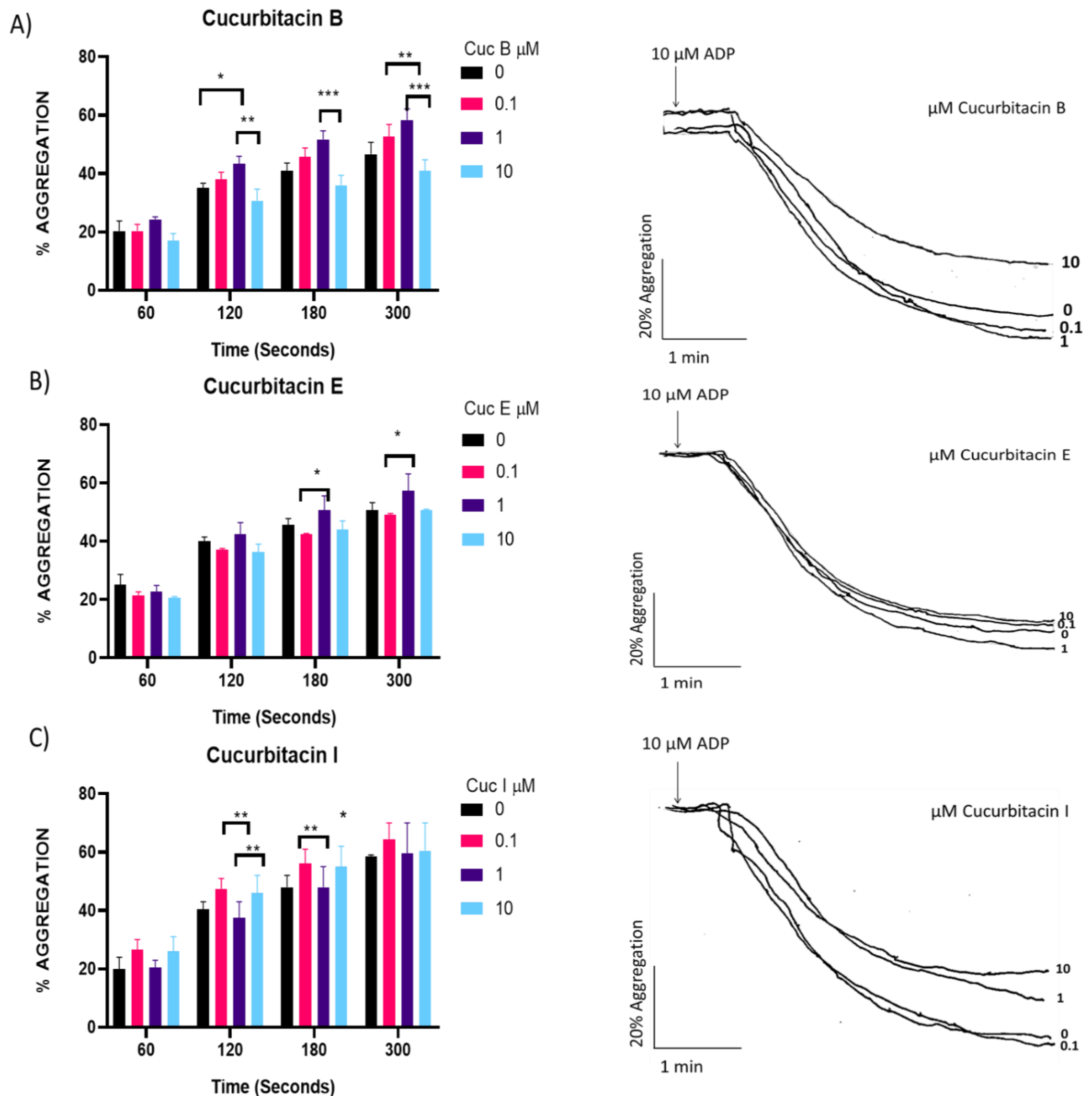

Figure 12. Cucurbitacin B, E and I inhibit real time platelet aggregation with ADP. *Human-washed platelets were pre-treated increasing concentrations of A) cucurbitacin B, B) cucurbitacin E or C) cucurbitacin I (0.1-10 µM) Representative traces shown. Tyrodes buffer with agonist and platelets used as a vehicle control to check that agonist was stimulating platelets. Stimulated with agonist ADP (10 µM) and aggregation was monitored for 5 minutes by real time optical light aggregometry. Data presented as % aggregation at the following time points (1, 2, 3 and 5 minutes) and expressed as mean + S.E.M for N≤4. * followed with bar indicates p<0.05 in comparison to other concentrations. Where normalised data shown, statistics were performed prior to normalisation. Data was analysed using two-way Anova.*

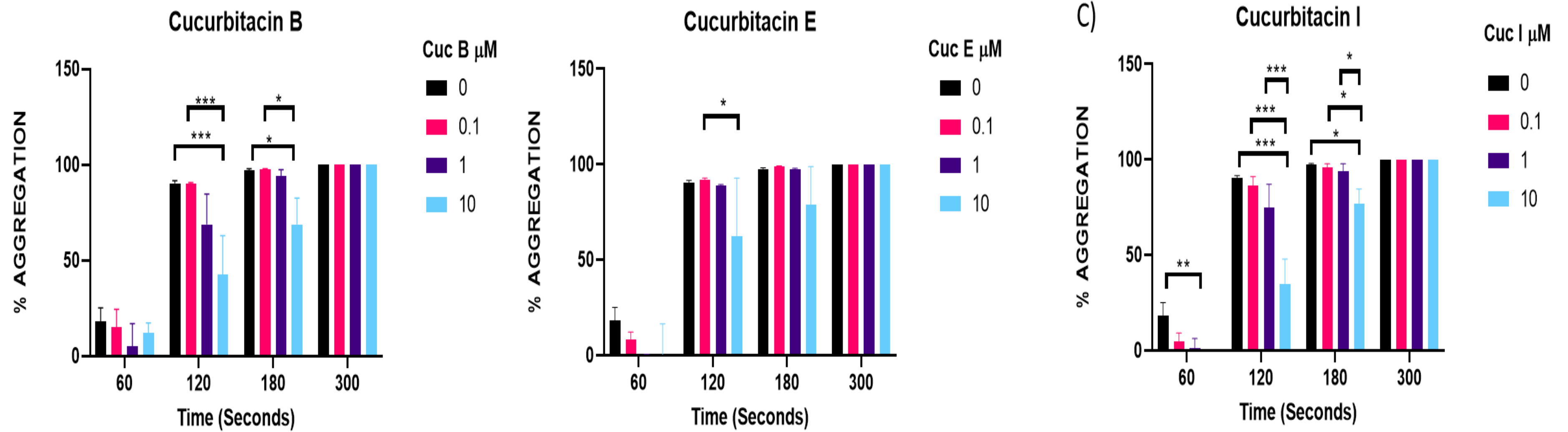

Figure 13. Cucurbitacin B, E and I inhibit real time platelet aggregation with collagen. *Human-washed platelets were pre-treated increasing concentrations of A) cucurbitacin B, B) cucurbitacin E or C) cucurbitacin I (0.1-10 µM). Blue bars represent vehicle, orange represents 0.1 µM, grey represents 1 µM and yellow represents 10 µM. Tyrodes buffer with agonist and platelets used as a vehicle control to check that agonist was stimulating platelets. Stimulated with agonist collagen (1µg/mL) and aggregation was monitored for 5 minutes by real time optical light aggregometry. Data presented as % aggregation at the following time points (1, 2, 3 and 5 minutes) and expressed as mean + S.E.M for N≤4. * indicates p<0.05 in comparison to vehicle controls, * followed with bar indicates p<0.05 in comparison to other concentrations. Where normalised data shown, statistics were performed prior to normalisation. Data was analysed using two-way Anova.*

4.3 Cucurbitacins inhibit platelet thrombus formation.

Having observed an inhibitory effect of cucurbitacins on platelet aggregation, the effect of cucurbitacins on thrombus formation on collagen was evaluated in human whole blood under flow conditions to see whether this inhibition in aggregation disrupted the ability of platelets to form stable thrombi formation. Blood was perfused over collagen (100 µg/mL) coated vena8Fluor+ biochips at an arterial shear rate 20 dyn/cm^2 for 10 minutes. Interestingly the fluorescence intensity remained similar following treatment with the cucurbitacins (10 µM) compared to vehicle. Fluorescence intensity is a marker of platelet adhesion indicating cucurbitacins do not alter the platelet ability to bind to collagen (Figure 14 C). However, fluorescence intensity does not distinguish between larger surface area and big stable thrombi which may explains the similar fluorescence between cucurbitacins and vehicle. Therefore, the surface area covered was also measured to determine thrombi generation. Compared to the vehicle, all the cucurbitacins were found to cause a larger surface area distribution indicating increased platelet adhesion, however larger aggregates were noted to have formed on vehicle treated compared to cucurbitacin treated samples (Figure 14 B). Observations made so far demonstrated a difference in thrombi morphology and platelet adhesion, indicating reduced thrombus formation, yet these differences were not quantifiable. The analysis methods used so far were unable to detect differences between stable and unstable thrombi formation. Therefore, the thrombus instability index Δ Sd/ Δ T (%) (Pugh et al., 2017) was used to determine whether treatment with cucurbitacins altered thrombus stability on collagen. This index measures any moving/unstable platelets/platelet aggregates that do not adhere to the collagen coated chips to form a stable thrombus. The instability index of thrombi following treatment with all three cucurbitacins compared to vehicle treated controls was significantly increased, indicating that the platelets treated with cucurbitacins were unstable and did not have the same ability as vehicle treated platelets to adhere to other platelets and form stable thrombi (Figure 14 D). Analysis of the image sequence taken show cucurbitacin treated thrombi to appear 'loose' which detach easily from the site of adhesion. Representative images taken at the end of each video shows that the size of thrombus is similar hence the similar fluorescence intensity, but the images show the difference in the formation and characteristics of the thrombus. The vehicle shows a uniform

and stable thrombus that seems to be stuck down whereas the cucurbitacins show an unstable, loose formation of thrombus (Figure 14 A).

Taken together this demonstrates that cucurbitacins reduce the ability of platelets to form stable thrombi.

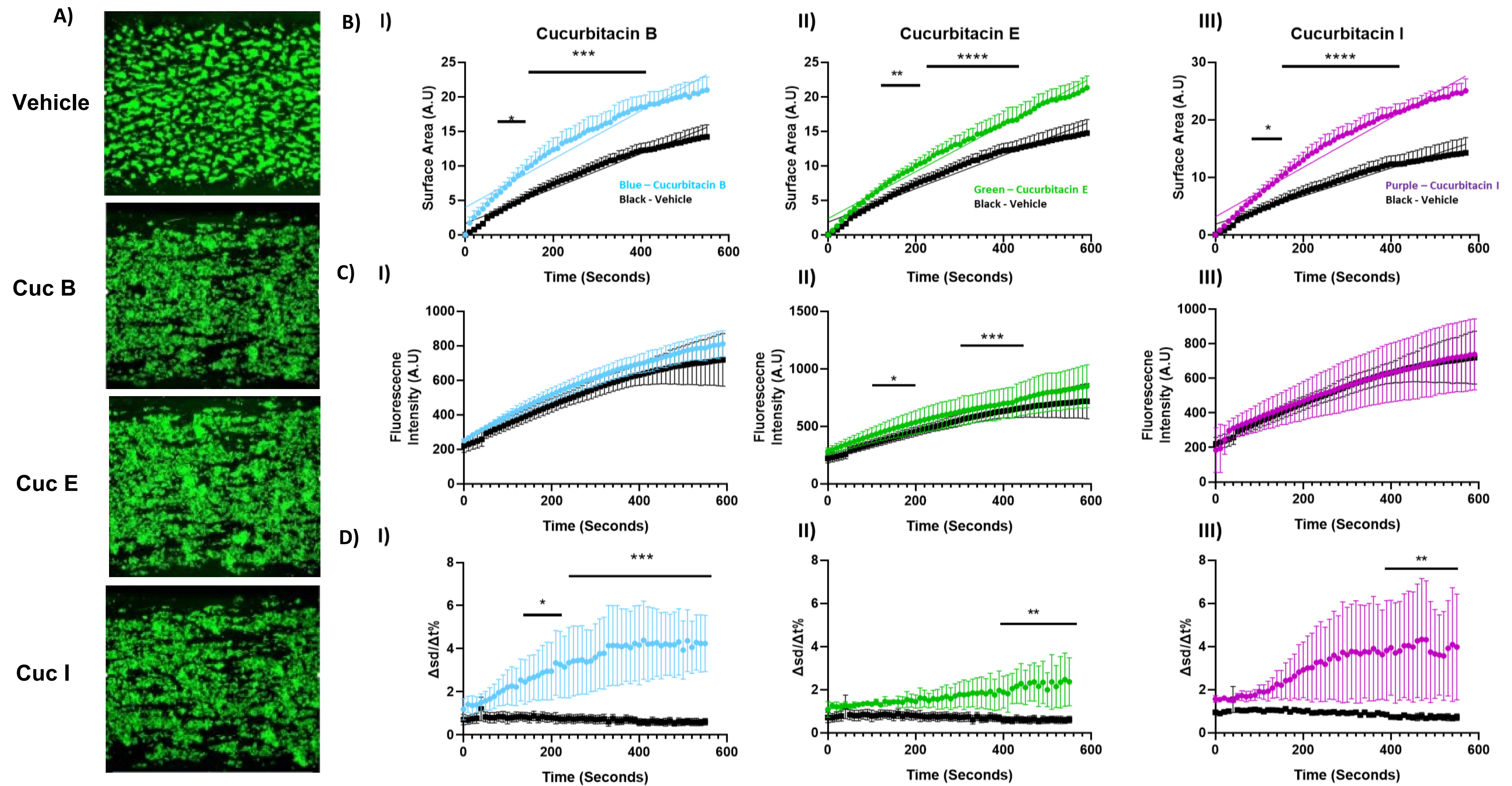

Figure 14: Cucurbitacins Inhibit platelet thrombus formation. *10µM DiOC6 labelled human whole blood was treated with 10 µM of Cucurbitacin i) B, ii) E or iii) I or vehicle (0.1% DMSO) for 10 minutes then perfused over collagen coated vena8Fluor+ biochips at an arterial shear rate of 20 dyn/cm^2 for 10 minutes. The channels were imaged on a Nikon A1-R confocal microscope to visualise thrombus formation then analysed using image J to determine surface area coverage and thrombus instability index (Δ Sd/ Δ T (%)). A) Representative images taken at end of each video. B) surface area coverage of cucurbitacins compared to vehicle C) Fluorescence intensity (A.U) and D) Thrombus instability index. Results are expressed as mean + S.E.M for n≥4. * indicates p<0.05 in comparison to vehicle controls, where normalised data shown, statistics were performed prior to normalisation. Data was analysed using two-way Anova.*

4.4 Cucurbitacin B, E and I inhibits platelet adhesion and spreading.

In platelets, integrins binding to their ligands such as αIIbβ3 to fibrinogen mediates platelet adhesion to matrix proteins. This leads to platelet adhesion, and integrin mediated outside-in signalling drives platelet shape change and spreading. Aggregation analysis indicates an inhibition of the ability of platelets to form integrin αIIbβ3-fibrinogen bridges, which are essential for platelet aggregation. Having identified a reduction in platelet aggregation and unstable thrombus formation, the effect of cucurbitacin on platelet adhesion and spreading was determined using platelet adhesion assays to ascertain whether Cucurbitacins reduce the activation of and signalling downstream of integrin αIIbβ3. Treatment of washed platelets with increasing concentrations of cucurbitacin B, E or I (0. 0.1, 0.3, 1, 3, 10 µM) (Figure 15, 16 and 17) caused a decrease in the ability of platelets to adhere to (cell count) and spread (surface area) on fibrinogen (Figure 15, 16 and 17 B). For example, treatment of platelets with 10µM cucurbitacin B caused an over 50% decrease in the number of platelets adhered to fibrinogen (259 +/-66.97) compared to vehicle treated platelets (549+/-153). 1 µM caused a 40% reduction with (333 +/- 46.33) adhered onto fibrinogen. Even the lowest concentration of cucurbitacin B tested, 0.1µM was capable of caused a 20% reduction in adhered platelets (542+/ - 111.0) compared to the vehicle (Figure 15A). Similarly, cucurbitacin's E (Figure 16 A) and I (Figure 17 A) show similar results with an over 60% reduction in platelets adhering to fibrinogen at 10µM, cucurbitacin E (133+/-84.21) compared to the vehicle treated platelets controls (919 +/-256.0) or 10µM cucurbitacin I (125+/- 59.65) compared to the vehicle control (773 +/- 256.6). Representative images of each concentration of Cucurbitacins visualises this inhibition of platelet adhesion to fibrinogen as it shows the decrease in the number of platelets adhered (Figure 15, 16 and 17 C). This indicates treatment with Cucurbitacins causes a dose dependent inhibition of platelet adhesion to fibrinogen, indicating a potential inhibition of integrin αIIβ3 signalling**.**

In addition to platelet adhesion to fibrinogen, the effect of cucurbitacins on the ability of platelets to undergo cytoskeletal changes and platelet spreading, a process driven by integrin αIIbβ3 was also determined. Total surface area covered by platelets was calculated and determined by correcting for number of platelets adhered. Treatment of platelets with either of the cucurbitacins B, E or I resulted in a decrease in the

surface area coverage on fibrinogen compared to the vehicle treated platelets. The % surface area (total area) covered by 10 µM cucurbitacin B treated platelets on fibrinogen was 2.5 +/- 0.8904 % compared to vehicle treated platelets which was 18.2+/-3.520 % (Figure 15 B). Equally, 10µM cucurbitacin E treated platelets covered 1.7+/- 0.7279 % of surface area on fibrinogen compared to vehicle treated platelets covered 18.5+/- 3.884 % on fibrinogen (Figure 16 B). This was also seen following pre-treatment with cucurbitacin I, as the % area covered by 10µM cucurbitacin I treated platelets was 3.2+/- 0.9740 % compared to the vehicle treated which was 19.9+/-2.465 % total area coverage (Figure 17 B). The representative images of phalloidin stained platelets treated with different concentrations of Cucurbitacins shows a decrease in platelet surface area coverage as concentration of cucurbitacin increases (Figure 15, 16 and 17 D).

This indicates that the Cucurbitacins prevent platelet shape change and spreading via inhibition of integrin outside in signalling or via alteration of platelet cytoskeletal rearrangements, which are essential for platelet spreading, including filopodia and lamellipodia formation.

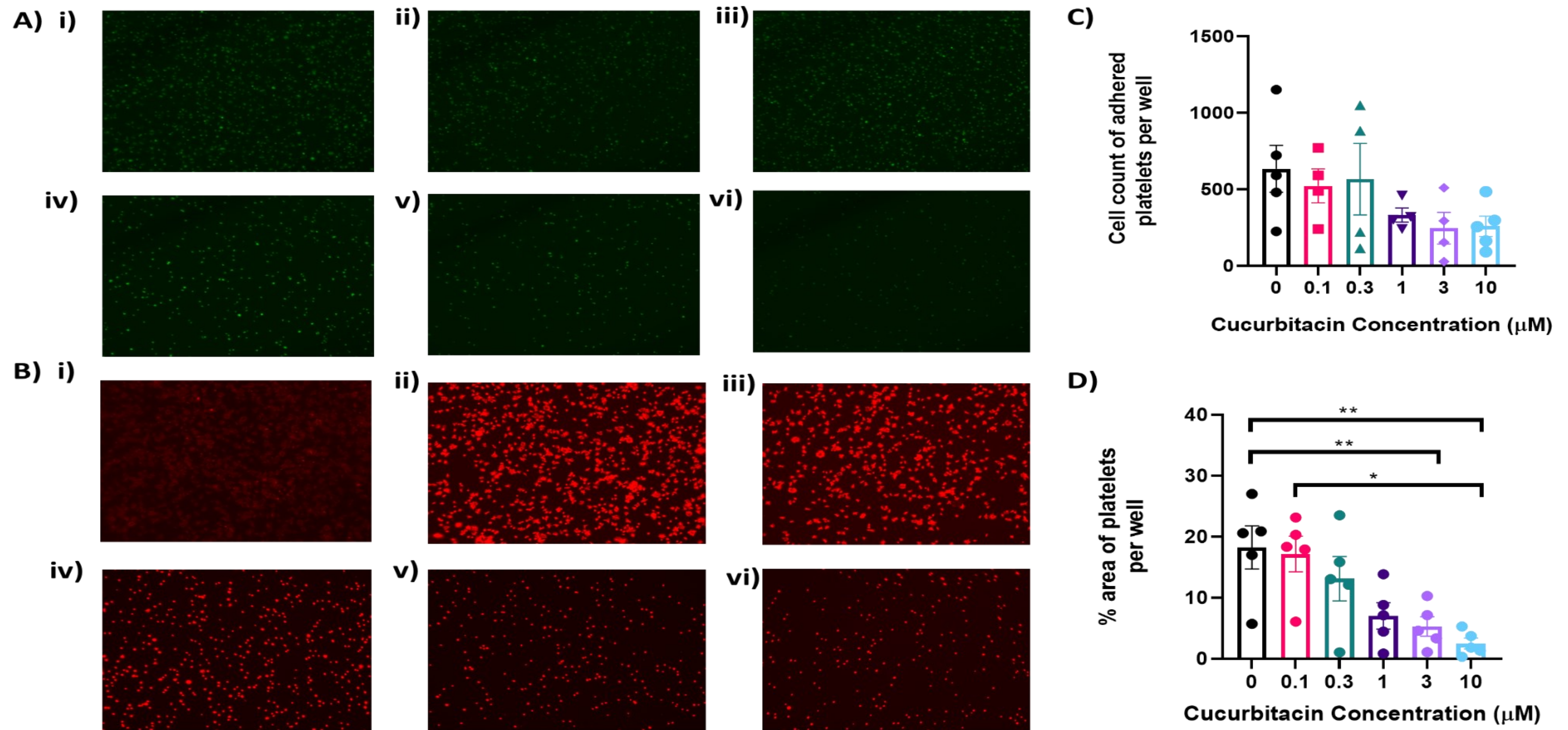

Figure 15. Cucurbitacin B inhibits platelet adhesion and spreading. *Human washed platelets treated i) tyrodes buffer vehicle control, or with different concentrations of cucurbitacin B, ii) 0.1 µM, iii) 0.3µM, iv) 1 µM, v) 3 µM, vi) 10µM, and left to adhere and spread on Fibrinogen 100 µg/mL or BSA coated wells of a 96 well plate at room temperature for 1 hour. Adhered platelets were fixed and labelled with DiOC6 (A) or rhodamine conjugated phalloidin (B) and fluorescence images of each well were captured with 20x objective lens with CELENA S Digital Imaging System and analysed by Image J software. Manual correction performed to ensure all individual platelets counted and prevent counting of overlapping platelets as one. A and B) Representative images of DiOC6 (A) or rhodamine-phalloidin (B) stained platelets adhered on fibrinogen. Data expressed as C) Corrected number of platelets adhered on fibrinogen (without non-specific binding of BSA) D) Total area covered by platelets per image Data expressed as mean + S.E.M for N≤5. * followed with bar indicates $p<0.05$ in comparison to other concentrations. Where normalised data shown, statistics were performed prior to normalisation. Data was analysed using one-way Anova*

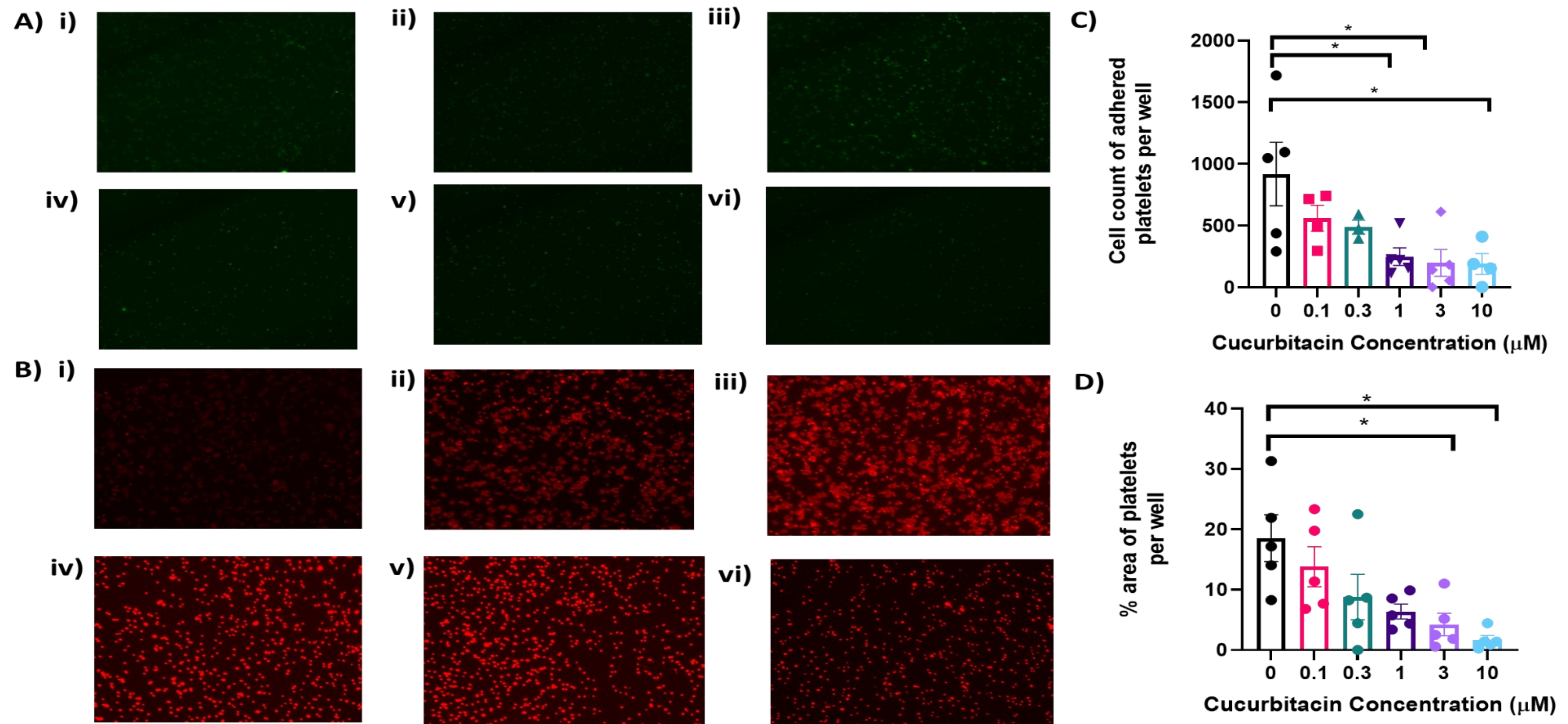

Figure 16. Cucurbitacin E inhibits platelet adhesion and spreading. *Human washed platelets treated i) tyrodes buffer vehicle control, or with different concentrations of cucurbitacin B, ii) 0.1 µ, iii) 0.3µM, iv) 1 µM, v) 3 µM, vi) 10µM, and left to adhere and spread on Fibrinogen 100 µg/mL or BSA coated wells of a 96 well plate at room temperature for 1 hour. Adhered platelets were fixed and labelled with DiOC6 (A) or rhodamine conjugated phalloidin (B) and fluorescence images of each well were captured with 20x objective lens with CELENA S Digital Imaging System and analysed by Image J software. Manual correction performed to ensure all individual platelets counted and prevent counting of overlapping platelets as one. A and B) Representative images of DiOC6 (A) or rhodamine-phalloidin (B) stained platelets adhered on fibrinogen. Data expressed as C) Corrected number of platelets adhered on fibrinogen (without non-specific binding of BSA) D) Total area covered by platelets per image Data expressed as mean + S.E.M for N≤5. * followed with bar indicates p<0.05 in comparison to other concentrations. Where normalised data shown, statistics were performed prior to normalisation. Data was analysed using one-way Anova*

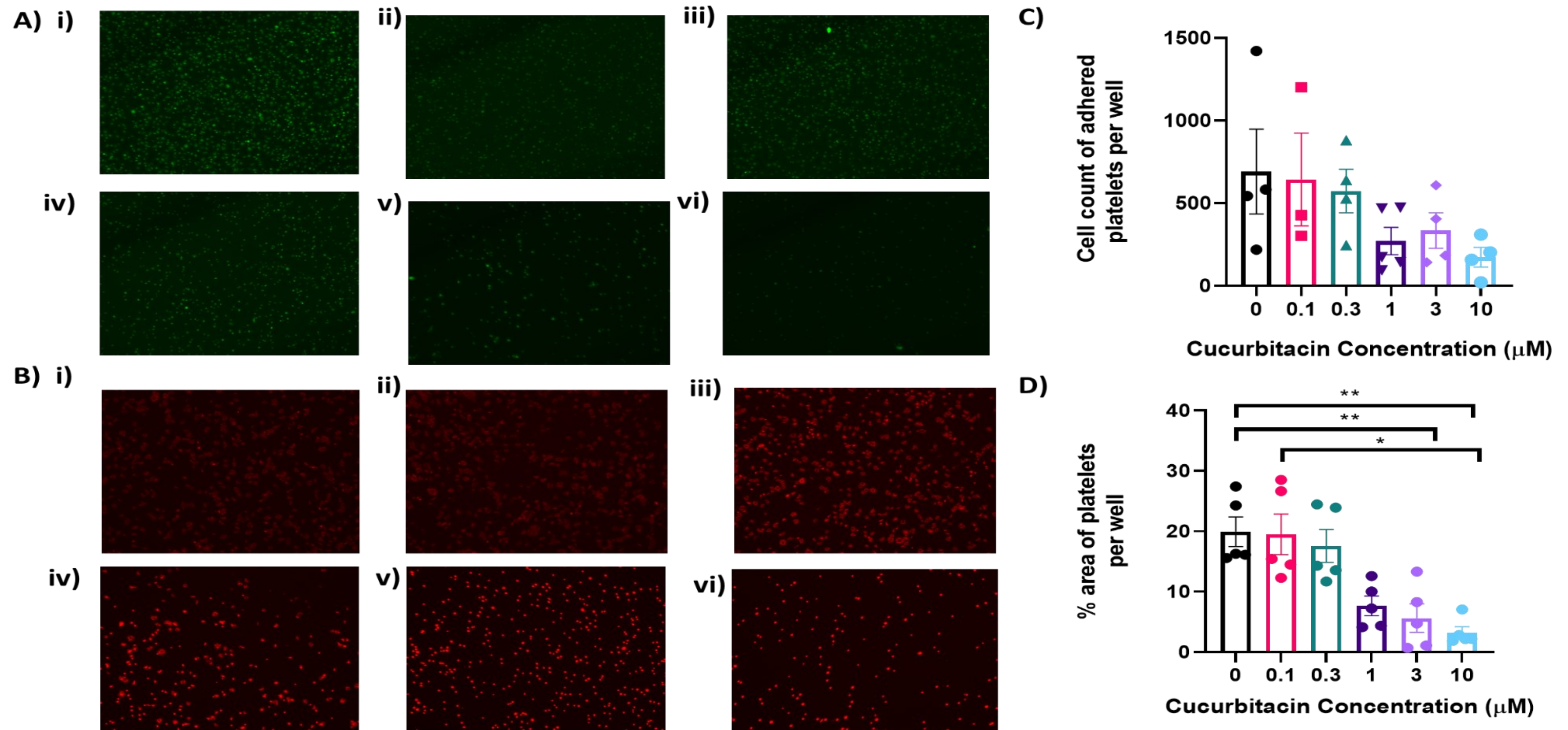

Figure 17. Cucurbitacin I inhibits platelet adhesion and spreading. *Human washed platelets treated i) tyrodes buffer vehicle control, or with different concentrations of cucurbitacin B, ii) 0.1 μM, iii) 0.3μM, iv) 1 μM, v) 3 μM, vi) 10μM, and left to adhere and spread on Fibrinogen 100 μg/mL or BSA coated wells of a 96 well plate at room temperature for 1 hour. Adhered platelets were fixed and labelled with DiOC6 (A) or rhodamine conjugated phalloidin (B) and fluorescence images of each well were captured with 20x objective lens with CELENA S Digital Imaging System and analysed by Image J software. Manual correction performed to ensure all individual platelets counted and prevent counting of overlapping platelets as one. A and B) Representative images of DiOC6 (A) or rhodamine-phalloidin (B) stained platelets adhered on fibrinogen. Data expressed as C) Corrected number of platelets adhered on fibrinogen (without non-specific binding of BSA) D) Total area covered by platelets per image Data expressed as mean + S.E.M for N≤5. * followed with bar indicates p<0.05 in comparison to other concentrations. Where normalised data shown, statistics were performed prior to normalisation. Data was analysed using one-way Anova.*

4.5 Cucurbitacins alter integrin αIIβ3 activation

Taking into consideration observations made in the earlier adhesion and thrombus formation assays, it is clear that cucurbitacins alter and inhibit adhesion of platelets on fibrinogen and thrombus formation. The main mediator for adhesion and aggregation is the integrin αIIβ3 thus; we looked at the levels of fibrinogen binding to platelets via integrin αIIβ3 following stimulation with ADP (10 µM) using flow cytometry. Platelet rich plasma diluted with 1/10 tyrodes buffer was treated with increasing concentrations of cucurbitacins (0.1, 1, 10 µM) and 0.1% DMSO/tyrodes as a vehicle control and stimulated with ADP (10 µM). We identified that as the concentration of cucurbitacins increases, there was a decrease in fibrinogen binding to platelets indicating a decrease in integrin αIIβ3 activation as fibrinogen (Figure 18). The median fluorescence intensity (M.F.I) for cucurbitacin B 10 µM was ~2.968 +/- 1.4 A.U compared to the vehicle which was ~9.016 +/- 1.8 A.U. Cucurbitacin E shows a similar trend in that the M.F.I for 10 µM of cucurbitacin E was ~ 2.9 +/- 1.5 A.U compared to vehicle of ~7.768 and 10 µM cucurbitacin I measured ~3.5 +/- 1.4 AU compared to the vehicle ~8.6 A.U. This supports the idea that cucurbitacins inhibits platelet adhesion and platelet aggregation due to a decrease in the activation of integrin αIIβ3.

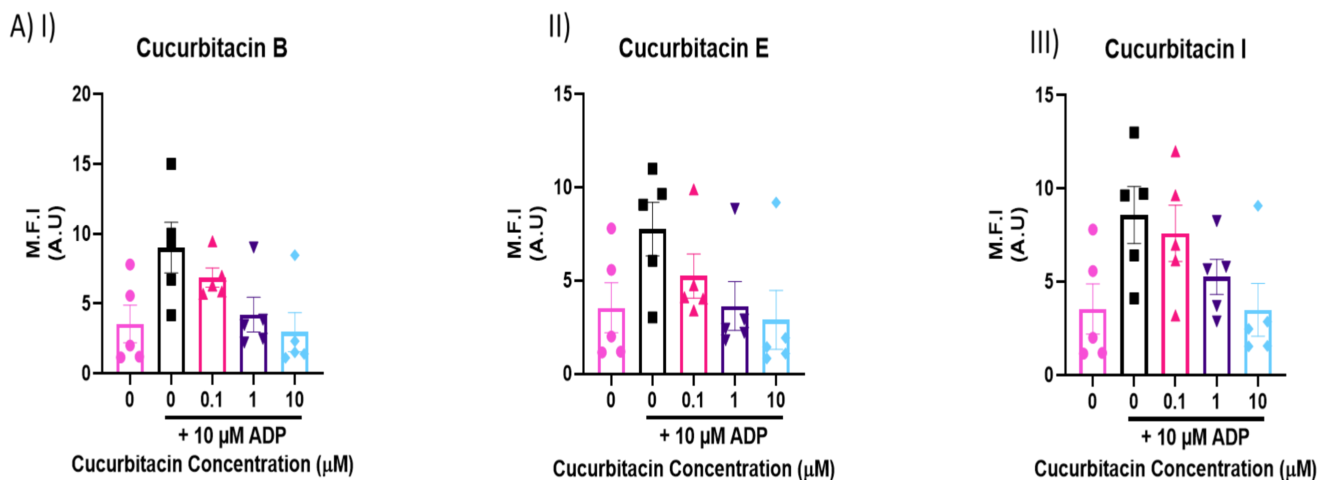

Figure 18: Cucurbitacins alter integrin αIIβ3 activation. *Platelet rich plasma diluted in 1/10 tyrodes were treated with increasing concentrations of i) cucurbitacin B, ii) cucurbitacin E or iii) cucurbitacin I (0.1, 1, 10µM) or vehicle control (0.1% DMSO/tyrodes). Samples were stimulated with ADP (10µM) and incubated with anti-fibrinogen antibody. One sample was untreated and unstimulated. MACSQuant (Miltenyi Biotec MACSQuant 16) flow cytometer and analysed using FlowJo software to determine median fluorescence intensity. A) Median fluorescence intensity (A.U). Data expressed as mean + S.E.M for N≤5. * indicates p<0.05 in comparison to vehicle controls, where normalised data shown, statistics were performed prior to normalisation. Data was analysed using Kruskall-wallis (parametric and non-parametric test.*

4.6 Cucurbitacin treatment increases actin polymerisation in platelets

In addition to alteration of integrin αIIbβ3 function, another possible reason for the observed inhibition of shape change could be changes to actin cytoskeleton dynamics, which are mediated by integrin activation but have also been shown to regulate integrin function. Following activation, platelets undergo shape change driven by rearrangements of the cytoskeleton. We observed a decrease in platelet spreading (surface area covered) following treatment with increasing cucurbitacin B, E or I (0, 0.1, 0.3, 1, 3, 10 µM) compared to vehicle treated control indicating that cucurbitacin also disrupts platelet shape change and cytoskeleton rearrangement (Figure 15, 16 and 17 B). Published studies have identified that some cucurbitacins inhibits cell motility by disrupting actin dynamics, and found that treatment with cucurbitacins results in accumulation of actin filaments in Madin-Darby canine kidney (MDCK) and B16-F1 mouse melanoma cell types (Knecht et al., 2010).

Washed platelets were treated with increasing concentrations of cucurbitacins (0.1, 1, 10 µM) or 0.1% DMSO/tyrodes as vehicle and stained with phalloidin (Alexa-488) and flow cytometry was performed to measure the amount of F- actin (polymerised actin) per platelet. No ADP was added to stimulate the cucurbitacin treated samples, only with untreated platelets as a positive control. Interestingly, we found that actin polymerisation was drastically amplified in cucurbitacins treated platelets compared to the vehicle and lower concentration of the cucurbitacins even in the absence of platelet agonist stimulation.

The median fluorescence intensity (M.F.I) for cucurbitacin B 10 µM was ~8.112 +/- 4 A.U compared to the vehicle which was ~0.659 +/- 0.16 A.U (figure 19 A I). Cucurbitacin E shows a similar trend in that the M.F.I for 10 µM of cucurbitacin E was ~ 5.8 +/- 1.9 A.U compared to vehicle of ~1.4 +/- 0.4 A.U (figure 19 A II) and 10 µM cucurbitacin I measured ~5.9 +/- 1 AU compared to the vehicle ~0.9 +/- 0.2 A.U (figure 19 A III). This increase is indicative of the increase of actin polymerisation, disrupting cytoskeleton dynamics and preventing platelet shape change. Another possible consequence of the actin disruption is alteration of integrin function as platelet shape change is important for αIIβ3 function.

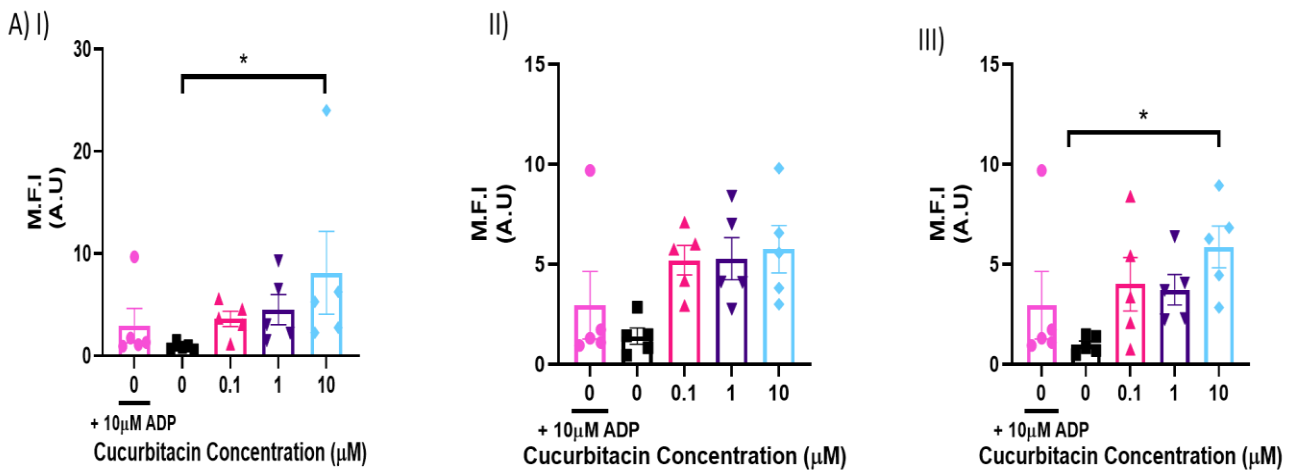

Figure 19: Cucurbitacins increases actin polymerisation. *Human washed platelets prepared at 8x10*^{8}*/mL in tyrodes HEPEs buffer were treated with increasing concentrations of cucurbitacins (0.1, 1, 10 µM) Cucurbitacin i) B, ii) E or iii) I or vehicle (0.1% DMSO). An ADP (10 µM) stimulated platelets sample was included as a positive control but was not used to stimulate cucurbitacin treated platelets. The samples were fixed with 2% paraformaldehyde and centrifuged then resuspended with BD Phosphoflow Perm Buffer III and left to incubate. The samples were washed and resuspended in tyrodes containing conjugated phalloidin (Alexa-488), flow cytometry was used to determine receptor surface expression of antibodies. MACSQuant (Miltenyi Biotec MACSQuant 16) flow cytometer and analysed using FlowJo software to determine median fluorescence intensity. A) Median fluorescence intensity (A.U). Data expressed as mean + S.E.M for N≤5. * followed with bar indicates* $p<0.05$ *in comparison to other concentrations. Where normalised data shown, statistics were performed prior to normalisation. Data was analysed using Kruskall-wallis (parametric and non-parametric test).*

4.7 Cucurbitacins increase myosin light chain phosphorylation.

To confirm whether the cucurbitacins were causing an alteration in actin polymerisation, we monitored the effect of cucurbitacins B, E and I on phosphorylation and activation of myosin light chain, which is essential for platelet shape change and occurs as a result of actin polymerisation. In support of the observations made of increased actin polymerisation in flow cytometry, as shown in Figure 18 an increase in the phosphorylation of myosin light chain at Ser-19 was also observed following treatment with increasing concentrations 0.1, 1 and 10 μM of cucurbitacin B, E or I compared to the vehicle control (tyrodes buffer with 0.1% DMSO) (Figure 20). This increase was not found to be statistically significant, but this could indicate that treatment of platelets with the cucurbitacins could alter cytoskeleton dynamics. This further supports that the cucurbitacins increases actin polymerisation and its components, disrupting cytoskeleton dynamics, platelet shape change, integrin function, platelet aggregation and stable thrombus formation.

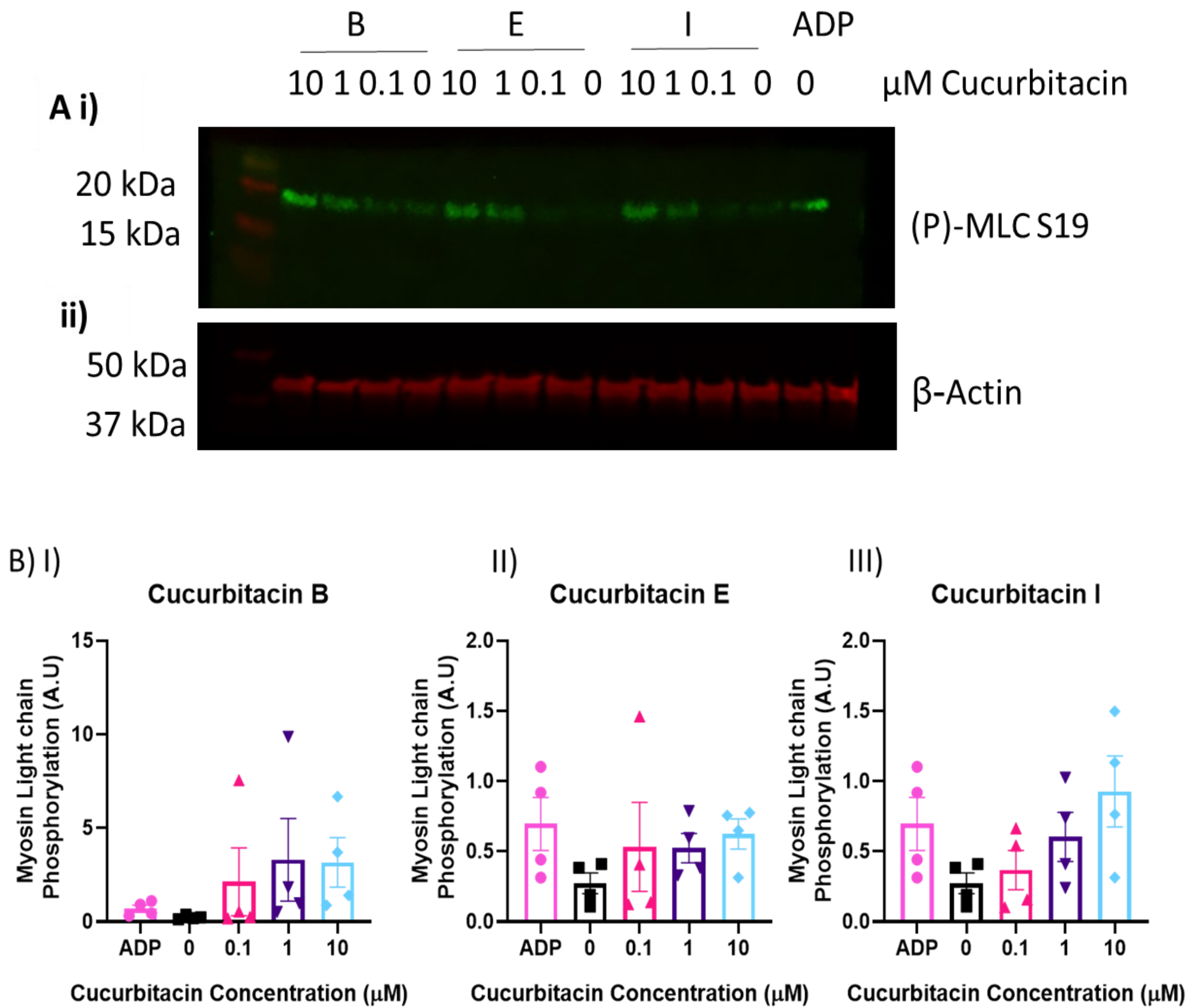

Figure 20: Cucurbitacins increase myosin light chain phosphorylation. *Human washed platelets treated with different concentrations of B) i) cucurbitacin B, ii) cucurbitacin E or iii) cucurbitacin I (0.1-10 µM) or, tyrodes buffer with 0.1% DMSO as vehicle control and left for incubation for 10 minutes, platelets stimulated with ADP (10 uM) were included as a positive control. The platelets were lysed with laemelli buffer and heated to 90°C for 5 minutes. Lysed platelets were run on pre-cast gradient 4-20% Tris-Bis gels, transferred onto Polyvinylidene difluoride (PVDF) membrane and probed overnight at 4°C using primary antibody Phospho-Myosin Light Chain 2 (Ser19). Anti-beta Actin antibody was used as a loading control. The proteins were detected using immunofluorescent secondary antibodies Goat anti-Rabbit IgG Secondary Antibody and Goat anti-Mouse IgG Secondary Antibody at 1;10000 for one hour and imaged using a Li-cor Odyssey Fc. Band intensity quantified using image J software and actin intensity was used to correct for equal loading. A) i) Representative blot for actin load in control shown. A) ii) Representative blot shown for Phospho-Myosin Light chain. B) Quantified Myosin light chain phosphorylation corrected for loading. The results are expressed as mean + S.E.M for N ≤ 4. Where normalised data shown, statistics were performed prior to normalisation. Data was analysed using one-way Anova.*

5 Discussion

Cardiovascular disease is the leading cause of death worldwide with several risk factors, including diet. MI and stroke occur due to the formation of a thrombus following platelet activation. Anti-platelet drugs and therapies are key treatments to aid the prevention and treatment of thrombosis. Many anti-platelet drugs developed work on altering different pathways of platelet function to stop the platelets ability to form a thrombus. Aspirin is one of the most commonly used anti-platelet drugs to treat thrombosis and works by irreversibly inactivating COX-1, preventing conversion of arachidonic acid to TXA2. This stops the platelet from being able to produce TXA2 a key secondary mediator of platelet activation (Awtry and Loscalzo, 2000). Clopidogrel is another anti-platelet drug that works differently to aspirin as it works by irreversibly inhibiting the ADP receptor P2Y12, blocking ADP from binding. Both of these drugs have shown signs of resistance in certain patients such as Gum et al (Gum et al., 2001) who found that among patients with cardiac arrest 5-9% were aspirin resistant and 23% were aspirin semi responders and Heptinstall et al (Heptinstall et al., 2004) who found that out of 12 patients treated with clopidogrel 4 showed resistance. The drugs are also associated with side effects, including gastric ulcers, renal failure, gastrointestinal problems, excessive bleeding and even cardiac events (Barrett et al., 2008). For this reason, alternative strategies are required to overcome these disadvantages of current anti-platelet therapies including drugs that treat alternative platelet activator pathways.

Targeting integrin function and integrin signalling has allowed for successful development of anti-platelet drugs against thrombosis as these agents work by altering integrin function, a crucial step for platelet activation and function. Integrins are important mediators for platelet signalling pathways including both platelet adhesion and aggregation (Nieswandt et al., 2009). The most widely expressed integrin, αIIbβ3, mediates platelet adhesion through the binding to its ligand fibrinogen via a process called outside-in signalling following 'inside-out' activation. This allows for platelets to aggregate as these integrin-fibrinogen-integrin interactions act as a bridge between platelets, thus allowing for platelet aggregation to occur resulting in formation of a thrombus (Estevez et al., 2015). However, despite their successes integrin targeted anti- platelet drugs developed to date to treat thrombotic

events provide limited protection as many result in excessive bleeding (Estevez et al., 2015). Integrin antagonists such as abciximab, eptifibatide and tirofiban have proven beneficial to preventing thrombosis but are associated with reduced efficacy and severe bleeding risk and therefore failed at clinical trial stages (Chew et al., 2001). Therefore, this means that new strategies for targeting integrin function could be vital in creating a successful efficacious anti-platelet therapy.

Integrin driven platelet shape change is important for the activation and function of platelets. Resting platelets are discoid in shape with a small surface area, once activated the platelet cytoskeleton under goes considerable rearrangement and increases the platelet surface area by extending filipodia and forming lamellipodia, this supports platelet adhesion and aggregation (Aslan et al., 2012). Cytoskeleton rearrangements and the formation of filipodia and lamellipodia occurs due to actin turnover and polymerisation. Targeting the cytoskeleton rearrangements which controls stable platelet adhesion and shape change could be a vital pathway to inhibit platelet function in thrombosis. Subsequently, this can result in the inhibition of platelet function as platelets are not able to undergo shape change.

There is increasing evidence of a link between a healthy diet and improvement in cardiovascular risk. Studies that suggest that a healthy diet has a positive impact on cardiovascular health include the Mediterranean diet. The diet is based on increased consumption of fruits, whole grains, nuts, green vegetables, extra virgin olive oil with low intakes in red meat and sweets (de Lorgeril et al., 1999). The study found that the diet lowered coronary heart disease. The suggestion that this healthy diet results in lowered cardiovascular risk prompted other studies to investigate individual foods in detail to see whether they present any properties that specifically lower cardiovascular risk. Many studies have researched different dietary compounds and have found them to have anti-platelet properties that work on different platelet pathways and processes (Vilahur and Badimon, 2013). Research suggests that polyphenols may reduce the risk of coronary heart disease (Vita, 2005). Observations into berries that have a high concentration of phenolic compound found that they have cardioprotective properties. They inhibited platelet aggregation with agonist ADP, lowered LDL cholesterol concentrations and decreased the total cholesterol (Erlund et al., 2008).

Our aims were to investigate cucurbitacins, which are tetracyclic triterpenoid compounds demonstrated to have anti-inflammatory (Jayaprakasam et al., 2003), anti-tumour (Kee and Hongtao, 2008) (Blaskovich et al., 2003), anti-atherosclerotic (Bernard and Olayinka, 2010) and anti-diabetic (Aharoni et al., 2005) activity. They are found in pumpkin, squash and cucumbers. Many derivates of the compound exist but we focussed on the most widely characterised cucurbitacin B, E and I derivatives and analysed the anti-platelet properties of these compounds. These compounds have been found to inhibit integrin function in cancer cell lines and alter actin polymerisation in other cell types which could be potential targets for platelet inhibition if similar activity is found in platelet treated cucurbitacins.

We found that each of the three cucurbitacin derivatives, Cucurbitacin B, E and I inhibited ADP and collagen induced platelet aggregation in a concentration dependant manner with increased levels of inhibition achieved at 10µM of cucurbitacin B, E and I (Figures 8, 9 10 and 11). Interestingly increasing the concentrations of agonists were not able to overcome the inhibitory effects of the different cucurbitacins. As cucurbitacins showed a possible inhibition of integrin function in previous studies, this evidence suggests that integrin function may be inhibited in platelets due to the importance of integrin in platelet aggregation. (Touihri-Barakati et al., 2017) (Gupta and Srivastava, 2014). A study found that cucurbitacin B inhibited the growth of breast cancer cell lines MDA-MB-231 and 4T-1 by inhibition of HER-2 (oncogenic receptor) - integrin signalling. They confirmed the interaction between HER-2 and ITGA6 and demonstrated that cucurbitacin B inhibited this interaction.

Furthermore, we identified that treatment with cucurbitacin B, E or I caused an alteration in thrombus formation compared to vehicle treated controls (Figure 14). Thrombi appeared less stable and did not appear to consolidate and form tight interactions. As aggregation was inhibited, we wanted to see whether this resulted in a decrease or inhibition of thrombus formation as integrin is vital for arterial thrombosis (Fuster et al., 1992). Comparison of fluorescence intensity and surface area of following treatment with cucurbitacin B, E or I indicated either an increase in or no alteration in thrombus formation compared to vehicle treated control. However, visually the thrombi formed were markedly different. Analysis of the

thrombus stability index identified that the thrombi formed from cucurbitacin treated platelets were unstable and loose suggesting that cucurbitacins form unstable thrombus that do not have the ability to be constant and will loosen, signifying that they can dissociate from the extracellular matrix or platelet aggregate as time goes on. Collagen is important for the adhesion of platelets to the vessel wall as they bind to integrin α2 β1 thus choosing it as an agonist to observe thrombus formation (Jackson and Schoenwaelder, 2003). Again, integrin function is important for thrombus formation as it is a consequence of the platelet aggregation. However, the adhesion to collagen seems unaffected as the results show the fluorescence intensity was similar, but the thrombi appearing unstable which suggest that the platelets are not able to aggregate with each other. One could propose that the integrin αIIbβ3 is specifically affected.

To determine whether this inhibition of stable thrombus formation was due to a specific cucurbitacin mediated inhibition of integrin αIIbβ3, adhesion and spreading assays on fibrinogen were performed. We identified a decrease in the number of cucurbitacin treated platelets that adhered to fibrinogen compared to vehicle treated control platelets (Figure 15, 16 and 17). Again, the highest concentration of cucurbitacins caused more of an inhibition of platelets to adhere to fibrinogen. In support of these findings one study found that cucurbitacin B was able to inhibit integrin α5β1 mediated cell adhesion, proliferation and migration of human glioblastoma U87 cell line. They found that the cucurbitacins affected the adhesion of cells to fibronectin. This suggested that the cucurbitacins affected cell adhesion by inhibition of the integrin to fibronectin (Touihri-Barakati et al., 2017). This could be a possible explanation as to why the platelets were not able to adhere via the integrin αIIbβ3 to fibrinogen as they previously show that ligand-integrin complexes were inhibited. To further understand the effect of cucurbitacins on the integrin αIIbβ3 to fibrinogen, we observed the activity of the integrin by measuring the fibrinogen binding to platelets in cucurbitacin treated platelets and vehicle treated platelets. Fibrinogen bound platelets decreased as the concentration of cucurbitacins increased (figure 18) which again highlights that the cucurbitacins inhibits the activity of the integrins in platelet function.

In addition to an inhibition in integrin function resulting in the lack of ability for the platelets to aggregate and form a stable thrombus, inhibition of integrin function would also impact the ability of platelets to be able to adhere and spread, and subsequently form the stable thrombus. We observed an inhibition of platelet adhesion and spreading following treatment with Cucurbitacins B, E or I, indicating irregularity in the ability of the actin cytoskeleton to undergo rearrangement, possibly via alteration of actin cytoskeleton dynamics. It is also known that αIIbβ3 outside-in signalling leads to rearrangement of cytoskeleton that are important for platelet spreading. Outside-in signalling via the integrin initiates the rearrangement of actin cytoskeleton in platelets is regulated by actin polymerisation by increasing rigidity of cytoskeleton to allow platelet shape change to occur and the actin depolarisation which allows platelet to aggregate and spread (Bearer et al., 2002).

Following treatment with cucurbitacins, we observed that cucurbitacin treated platelets lost the ability to spread compared to the vehicle control treated platelets. We observed a decrease in surface area coverage on fibrinogen and the average size of platelets following cucurbitacin treatment. This indicates that treatment with cucurbitacins arrested the cytoskeleton rearrangement as they were no longer able to spread. This points towards the irregularity in actin polymerisation as the platelets were not able to form the extensions - lamellipodia and filipodia which derive from actin polymerisation required for platelet aggregation and adhesion (Bearer et al., 2002). This correlates with the other observations made whereby the platelets were not able to aggregate or adhere to fibrinogen, both processes require the cytoskeletal rearrangements that lead to platelet shape change.

To see whether this inhibition of platelet spreading was due to alterations in actin turnover, we measured actin polymerisation in cucurbitacin treated platelets using phalloidin, which binds to polymerised filamentous F-actin and found that it was drastically increased in comparison to the vehicle (Figure 19). This was not expected as cucurbitacins mechanism of action thus far was an inhibitory effect, so we expected it to inhibit actin polymerisation rather than increase.

Cytoskeletal actin rearrangements are regulated by myosin light chain phosphorylation. The phosphorylation of myosin light chain is essential for platelet shape change. They facilitate the binding of myosin to actin-filaments which result in

the contraction of the platelet and regulation of cytoskeleton rearrangements (Johnson et al., 2007). Activated platelets contract by making the clot rigid allowing for the clot shape to remain uniform. We found that similar to the observations made with actin polymerisation, treatment with cucurbitacins caused an increase in myosin light chain phosphorylation compared to vehicle treated controls (Figure 20).

A balance between the monomeric (globular) G-actin and (filament) F-actin forms of actin maintain the actin cytoskeleton in platelets and platelet shape. Investigation of the effect of cucurbitacin treatment on the actin cytoskeleton of other cell types has shown contrasting effects. Some studies have found that cucurbitacins caused polymerisation of actin. Observations in endothelial cells indicate that cucurbitacin E causes unwarranted depolymerisation of F-actin such as (M. D. Duncan and Duncan, 1997) and the loss of stress fibres. A study by Graness (Graness et al., 2006) et al , reported cucurbitacin I mediated disassembly of F- actin fibres that reorganised rather into patches of F-actin in fibroblasts. These observations indicate cucurbitacins have similar effects to Cytochalasin D and other inhibitors of actin polymerisation. In contrast, there are several other studies suggest treatment with cucurbitacin's cause an increase in actin polymerisation which are more in line with the results described here. One study for example demonstrated that treatment with cucurbitacin E caused an increase in F-actin polymerised filaments with a decrease in G-actin observed in prostate carcinoma cells resulting in an accumulation of actin and morphological changes to the cells (K. L. Duncan et al., 1996). Similarly, another study found that cucurbitacin B increased F-actin polymerisation and actin aggregates in myeloid leukaemia cell lines (Haritunians et al., 2008). These results are similar to the findings observed in cells following treatment with jasplakinolide, an actin-stabilising drug. Treatment with Jasplakinolide has been shown to increase the formation of F-actin aggregates in *Dictyostelium* amoebae which may be due to the inability of the F-actin in the cells to depolymerise at a normal rate (Lee et al., 1998). Interestingly a recent study investigating the effect actin polymerisation on platelet function found that jasplakinolide-treated platelets showed impairment to αIIbβ3 activation, inhibited platelet aggregation and platelet spreading, indicating increased actin polymerisation can negatively regulate integrin αIIbβ3 function, (Bury et al., 2016) which are similar to results we observed following treatment with cucurbitacins B, E and I.

6 Conclusion

In conclusion, cucurbitacin B, E and I elicit anti-platelet activity including inhibition of platelet aggregation, platelet adhesion and stable thrombus formation by disrupting actin polymerisation and turnover thereby inhibiting integrin αIIbβ3 activation (Figure 21). This subsequently prevents the formation of integrin- fibrinogen bridges platelet aggregation and stable thrombus formation. It is unclear how the cucurbitacin's alter actin polymerisation, more research into different aspects of actin dynamic such as the effect of cucurbitacins on the regulators of actin polymerisation and depolymerisation is required to conclude the mode of action of cucurbitacin's in platelet function. Nevertheless, cucurbitacins offer a potentially exciting novel anti-platelet therapeutic agent.

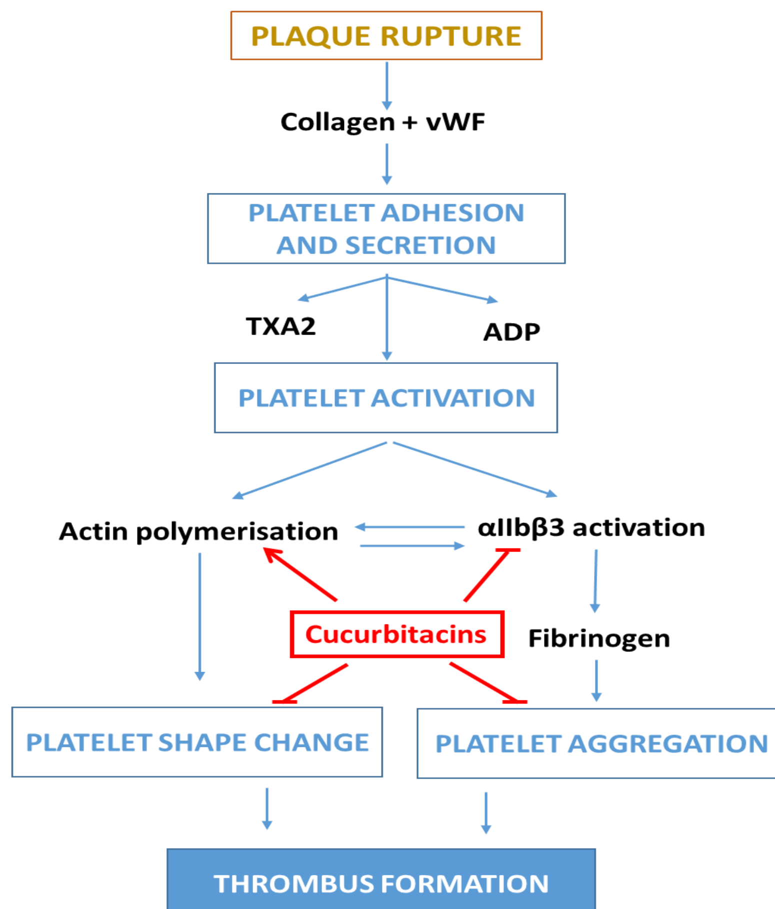

Figure 21: Cucurbitacin mechanism of action in inhibiting platelets. *Following plaque rupture, the platelet adheres via collagen and vWF to vessel wall and activation is enhanced through the secretion of ADP and TXA2 which bind to their receptors on platelet surface. Cucurbitacins inhibit platelet function by inhibiting integrin αIIbβ3 activity preventing platelets from aggregating and undergoing shape change. Cucurbitacins also disrupt actin polymerisation and platelet shape change and stable adhesion and thrombus formation.*

7 References

Aharoni, A., Jongsma, M. A. and Bouwmeester, H. J. (2005) 'Volatile science? Metabolic engineering of terpenoids in plants.' *Trends in plant science*, 10(12) pp. 594-602.

Alghasham, A. A. (2013) 'Cucurbitacins–a promising target for cancer therapy.' *International journal of health sciences*, 7(1) p. 77.

Algra, A. M. (2013) 'Towards a global brief on aspirin.' *Lancet (London, England)*, 381(9875) pp. 1344-1345.

Anand, S. S., Hawkes, C., De Souza, R. J., Mente, A., Dehghan, M., Nugent, R., Zulyniak, M. A., Weis, T., et al. (2015) 'Food consumption and its impact on cardiovascular disease: importance of solutions focused on the globalized food system: a report from the workshop convened by the World Heart Federation.' *Journal of the American College of Cardiology*, 66(14) pp. 1590-1614.

Anfossi, G., Russo, I., Massucco, P., Mattiello, L., Cavalot, F., Balbo, A. and Trovati, M. (2002) 'Adenosine increases human platelet levels of 3′, 5′-cGMP through nitric oxide: Possible role in its antiaggregating effect.' *Thrombosis Research*, 105(1) pp. 71-78.

Antonipillai, J., Rigby, S., Bassler, N., Peter, K. and Bernard, O. (2018) 'Inhibition of platelet function by targeting the platelet cytoskeleton via the LIMK-signaling pathway.' *BioRxiv*, p. 438051.

Aslan, J. E., Itakura, A., Gertz, J. M. and McCarty, O. J. (2012) 'Platelet shape change and spreading.' *In Platelets and Megakaryocytes*. Springer, pp. 91-100.

Awtry, E. H. and Loscalzo, J. (2000) 'Aspirin.' *Circulation*, 101(10) pp. 1206-1218.

Barbaresko, J., Koch, M., Schulze, M. B. and Nöthlings, U. (2013) 'Dietary pattern analysis and biomarkers of low-grade inflammation: a systematic literature review.' *Nutrition reviews*, 71(8) pp. 511-527.

Barrett, N., Holbrook, L., Jones, S., Kaiser, W., Moraes, L., Rana, R., Sage, T., Stanley, R., et al. (2008) 'Future innovations in anti-platelet therapies.' *British journal of pharmacology*, 154(5) pp. 918-939.

Bearer, E., Prakash, J. and Li, Z. (2002) 'Actin dynamics in platelets.' *International review of cytology*, 217 pp. 137-182.

Beckman, C. H. (2000) 'Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants?' *Physiological and molecular plant pathology*, 57(3) pp. 101-110.

Behan, M. and Storey, R. (2004) 'Antiplatelet therapy in cardiovascular disease.' *Postgraduate medical journal*, 80(941) pp. 155-164.

Berger, J. S., Roncaglioni, M. C., Avanzini, F., Pangrazzi, I., Tognoni, G. and Brown, D. L. (2006) 'Aspirin for the primary prevention of cardiovascular events in women and men: a sex-specific meta-analysis of randomized controlled trials.' *Jama*, 295(3) pp. 306-313.

Bernard, S. A. and Olayinka, O. A. (2010) 'Search for a novel antioxidant, anti-inflammatory/analgesic or anti-proliferative drug: Cucurbitacins hold the ace.' *Journal of Medicinal Plants Research*, 4(25) pp. 2821-2826.

Berndt, M. C., Metharom, P. and Andrews, R. K. (2014) 'Primary haemostasis: newer insights.' *Haemophilia*, 20 pp. 15-22.

Blair, P. and Flaumenhaft, R. (2009) 'Platelet α-granules: basic biology and clinical correlates.' *Blood reviews*, 23(4) pp. 177-189.

Blaskovich, M. A., Sun, J., Cantor, A., Turkson, J., Jove, R. and Sebti, S. M. (2003) 'Discovery of JSI-124 (cucurbitacin I), a selective Janus kinase/signal transducer and activator of transcription 3 signaling pathway inhibitor with potent antitumor activity against human and murine cancer cells in mice.' *Cancer research*, 63(6) pp. 1270-1279.

Bledzka, K., Smyth, S. S. and Plow, E. F. (2013) 'Integrin αIIbβ3: from discovery to efficacious therapeutic target.' *Circulation research*, 112(8) pp. 1189-1200.

Body, S. C. (1996) 'Platelet activation and interactions with the microvasculature.' *Journal of cardiovascular pharmacology*, 27 pp. 13-25.

Bury, L., Falcinelli, E., Chiasserini, D., Springer, T. A., Italiano, J. E. and Gresele, P. (2016) 'Cytoskeletal perturbation leads to platelet dysfunction and thrombocytopenia in variant forms of Glanzmann thrombasthenia.' *Haematologica*, 101(1) pp. 46-56.

Cannon, C. P., Husted, S., Harrington, R. A., Scirica, B. M., Emanuelsson, H., Peters, G., Storey, R. F. and Investigators, D.-. (2007) 'Safety, tolerability, and initial efficacy of AZD6140, the first reversible oral adenosine diphosphate receptor antagonist, compared with clopidogrel, in patients with non–ST-segment elevation acute coronary syndrome: primary results of the DISPERSE-2 trial.' *Journal of the American College of Cardiology*, 50(19) pp. 1844-1851.

Casas, R., Castro-Barquero, S., Estruch, R. and Sacanella, E. (2018) 'Nutrition and cardiovascular health.' *International journal of molecular sciences*, 19(12) p. 3988.

Chen, J. C., Chiu, M. H., Nie, R. L., Cordell, G. A. and Qiu, S. X. (2005) 'Cucurbitacins and cucurbitane glycosides: structures and biological activities.' *Natural product reports*, 22(3) pp. 386-399.

Chew, D. P., Bhatt, D. L., Sapp, S. and Topol, E. J. (2001) 'Increased mortality with oral platelet glycoprotein IIb/IIIa antagonists: a meta-analysis of phase III multicenter randomized trials.' *Circulation*, 103(2) pp. 201-206.

Clark, H. (2013) 'NCDs: a challenge to sustainable human development.' *Lancet (London, England)*, 381(9866) pp. 510-511.

Dakeng, S., Duangmano, S. and Jiratchariyahul, W. (2012) 'U-Pratya, Y.; Bogler, O.; Patmasriwat, P. Inhibition of Wnt signaling by cucurbitacin B in breast cancer cells: Reduction of Wnt-associated proteins and reduced translocation of galectin-3 mediated β-catenin to the nucleus.' *J. Cell Biochem*, 113 pp. 49-60.

de Groot, P. G., Urbanus, R. T. and Roest, M. (2012) 'Platelet interaction with the vessel wall.' *In Antiplatelet Agents*. Springer, pp. 87-110.

de Lorgeril, M., Salen, P., Martin, J.-L., Monjaud, I., Delaye, J. and Mamelle, N. (1999) 'Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study.' *Circulation*, 99(6) pp. 779-785.

Duncan, K. L., Duncan, M. D., Alley, M. C. and Sausville, E. A. (1996) 'Cucurbitacin E-induced disruption of the actin and vimentin cytoskeleton in prostate carcinoma cells.' *Biochemical pharmacology*, 52(10) pp. 1553-1560.

Duncan, M. D. and Duncan, K. L. (1997) 'Cucurbitacin E targets proliferating endothelia.' *Journal of surgical research*, 69(1) pp. 55-60.

Erlund, I., Koli, R., Alfthan, G., Marniemi, J., Puukka, P., Mustonen, P., Mattila, P. and Jula, A. (2008) 'Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol.' *The American journal of clinical nutrition*, 87(2) pp. 323-331.

Esposito, K., Ciotola, M. and Giugliano, D. (2006) 'Mediterranean diet, endothelial function and vascular inflammatory markers.' *Public health nutrition*, 9(8A) pp. 1073-1076.

Estevez, B. and Du, X. (2017) 'New concepts and mechanisms of platelet activation signaling.' *Physiology*, 32(2) pp. 162-177.

Estevez, B., Shen, B. and Du, X. (2015) 'Targeting integrin and integrin signaling in treating thrombosis.' *Arteriosclerosis, thrombosis, and vascular biology*, 35(1) pp. 24-29.

Fitch-Tewfik, J. and Flaumenhaft, R. (2013) 'Platelet granule exocytosis: a comparison with chromaffin cells.' *Frontiers in endocrinology*, 4 p. 77.

Fox, J. E. (1993) 'Regulation of platelet function by the cytoskeleton.' *In Mechanisms of platelet activation and control*. Springer, pp. 175-185.

French, D. L. and Seligsohn, U. (2000) 'Platelet glycoprotein IIb/IIIa receptors and Glanzmann's thrombasthenia.' *Arteriosclerosis, thrombosis, and vascular biology*, 20(3) pp. 607-610.

Fuentes, E., Caballero, J., Alarcon, M., Rojas, A. and Palomo, I. (2014) 'Chlorogenic acid inhibits human platelet activation and thrombus formation.' *PloS one*, 9(3) p. e90699.

Fuentes, E., Castro, R., Astudillo, L., Carrasco, G., Alarcón, M., Gutiérrez, M. and Palomo, I. (2012) 'Bioassay-guided isolation and HPLC determination of bioactive compound that relate to the antiplatelet activity (adhesion, secretion, and aggregation) from Solanum lycopersicum.' *Evidence-Based Complementary and Alternative Medicine*, 2012

Fuentes Q, E., Fuentes Q, F., Andrés, V., Pello, O. M., de Mora, J. F. and Palomo G, I. (2013) 'Role of platelets as mediators that link inflammation and thrombosis in atherosclerosis.' *Platelets*, 24(4) pp. 255-262.

Fuster, V., Badimon, L., Badimon, J. J. and Chesebro, J. H. (1992) 'The pathogenesis of coronary artery disease and the acute coronary syndromes.' *New England journal of medicine*, 326(5) pp. 310-318.

Gachet, C. and Hechler, B. (2005) *The platelet P2 receptors in thrombosis.* Vol. 31: Copyright© 2005 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New ….

Ghoshal, K. and Bhattacharyya, M. (2014) 'Overview of platelet physiology: its hemostatic and nonhemostatic role in disease pathogenesis.' *The Scientific World Journal*, 2014

Graness, A., Poli, V. and Goppelt-Struebe, M. (2006) 'STAT3-independent inhibition of lysophosphatidic acid-mediated upregulation of connective tissue growth factor (CTGF) by cucurbitacin I.' *Biochemical pharmacology*, 72(1) pp. 32-41.

Gregg, D. and Goldschmidt-Clermont, P. J. (2003) 'Platelets and cardiovascular disease.' *Circulation*, 108(13) pp. e88-e90.

Gry, J. (2006) *Cucurbitacins in plant food.* Nordic Council of Ministers.

Gum, P. A., Kottke-Marchant, K., Poggio, E. D., Gurm, H., Welsh, P. A., Brooks, L., Sapp, S. K. and Topol, E. J. (2001) 'Profile and prevalence of aspirin resistance in patients with cardiovascular disease.' *The American journal of cardiology*, 88(3) pp. 230-235.

Gupta, P. and Srivastava, S. K. (2014) 'Inhibition of HER2-integrin signaling by Cucurbitacin B leads to in vitro and in vivo breast tumor growth suppression.' *Oncotarget*, 5(7) p. 1812.

Guthrie, R. (2011) 'Review and management of side effects associated with antiplatelet therapy for prevention of recurrent cerebrovascular events.' *Advances in therapy*, 28(6) p. 473.

Hamilos, M., Petousis, S. and Parthenakis, F. (2018) 'Interaction between platelets and endothelium: from pathophysiology to new therapeutic options.' *Cardiovascular diagnosis and therapy*, 8(5) p. 568.

Haritunians, T., Gueller, S., Zhang, L., Badr, R., Yin, D., Xing, H., Fung, M. C. and Koeffler, H. P. (2008) 'Cucurbitacin B induces differentiation, cell cycle arrest, and actin cytoskeletal alterations in myeloid leukemia cells.' *Leukemia research*, 32(9) pp. 1366-1373.

Hennekens, C. H., Sechenova, O., Hollar, D. and Serebruany, V. L. (2006) 'Dose of aspirin in the treatment and prevention of cardiovascular disease: current and future directions.' *Journal of cardiovascular pharmacology and therapeutics*, 11(3) pp. 170-176.

Heptinstall, S., Glenn, J. R., May, J. A., Storey, R. F. and Wilcox, R. G. (2004) 'Clopidogrel resistance.' *Catheterization and cardiovascular interventions: official journal of the Society for Cardiac Angiography & Interventions*, 63(3) pp. 397-398.

Hou, Y., Carrim, N., Wang, Y., Gallant, R. and Marshall, A. (2015) 'Ni H. Platelets in hemostasis and thrombosis: novel mechanisms of fibrinogen-independent platelet aggregation and fibronectin-mediated protein wave of hemostasis.' *Journal of biomedical research*, 29(6) p. 437.

Huang, J., Li, X., Shi, X., Zhu, M., Wang, J., Huang, S., Huang, X., Wang, H., et al. (2019) 'Platelet integrin αIIbβ3: signal transduction, regulation, and its therapeutic targeting.' *Journal of hematology & oncology*, 12(1) p. 26.

Induruwa, I., Moroi, M., Bonna, A., Malcor, J. D., Howes, J. M., Warburton, E., Farndale, R. and Jung, S. (2018) 'Platelet collagen receptor Glycoprotein VI-dimer recognizes fibrinogen and fibrin through their D-domains, contributing to platelet adhesion and activation during thrombus formation.' *Journal of Thrombosis and Haemostasis*, 16(2) pp. 389-404.

Italiano Jr, J. and Shivdasani, R. (2003) 'Megakaryocytes and beyond: the birth of platelets.' *Journal of Thrombosis and Haemostasis*, 1(6) pp. 1174-1182.

Jackson, S. P. and Schoenwaelder, S. M. (2003) 'Antiplatelet therapy: in search of the'magic bullet'.' *Nature Reviews Drug Discovery*, 2(10) pp. 775-789.

Jayaprakasam, B., Seeram, N. P. and Nair, M. G. (2003) 'Anticancer and antiinflammatory activities of cucurbitacins from Cucurbitaandreana.' *Cancer letters*, 189(1) pp. 11-16.

Jin, Y.-R., Han, X.-H., Zhang, Y.-H., Lee, J.-J., Lim, Y., Chung, J.-H. and Yun, Y.-P. (2007) 'Antiplatelet activity of hesperetin, a bioflavonoid, is mainly mediated by inhibition of PLC-γ2 phosphorylation and cyclooxygenase-1 activity.' *Atherosclerosis*, 194(1) pp. 144-152.

Jiroušková, M., Jaiswal, J. K. and Coller, B. S. (2007) 'Ligand density dramatically affects integrin αIIbβ3-mediated platelet signaling and spreading.' *Blood, The Journal of the American Society of Hematology*, 109(12) pp. 5260-5269.

Johnson, G. J., Leis, L., Krumwiede, M. and White, J. (2007) 'The critical role of myosin IIA in platelet internal contraction.' *Journal of Thrombosis and Haemostasis*, 5(7) pp. 1516-1529.

Johnston-Cox, H. A., Yang, D. and Ravid, K. (2011) 'Physiological implications of adenosine receptor-mediated platelet aggregation.' *Journal of cellular physiology*, 226(1) pp. 46-51.

Kaushik, U., Aeri, V. and Mir, S. R. (2015) 'Cucurbitacins–an insight into medicinal leads from nature.' *Pharmacognosy reviews*, 9(17) p. 12.

Kee, H. and Hongtao, X. (2008) 'Methods of inducing apoptosis in Cancer treatment by using Cucurbitacins.' *US2008/0207578A1*, 28

Knecht, D. A., LaFleur, R. A., Kahsai, A. W., Argueta, C. E., Beshir, A. B. and Fenteany, G. (2010) 'Cucurbitacin I inhibits cell motility by indirectly interfering with actin dynamics.' *PLoS One*, 5(11) p. e14039.

Koupenova, M., Kehrel, B. E., Corkrey, H. A. and Freedman, J. E. (2017) 'Thrombosis and platelets: an update.' *European heart journal*, 38(11) pp. 785-791.

Lee, E., Shelden, E. A. and Knecht, D. A. (1998) 'Formation of F-actin aggregates in cells treated with actin stabilizing drugs.' *Cell motility and the cytoskeleton*, 39(2) pp. 122-133.

Ley, K., Rivera-Nieves, J., Sandborn, W. J. and Shattil, S. (2016) 'Integrin-based therapeutics: biological basis, clinical use and new drugs.' *Nature reviews Drug discovery*, 15(3) p. 173.

Ludovici, V., Barthelmes, J., Nagele, M. P., Flammer, A. J. and Sudano, I. (2018) 'Polyphenols: anti-platelet nutraceutical?' *Current Pharmaceutical Design*, 24(2) pp. 146-157.

Mathur, A., Correia, S. R., Dmitrieff, S., Gibeaux, R., Kalinina, I., Quidwai, T., Ries, J. and Nedelec, F. (2018) 'Cytoskeleton mechanics determine resting size and activation dynamics of platelets.' *bioRxiv*, p. 413377.

Minors, D. S. (2007) 'Haemostasis, blood platelets and coagulation.' *Anaesthesia & intensive care medicine*, 8(5) pp. 214-216.

Murugappa, S. and Kunapuli, S. P. (2006) 'The role of ADP receptors in platelet function.' *Front Biosci*, 11 pp. 1977-1986.

Musza, L. L., Speight, P., McElhiney, S., Barrow, C. J., Gillum, A. M., Cooper, R. and Killar, L. M. (1994) 'Cucurbitacins, cell adhesion inhibitors from Conobea scoparioides.' *Journal of natural products*, 57(11) pp. 1498-1502.

Ni, H. and Freedman, J. (2003) 'Platelets in hemostasis and thrombosis: role of integrins and their ligands.' *Transfusion and Apheresis Science*, 28(3) pp. 257-264.

Nieswandt, B., Varga-Szabo, D. and Elvers, M. (2009) 'Integrins in platelet activation.' *Journal of Thrombosis and Haemostasis*, 7 pp. 206-209.

Nishijima, K., Kiryu, J., Tsujikawa, A., Miyamoto, K., Honjo, M., Tanihara, H., Nonaka, A., Yamashiro, K., et al. (2004) 'Platelets adhering to the vascular wall mediate postischemic leukocyte–endothelial cell interactions in retinal microcirculation.' *Investigative ophthalmology & visual science*, 45(3) pp. 977-984.

O'Kennedy, N., Raederstorff, D. and Duttaroy, A. K. (2017) 'Fruitflow®: the first European Food Safety Authority-approved natural cardio-protective functional ingredient.' *European journal of nutrition*, 56(2) pp. 461-482.

Periayah, M. H., Halim, A. S. and Saad, A. Z. M. (2017) 'Mechanism action of platelets and crucial blood coagulation pathways in hemostasis.' *International journal of hematology-oncology and stem cell research*, 11(4) p. 319.

Polasek, J. (2005) 'Platelet secretory granules or secretory lysosomes?' *Platelets*, 16(8) pp. 500-501.

Pugh, N., Maddox, B. D., Bihan, D., Taylor, K. A., Mahaut-Smith, M. P. and Farndale, R. W. (2017) 'Differential integrin activity mediated by platelet collagen receptor engagement under flow conditions.' *Thrombosis and haemostasis*, 117(8) p. 1588.

Quinn, M. J. and Fitzgerald, D. J. (1999) 'Ticlopidine and clopidogrel.' *Circulation*, 100(15) pp. 1667-1672.

Rivera, J., Lozano, M. L., Navarro-Núñez, L. and Vicente, V. (2009) 'Platelet receptors and signaling in the dynamics of thrombus formation.' *haematologica*, 94(5) pp. 700-711.

Ruggeri, Z. M. (1997) 'Mechanisms initiating platelet thrombus formation.' *Thrombosis and haemostasis*, 78(1) pp. 611-616.

Sambrano, G. R., Weiss, E. J., Zheng, Y.-W., Huang, W. and Coughlin, S. R. (2001) 'Role of thrombin signalling in platelets in haemostasis and thrombosis.' *Nature*, 413(6851) p. 74.

Sandmann, R. and Köster, S. (2016) 'Topographic cues reveal two distinct spreading mechanisms in blood platelets.' *Scientific reports*, 6 p. 22357.

Shattil, S. J., Kim, C. and Ginsberg, M. H. (2010) 'The final steps of integrin activation: the end game.' *Nature reviews Molecular cell biology*, 11(4) pp. 288-300.

Singh, I., Mok, M., Christensen, A.-M., Turner, A. H. and Hawley, J. A. (2008) 'The effects of polyphenols in olive leaves on platelet function.' *Nutrition, metabolism and cardiovascular diseases*, 18(2) pp. 127-132.

Sonmez, O. and Sonmez, M. (2017) 'Role of platelets in immune system and inflammation.' *Porto Biomedical Journal*, 2(6) pp. 311-314.

Stalker, T. J., Newman, D. K., Ma, P., Wannemacher, K. M. and Brass, L. F. (2012) 'Platelet signaling.' *In Antiplatelet Agents*. Springer, pp. 59-85.

Storey, R. F., Sanderson, H. M., White, A. E., May, J. A., Cameron, K. E. and Heptinstall, S. (2000) 'The central role of the P2T receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity.' *British journal of haematology*, 110(4) pp. 925-934.

Talhaoui, N., Gómez-Caravaca, A., León, L., De La Rosa, R. and Segura-Carretero, A. 'Fernández-Gutiérrez. 2014. Determination of phenolic compounds of 'Sikitita'olive leaves by HPLC-DAD-TOF-MS. Comparison with its parents 'Arbequina'and 'Picual'olive leaves.' *LWT-Food Sci. Technol*, 58(1) pp. 28-34.

Tannin-Spitz, T., Bergman, M. and Grossman, S. (2007) 'Cucurbitacin glucosides: Antioxidant and free-radical scavenging activities.' *Biochemical and Biophysical Research Communications*, 364(1) pp. 181-186.

Thomas, M. R. and Storey, R. F. (2015) 'The role of platelets in inflammation.' *Thrombosis and haemostasis*, 114(09) pp. 449-458.

Touihri-Barakati, I., Kallech-Ziri, O., Ayadi, W., Kovacic, H., Hanchi, B., Hosni, K. and Luis, J. (2017) 'Cucurbitacin B purified from Ecballium elaterium (L.) A. Rich from Tunisia inhibits α5β1 integrin-mediated adhesion, migration, proliferation of human glioblastoma cell line and angiogenesis.' *European journal of pharmacology*, 797 pp. 153-161.

Triggers Mackman, N. (2008) 'Targets and treatments for thrombosis.' *Nature*, 451 pp. 914-918.

Vaiyapuri, S., Ali, M. S., Moraes, L. A., Sage, T., Lewis, K. R., Jones, C. I. and Gibbins, J. M. (2013) 'Tangeretin regulates platelet function through inhibition of phosphoinositide 3-kinase and cyclic nucleotide signaling.' *Arteriosclerosis, thrombosis, and vascular biology*, 33(12) pp. 2740-2749.

Vaiyapuri, S., Roweth, H., Ali, M. S., Unsworth, A. J., Stainer, A. R., Flora, G. D., Crescente, M., Jones, C. I., et al. (2015) 'Pharmacological actions of nobiletin in the modulation of platelet function.' *British journal of pharmacology*, 172(16) pp. 4133-4145.

Valgimigli, M., Bueno, H., Byrne, R. A., Collet, J.-P., Costa, F., Jeppsson, A., Jüni, P., Kastrati, A., et al. (2018) '2017 ESC focused update on dual antiplatelet therapy in coronary artery disease developed in collaboration with EACTS.' *European journal of cardio-thoracic surgery*, 53(1) pp. 34-78.

Vilahur, G. and Badimon, L. (2013) 'Antiplatelet properties of natural products.' *Vascular Pharmacology*, 59(3-4) pp. 67-75.

Vita, J. A. (2005) 'Polyphenols and cardiovascular disease: effects on endothelial and platelet function.' *The American journal of clinical nutrition*, 81(1) pp. 292S-297S.

Warner, T. D., Nylander, S. and Whatling, C. (2011) 'Anti-platelet therapy: cyclo-oxygenase inhibition and the use of aspirin with particular regard to dual anti-platelet therapy.' *British journal of clinical pharmacology*, 72(4) pp. 619-633.

Wijeyeratne, Y. D. and Heptinstall, S. (2011) 'Anti-platelet therapy: ADP receptor antagonists.' *British journal of clinical pharmacology*, 72(4) pp. 647-657.

Yuan, G., Wahlqvist, M. L., He, G., Yang, M. and Li, D. (2006) 'Natural products and anti-inflammatory activity.' *Asia Pacific journal of clinical nutrition*, 15(2)

Yun, S.-H., Sim, E.-H., Goh, R.-Y., Park, J.-I. and Han, J.-Y. (2016) 'Platelet activation: the mechanisms and potential biomarkers.' *BioMed research international*, 2016

Yusuf, S. (2001) 'Clopidogrel in Unstable Angina to Prevent Recurrent Events Trial Investigators. Effect of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation.' *N Engl J Med*, 345 pp. 494-502.

8 Appendix

20/08/2020

Project Title: Investigation of the anti-platelet properties of dietary cucurbitacins

EthOS Reference Number: 14849

Ethical Opinion

Dear Badrija Khalifa,

The above amendment was reviewed by the Science and Engineering Research Ethics and Governance Committee and, on the 20/08/2020, was given a favourable ethical opinion. The approval is in place until 01/01/2021.

Conditions of favourable ethical opinion

Application Documents

Document Type	File Name	Date	Version
Additional Documentation	Participant-Information-Sheet AMENDMENT 13092020	13/08/2020	1
Additional Documentation	COVID-19 questionnaire BK	13/08/2020	1
Additional Documentation	Human Participant Research Guidance Final FSE	18/08/2020	1

The Science and Engineering Research Ethics and Governance Committee favourable ethical opinion is granted with the following conditions

Adherence to Manchester Metropolitan University's Policies and procedures

This ethical approval is conditional on adherence to Manchester Metropolitan University's Policies, Procedures, guidance and Standard Operating procedures. These can be found on the Manchester Metropolitan University Research Ethics and Governance webpages.

Amendments

If you wish to make further changes to this approved application, you will be required to submit an amendment. Please visit the Manchester Metropolitan University Research Ethics and Governance webpages or contact your Faculty research officer for advice around how to do this.

We wish you every success with your project.

Science and Engineering Research Ethics and Governance Committee

Science and Engineering Research Ethics and Governance Committee

For help with this application, please first contact your Faculty Research Officer. Their details can be found here

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8.1 Step by Step guide for platelet adhesion analysis in Image J

Step 1: Open up Image J (Figure 6 A) Identify the image that needs to be analysed by clicking the 'file' drop down menu and 'open'.

Step 2: Create, the threshold for image analyse by clicking the 'image' drop down (figure 6 A) and clicking on 'adjust' which opens another drop down menu and the option of threshold is there. This will open an extension tab called 'threshold' (Figure 6 B).

Step 3: The original image will change to either red and black or white and black. To analyse DIOC6 image, select black and white to enable comparison with the original monochrome image (figure 6 C). To analyse phalloidin image, select red and black to enable comparison with the original monochrome image.

Step 4: The extension tab enables the background to be removed (Figure 6 D). Using the second bar adjust until the individual platelet's threshold mask looks the same size as the platelets in the original image (Figure 6 E).

Step 5: Once the desired image is reached, click 'apply' (figure 6 D), the image should convert to a white background and black platelet (Figure 6 F). Exit the threshold tab.

Step 6: Select 'analyse' from the drop down menu (Figure 6 A) click 'analyse particles' (Figure 6 G). This allows image j to recognise what needs to be analysed by adjusting the size or circularity. Select the outlines option which will identify how many platelets were counted and where they are on the image (figure 6 G).

Step 7: Once finished, click 'ok' option and the results; a summary of the results (Figure 6H) and outlined/highlighted image (Figure 6 H) shows up. The data is expressed as the total number of platelets, the total area of the platelets and average size of the platelets (Figure 6H).

8.2 Step by step guide for Δ Sd/ Δ T (%) analysis in Image J

Nikon ND2 plugin for image J was used to open the ND2 video file (Figure 7A), and the converted into an image sequence (Figure 7B). Frames at 10 second intervals were analysed (Figure 7C) to enable the distinction of movement (instability index) of the platelets.

Step 1: To analyse, the image sequence is converted to an 8 bit to allow for a threshold to be created (Figure 7D). A threshold is selected that catches all of the platelets adhered whilst minimising background. Threshold is kept the same across treatment conditions per experiment. The video is then analysed for % surface area coverage of the thrombus.

Step 2: The image sequence was then further analysed to find the Δ Sd/ Δ T (%) which is a record of the change in surface distribution with time. To do this, the threshold image sequence is duplicated in ImageJ, then one slice at the end of one duplicate is deleted and a slice at the beginning of the other duplicate is deleted (Figure 7E).

Step 3: Both image sequences should have the same number of frames and can now be subtracted by one another using the image calculator tab (Figure 7F). The subtracted image sequence now visualises thrombi/platelets that are moving/unstable, whilst anything 'stable'/stationary is eliminated by the subtraction. Figure 7G is a representative of the image without subtracting the image from one another and with all the thrombi stuck down. Figure 7H is the subtracted video that has been analysed.

Step 4: The Image is then reanalysed using 'Analyse particles' and surface area is representative of Δ Sd/ Δ T (%) which is the δ in surface distribution relative to change in time.