Beneficial effects of eucalyptol (1,8-cineole) on the modulation of platelet reactivity, thrombus formation and platelet-mediated inflammatory responses

A thesis submitted for the degree of Doctor of Philosophy

By Kahdr Alatawi

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DECLARATION

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

Signed: Kahdr Alatawi
Date: December 2020

Abstract

1,8-cineole, a monoterpenoid is a major component of eucalyptus oil and has been proven to possess numerous beneficial effects in humans. Notably, 1,8-cineole is the primary active ingredient of a clinically approved drug, Soledum® which is being mainly used for the maintenance of sinus and respiratory health. Due to its clinically valuable properties, 1,8-cineole has gained significant scientific interest over the recent years specifically to investigate its anti-inflammatory and antioxidant effects. However, the impact of 1,8-cineole on the modulation of platelet activation, thrombosis and haemostasis was not fully established. Therefore, in this study, we demonstrate the effects of 1,8-cineole on agonists-induced platelet activation, thrombus formation under arterial flow conditions and haemostasis in mice. 1,8-cineole selectively inhibits platelet activation stimulated by glycoprotein VI (GPVI) agonists such as collagen and cross-linked collagen-related peptide (CRP-XL), while it displays only a minimal inhibitory effect at higher concentrations (e.g., 100 μM) on thrombin or ADP-induced platelet aggregation. It inhibited inside-out signalling to integrin αIIbβ3 and outside-in signalling triggered by the same integrin as well as granule secretion and intracellular calcium mobilisation in platelets. 1,8-cineole affected thrombus formation on collagen-coated surface under arterial flow conditions and displayed a minimal effect on haemostasis of mice at a lower concentration of 6.25 μM. Notably, 1,8-cineole was found to be non-toxic to platelets up to 50 μM concentration. The potential anti-inflammatory effects of 1,8-cineole was investigated to evaluate its effects in platelet mediate inflammatory response. 1,8-cineole exhibit inhibitory effects in various of inflammatory cytokines secreted from activated platelets as well as cause a reduction in platelet-leukocyte formation. The investigation on the molecular mechanisms through which 1,8-cineole inhibits platelet function suggests that this compound affects

signalling mediated by various molecules such as AKT, LAT, Src and cAMP in platelets. Based on these results, we conclude that 1,8-cineole may act as a potential therapeutic agent to control unwarranted platelet reactivity under various pathophysiological settings.

ACKNOWLEDGEMENTS

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Effect of 1,8-cineole in the modulation of platelet activation, thrombus formation and haemostasis

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ABBREVIATIONS

ACD – acid citrate dextrose
ADP – adenosine diphosphate
ANOVA – analysis of variance
ApoE – apolipoprotein E
ATP – adenosine triphosphate
AU – arbitrary units
BSA – bovine serum albumin
BTK – Bruton's tyrosine kinase
Ca^{2+} – calcium ion
$CaCl_2$ – calcium chloride
CalDAG-GEFI – Ca^{2+} and DAG-regulated guanine nucleotide exchange factor I
CAM-1 – cell adhesion molecule
cAMP – cyclic adenosine monophosphate
CD62P – P-selectin
cGMP – cyclic guanosine monophosphate
CHD – coronary heart disease
CLEC-2 – C-type lectin-like receptor 2
cm_2 – square centimetre
CO_2 – carbon dioxide
COX – cyclooxygenase
CRP-XL – cross-linked collagen-related peptide
Csk – C-terminal Src kinase
CVD – cardiovascular disease
Cy5 – cyanine 5 dye
C57BL/6 – C57 Black 6 mice
DAG - diacylglycerol
DIC – disseminated intravascular coagulation
DioC6 – 3,3'-dihexyloxacarbocyanine iodide
DNA – deoxyribonucleic acid
DTS – dense tubular system
EGTA – ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid
eNOS – endothelial NOS
ERK – extracellular signal-regulated kinase
FcR – Fc receptor
FITC – fluorescein isothiocyanate
FSC – forward scatter
g – g-force
g – grams
Gads – Grb2 related adaptor protein downstream of Shc
GDP – guanosine diphosphate
GEF – guanine nucleotide exchange factor
GP – glycoprotein
GPCR – G-protein coupled receptor
GPO – Gly-Pro-Hyp
GTP – guanosine triphosphate
h – hour(s)
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HUVEC – human umbilical vein endothelial cell

IC_{50} – half maximal inhibitory concentration
Ig – immunoglobulin
iNOS – inducible NOS
IP receptor – prostaglandin receptor
IP_3 – inositol triphosphate
IP3R – inositol triphosphate receptor
ITAM – immunoreceptor tyrosine-based activation motif
KCl – potassium chloride
kg – kilogram
LAT – linker for activation of T cells
LDL – low density lipoprotein
LTA – light transmission aggregometry
M – molar
MAPK – mitogen-activated protein kinase
mg – milligram
Mg^{2+} - magnesium ion
$MgCl_2$ – magnesium chloride
mg/kg – milligrams/kilogram
mL – millilitre
MLC – myosin light chain
mm – millimetre(s)
mmol/L – millimolar
mRNA – messenger RNA
µg – microgram
µL – microliter
µM – micro-molar
NaCl – sodium chloride
$NaHCO_3$ – sodium bicarbonate
Na_2HPO_4 – disodium phosphate
nm – nanometre
nM - nanomolar
NO – nitric oxide
NOS – nitric oxide synthase
OCS – open canalicular system
OH – hydroxyl group
Orai1 – calcium-release activated calcium modulator 1
PAR – protease activated receptor
PBS – phosphate buffered saline
PDGF – platelet derived growth factor
PE – phycoerythrin
PECAM-1 – platelet endothelial cell adhesion molecule 1
PF4 – platelet factor 4
PGI_2 - prostacyclin
PH – pleckstrin homology
PIP2 – phosphatidylinositol 4,5-bisphosphate
PIP3 – phosphatidylinositol 3,4,5-trisphosphate
PI3K – phosphoinositide 3-kinase
PKA – cAMP-dependent protein kinase
PKC – protein kinase C
PKG – cGMP-dependent protein kinase
PLC – phospholipase C
PRP – platelet rich plasma
PVDF – polyvinylidene fluoride
RBC – red blood cell

RPM – revolutions per minute
s – second(s)
s^{-1} – per second (reciprocal second)
SDS – sodium dodecyl sulphate
SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM – standard error of the mean
SFK – Src-family kinase
sGC – soluble guanylyl cyclase
SH2 – Src homology 2
SH3 – Src homology 3
SLP-76 – SH2 domain containing leukocyte protein of 76 kDa
SOCE – store operated calcium entry
SSC – side scatter
STIM1 – stromal interaction molecule 1
Syk – spleen tyrosine kinase
TBS-T – Tris buffered saline with Tween 20
TGF-b - transforming growth factor b
TGX – Tris-glycine eXtended
TP – thromboxane receptor
TRPC6 – transient receptor potential cation channel subfamily C member 6
TXA_2 – thromboxane A_2
U/mL – unit/millilitre
U46619 – 9,11-Dideoxy-11a,9a-epoxymethanoprostaglandin
v/v – volume/volume
vWF – von Willebrand Factor
w/v – weight/volume
Y – tyrosine
^oC – degrees centigrade

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Effect of 1,8-cineole in the modulation of platelet activation, thrombus formation and haemostasis

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Abstract: 1,8-cineole, a monoterpenoid is a major component of eucalyptus oil and has been proven to possess numerous beneficial effects in humans. Notably, 1,8-cineole is the primary active ingredient of a clinically approved drug, Soledum® which is being mainly used for the maintenance of sinus and respiratory health. Due to its clinically valuable properties, 1,8-cineole has gained significant scientific interest over the recent years specifically to investigate its anti-inflammatory and antioxidant effects. However, the impact of 1,8-cineole on the modulation of platelet activation, thrombosis and haemostasis was not fully established. Therefore, in this study, we demonstrate the effects of 1,8-cineole on agonists-induced platelet activation, thrombus formation under arterial flow conditions and haemostasis in mice. 1,8-cineole selectively inhibits platelet activation stimulated by glycoprotein VI (GPVI) agonists such as collagen and cross-linked collagen-related peptide (CRP-XL), while it displays only a minimal inhibitory effect at higher concentrations (e.g. 100 μM) on thrombin or ADP-induced platelet aggregation. It inhibited inside-out signalling to integrin αIIbβ3 and outside-in signalling triggered by the same integrin as well as granule secretion and intracellular calcium mobilisation in platelets. 1,8-cineole affected thrombus formation on collagen-coated surface under arterial flow conditions, and displayed a significant effect on haemostasis of mice at a lower concentration of 6.25 μM. Notably, 1,8-cineole was found to be non-toxic to platelets up to 50 μM concentration. The investigation on the molecular mechanisms through which 1,8-cineole inhibits platelet function suggests that this compound affects signalling mediated by various molecules such as AKT, LAT, Src and cAMP in platelets although we cannot rule out the involvement of other molecules/mechanisms. Based on these results, we conclude that 1,8-cineole may act as a potential therapeutic agent to control unwarranted platelet reactivity under various pathophysiological settings.

Keywords: 1,8-cineole; Platelets; Collagen; Haemostasis; Thrombosis; Platelet reactivity; Signalling.

1. Introduction

Cardiovascular diseases (CVD) specifically thrombotic conditions such as ischemic stroke and heart attacks are the leading cause of death around the modern world. Platelets are small anucleated circulating blood cells and they play an essential role in the maintenance of haemostasis through blood clotting upon vascular injury (Gibbins 2004). However, the inappropriate activation of platelets during pathological conditions such as upon the rupture of atherosclerotic plaque results in the formation blood clots (thrombi) within the vasculature leading to reduced/blocked blood supply to vital organs such as the heart and brain. Therefore, platelets act as a powerful therapeutic target to prevent/treat thrombotic diseases and indeed, anti-platelet drugs such as aspirin and clopidogrel play predominant roles in the treatment, management and prevention of these conditions. However, the currently available anti-platelet drugs present serious side effects such as gastrointestinal toxicity, bleeding and tolerance in some patients. Hence, there is a huge unmet clinical need for developing more effective

and safer anti-platelet drugs in order to prevent and treat thrombotic diseases (Barrett, Holbrook et al. 2008).

Naturally available small molecules derived from plants have remained as a major source of active pharmaceutical ingredients for drug discoveries (Cragg and Newman 2013). While the impact of a broad spectrum of flavonoids on the modulation of platelet activation has been established in detail, the research on the modulatory effects of terpenoids on platelets is limited. Essential oils are volatile plant secondary metabolites containing a complex mixture of monoterpenoids, sesquiterpenoids and other oxygenated hydrocarbons such as aldehydes, ketones, epoxides, alcohols, and esters (Dagli, Dagli et al. 2015, Dhifi, Bellili et al. 2016). They are mainly extracted from flowers, fruits, roots, barks, stem and leaves of the selected plant by various methods. Essential oils play critical roles in many industries such as for the production of cosmetics, perfumes, food materials and pharmaceuticals (Burt 2004, Reis and Jones 2017). Essential oils from plant sources such as lemongrass, black cumin, garlic and cinnamon possess numerous chemicals with therapeutic effects such as anti-inflammatory and analgesic properties (Edris 2007). 1,8-cineole (also known as 'eucalyptol'), a principal constituent of eucalyptus oil which is one such essential oil that has been increasingly used in medical applications due to its pharmacological effects (Sadlon and Lamson 2010, Dhakad, Pandey et al. 2018). Chemically, it is a bicyclic monoterpene (Brown, Garver et al. 2017) and has been reported to possess anti-inflammatory, antimicrobial and antioxidant effects in several pathological conditions including respiratory, cardiovascular and neurodegenerative diseases (Seol and Kim 2016). Indeed, 1,8-cineole is the active ingredient of Soledum®, a clinically approved medication for the treatment of respiratory tract diseases such as chronic sinusitis, bronchitis, chronic obstructive pulmonary disease and asthma (Juergens, Dethlefsen et al. 2003, Seol and Kim 2016). Some *in vitro* studies have shown that 1,8-cineole exhibits anti-inflammatory activities by inhibiting the production of cytokines such as TNF-α and interleukin (IL)-1β in monocytes when stimulated with bacterial lipopolysaccharides (LPS) (Juergens, Engelen et al. 2004). It has been reported that 1,8-cineole is also able to reduce hypersecretion of mucus in airways during asthma (Juergens, Engelen et al. 2004). Furthermore, 1,8-cineole has been found to inhibit the activity of specific pro-inflammatory mediators of NF-κB-signalling by preventing the nuclear translocation of p65 (Greiner, Müller et al. 2013). Intrigued by the broad range of beneficial effects of 1,8-cineole, here, we demonstrate its effects in the modulation of platelet activation, thrombus formation and haemostasis.

2. Results

2.1 1,8-cineole selectively inhibits platelet aggregation induced by GPVI agonists

To determine the impact of 1,8-cineole on the modulation of platelet activation, aggregation assays were performed using various agonists. Human isolated platelets were incubated with a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole (6.25 μM - 100 μM) for five minutes prior to stimulation with different concentrations of GPVI agonists such as collagen and cross-linked collagen-related peptide (CPR-XL). At higher concentrations (50 and 100 μM), 1,8-cineole significantly inhibited (by around 50%) 1 μg/mL collagen-activated platelet aggregation (Figure 1A and 1B). However, when the concentration of collagen was reduced to 0.5 μg/mL, 1,8-cineole displayed significant inhibitory effects at all the concentrations used (Figure 1C and 1D). Notably, 100 μM of 1,8-cineole has completely inhibited collagen-induced platelet aggregation, while the other concentrations have shown around 50-80% inhibition. Since collagen binds to both GPVI and integrin α2β1 receptors to activate platelets, a selective agonist for GPVI, CRP-XL was then used to determine the precise effects of 1,8-cineole via this receptor. Similar to collagen, CRP-XL (1 μg/mL) induced platelet aggregation was largely reduced by higher concentrations of 1,8-cineole (Figure 1E and 1F), although at a lower (0.5 μg/mL) concentration of CRP-XL, all the concentrations of 1,8-cineole inhibited platelet aggregation in a concentration-dependent manner (Figure 1G and 1H).

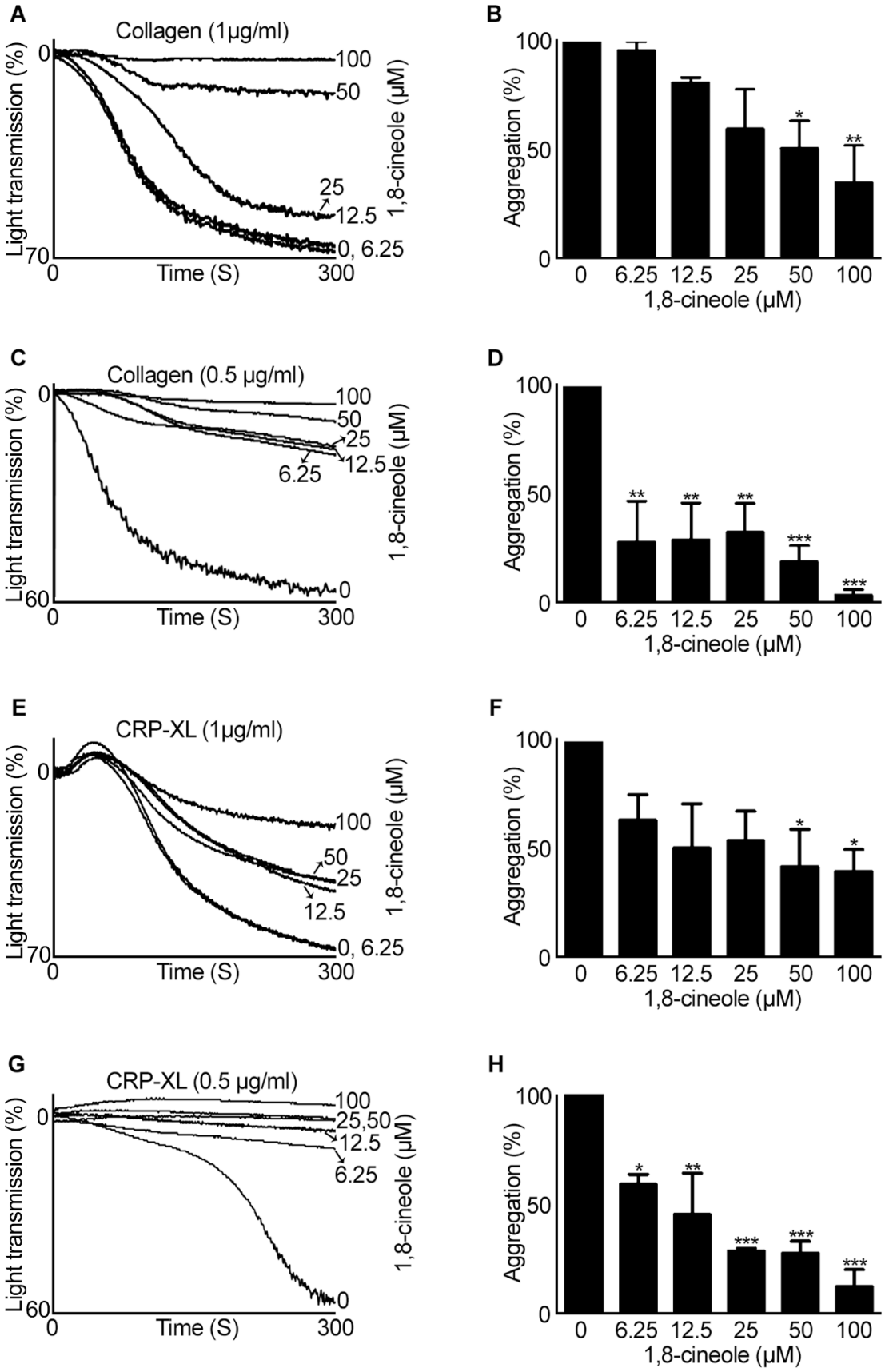

Figure 1. Effect of 1,8-cineole on GPVI agonists-stimulated aggregation in human isolated platelets. A vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole were incubated with human isolated platelets for 5 minutes prior to stimulation of aggregation with 1 µg/mL (**A** and **B**) or 0.5 µg/mL (**C** and **D**) collagen, and 1 µg/mL (**E** and **F**) or 0.5 µg/mL (**G** and **H**) CRP-XL. The level of

aggregation was monitored for 5 minutes in an optical aggregometer. The aggregation traces shown are representative of four separate experiments. The percentage of aggregation for 1,8-cineole-treated samples was calculated by considering the level of aggregation obtained with the vehicle control (0) as 100%. Data represent mean ± SEM (n=4). The *p* values shown (*$p<0.05$, **$p<0.01$ and ***$p<0.001$) are as calculated by one-way ANOVA followed by Bonferroni *post-hoc* test.

Following the determination of the inhibitory effects of 1,8-cineole on isolated platelets, its effects in the modulation of platelet activation was determined in the presence of plasma proteins using human platelet-rich plasma (PRP). Similar to its effects in isolated platelets, 1,8-cineole did not show large inhibitory effects on 1 μg/mL collagen (Figure 2A and 2B)- and CRP-XL (Figure 2E and 2F)-induced platelet aggregation. However, when the concentration of collagen (Figure 2C and 2D)- and CRP-XL (Figure 2G and 2H) was reduced to 0.5 μg/mL, 1,8-cineole inhibited platelet aggregation at all the concentrations tested.

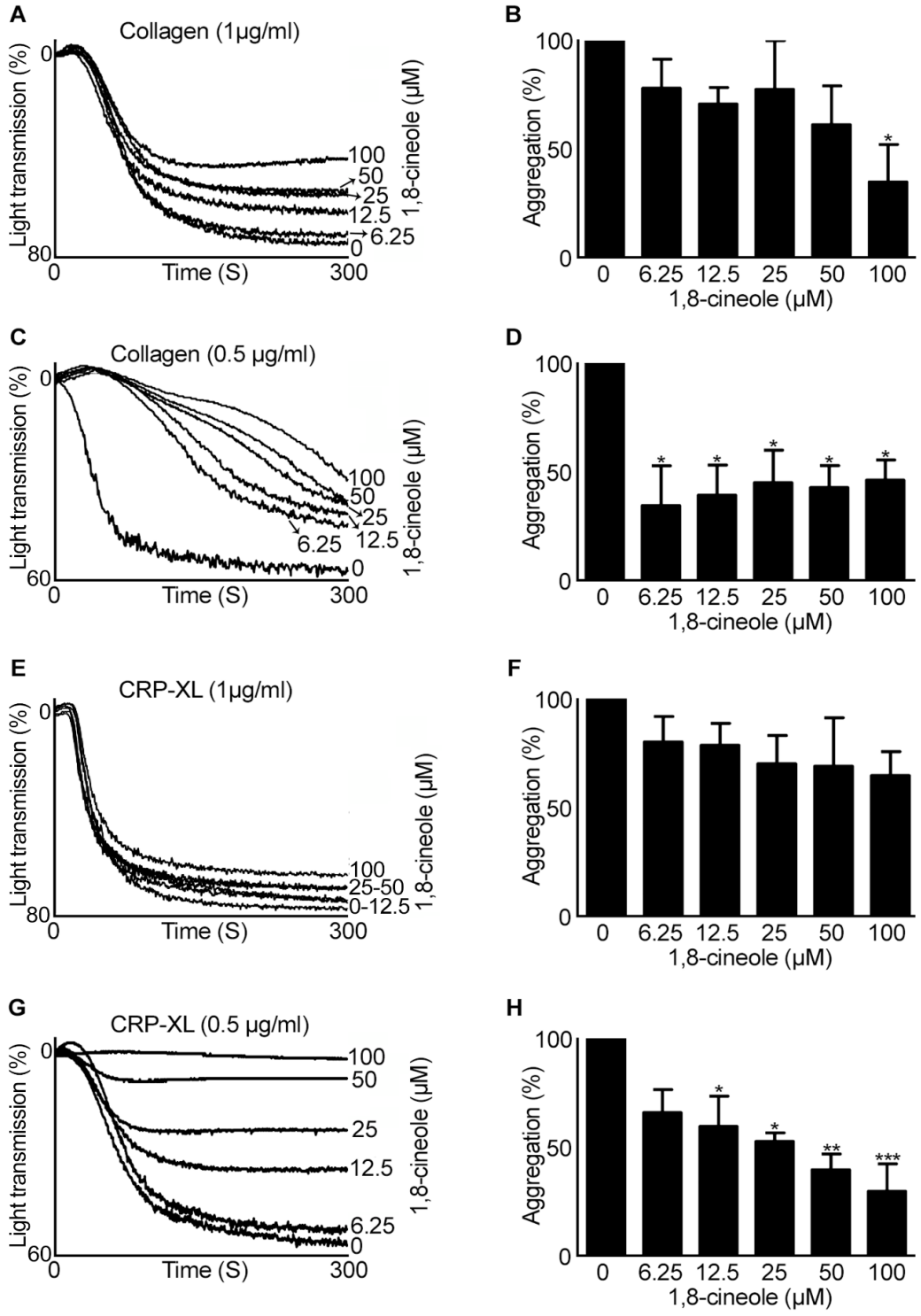

Figure 2: Impact of 1,8-cineole on GPVI agonists-stimulated aggregation in human PRP. Human PRP was treated with a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole for 5 minutes prior to stimulation of aggregation with 1 µg/mL (**A** and **B**) or 0.5 µg/mL (**C** and **D**) of collagen, and 1 µg/mL (**E** and **F**) or 0.5 µg/mL (**G** and **H**) of CRP-XL for 5 minutes. The aggregation traces shown are representative of four separate experiments. The percentage of aggregation for 1,8-cineole-treated samples was calculated by considering the level of aggregation obtained with the vehicle control (0) as 100%. Data represent mean ± SEM (n=4). The *p* values shown (*p<0.05, **p<0.01 and ***p<0.001) are as calculated by one-way ANOVA followed by Bonferroni *post-hoc* test.

Furthermore, to analyse if 1,8-cineole is able to inhibit platelet aggregation induced by agonists that stimulate platelet activation via G protein-coupled receptors (GPCRs), aggregation assays using thrombin in human isolated platelets and ADP in human PRP were performed. Interestingly, 1,8-cineole did not largely affect thrombin (Figure 3A-3B)- or ADP (Figure 3C-3F)-induced platelet aggregation at any of the concentrations used except around 20-30% inhibition observed at 100 μM when thrombin (0.1 U/ml) and ADP (2.5 μM) were used. Together, these results suggest that 1,8-cineole mainly inhibits platelet aggregation induced by collagen and CRP-XL. However, we cannot rule out the possibilities of the inhibitory effects of 1,8-cineole on low concentrations of GPCR agonists over a prolonged exposure period. Here, we tested up to 20 minutes of pre-incubation of platelets with 1,8-cineole, however, no significant effects were observed at lower concentrations of 1,8-cineole (data not shown). Furthermore, only thrombin and ADP were tested in this study, and it may be possible for 1,8-cineole to inhibit other GPCR agonists-induced platelet activation. Therefore, these aspects should be analysed in future studies.

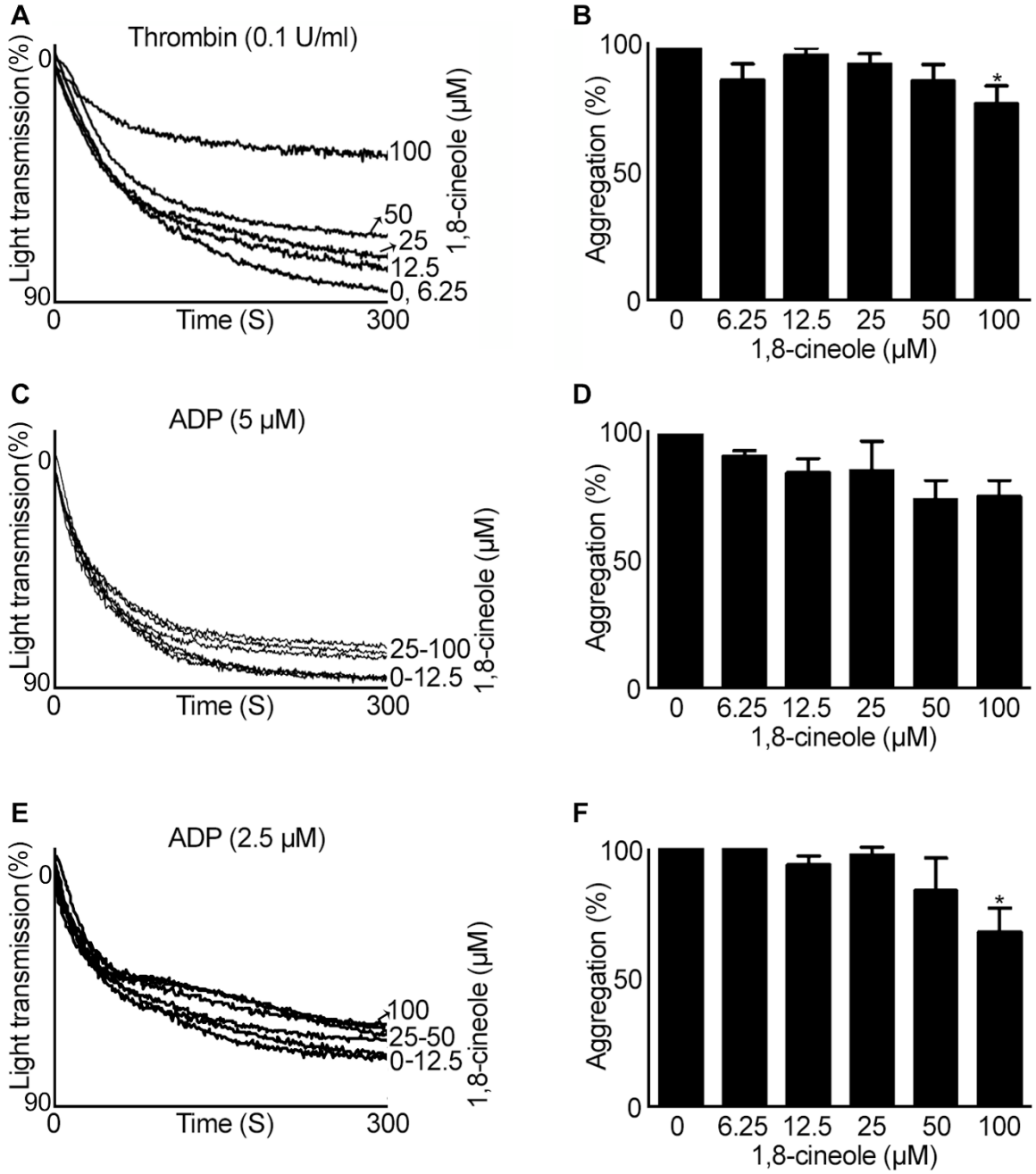

Figure 3: Effect of 1,8-cineole on thrombin- and ADP-stimulated aggregation in human platelets. Human isolated platelets were incubated with a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole for 5 minutes prior to stimulation of aggregation with 0.1 U/mL thrombin (**A** and **B**). Similarly, human PRP was incubated with various concentrations of 1,8-cineole for 5 minutes prior to activation of platelet aggregation with 5 μM (**C** and **D**) or 2.5 μM (**E** and **F**) ADP. The level of aggregation was monitored for 5 minutes by optical aggregometry. The aggregation traces shown are representative of three separate experiments. The percentage of aggregation for 1,8-cineole-treated samples was calculated by considering the level of aggregation obtained with the vehicle control (0) as 100%. Data represent mean ± SEM (n=3). The *p* values shown (*$p<0.05$) are as calculated by one-way ANOVA followed by Bonferroni *post-hoc* test.

2.2 Inside-out signalling to integrin αIIbβ3 is affected by 1,8-cineole

Platelet aggregation is dependent on the conformational changes to integrin αIIbβ3 (a highly abundant platelet surface receptor (Shattil, Kashiwagi et al. 1998)) induced by inside-out signalling in

platelets in order to transform the receptor from a low-affinity state to a high affinity binding state for fibrinogen and von Willebrand factor (vWF) (Shattil, Kashiwagi et al. 1998, Nieswandt, Varga-Szabo et al. 2009). To determine the effect of 1,8-cineole on this critical event, the level of fibrinogen binding as a marker for inside-out signalling to integrin αIIbβ3 was measured in platelets upon stimulation with CRP-XL (0.5 μg/mL). 1,8-cineole showed significant reduction in the level of fibrinogen binding in a concentration dependent manner in both human isolated platelets and PRP (Figure 4A). Higher concentrations of 1,8-cineole (>25 μM) displayed over 80% inhibition in isolated platelets, while the lower concentrations such as 12.5 μM and 6.25 μM exhibited up to 50% inhibition. When PRP was used, except 6.25 μM, all the other concentrations of 1,8-cineole displayed clear inhibitory effects of around 40-70%. Similar to aggregation assays, the level of 5 μM ADP-induced fibrinogen binding was not affected by various concentrations of 1,8-cineole following 20 minutes incubation (data not shown). These results demonstrate that 1,8-cineole is able to affect the CRP-XL-induced inside-out signalling to integrin αIIbβ3, and thereby controls the level of fibrinogen binding on the platelet surface and subsequent platelet aggregation.

2.3 1,8-cineole affects granule secretion in platelets

Platelets release several effector molecules from their granules upon stimulation with agonists in order to augment platelet activation and thrombus formation (White and Estensen 1972). Platelets contain different granules such as α-granules, dense granules and lysosomes. α-granules stores mainly proteins such as fibrinogen, vWF and P-selectin, whereas small organic molecules such as ADP, ATP and serotonin are stored in dense granules (Blair and Flaumenhaft 2009, Whiteheart 2011). The effect of 1,8-cineole on α-granule secretion in platelets was assessed by measuring the level of P-selectin exposure on the platelet surface upon stimulation with CRP-XL (0.5 μg/mL) using flow cytometry. The level of P-selectin exposure was significantly reduced in the presence of 1,8-cineole at all concentrations (6.25 μM - 50 μM) tested in human isolated platelets and at concentrations between 12.5 μM - 50 μM in human PRP (Figure 4B). Similarly, the effect of 1,8-cineole on dense granule secretion upon platelet activation was analysed by measuring the level of ATP secretion using lumi-aggregometer. Human isolated platelets were incubated with luciferase-luciferin chrono-lume reagent in the presence and absence of different concentrations of 1,8-cineole prior to stimulation with CRP-XL (0.5 μg/mL). Indeed, 1,8-cineole has displayed significant inhibitory effects on dense granule secretion at all the concentrations tested (Figure 4C and 4D). These results demonstrate the effects of 1,8-cineole on the modulation of granule secretion in platelets.

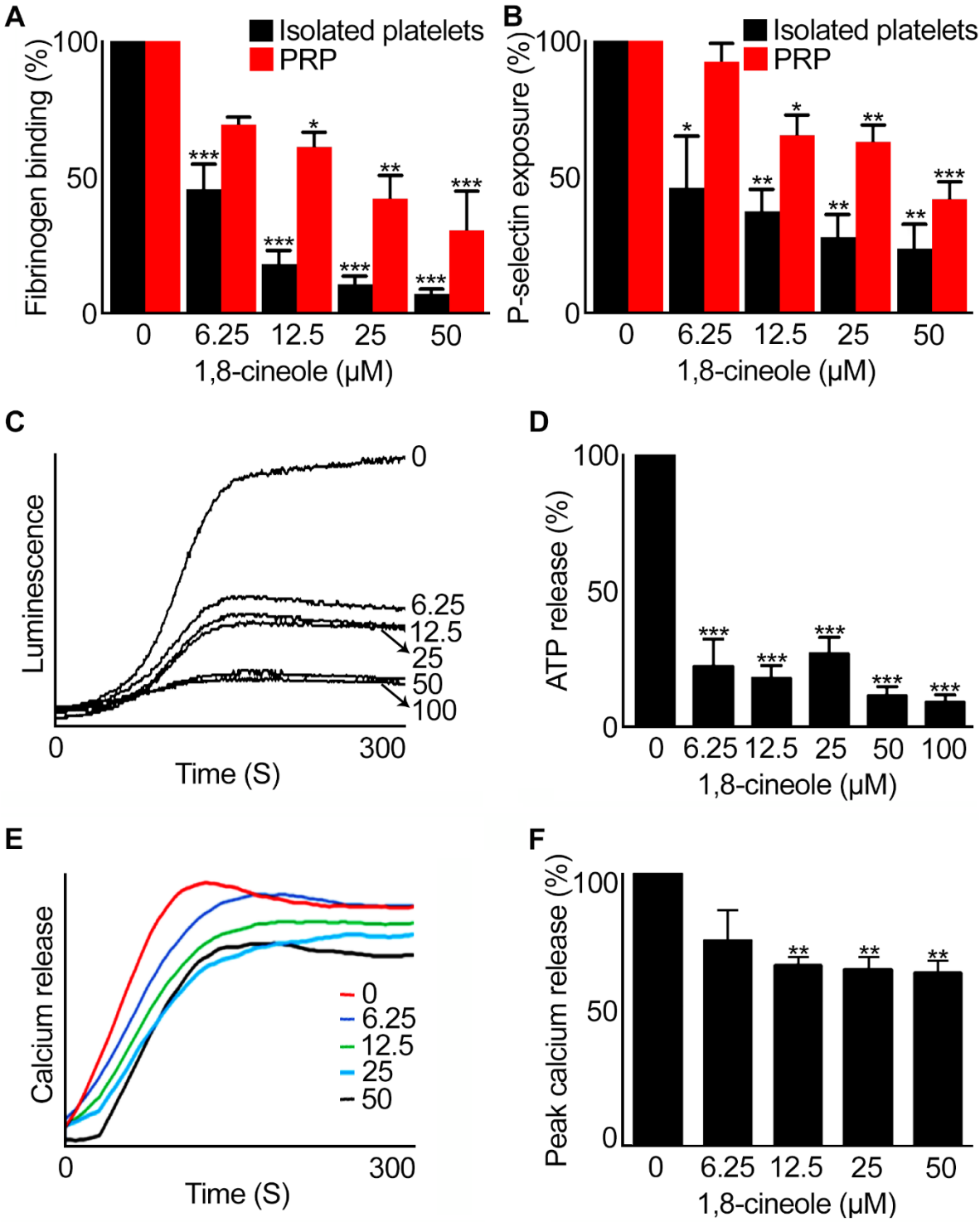

Figure 4: Effect of 1,8-cineole on inside-out signalling to integrin αIIbβ3, granule secretion and intracellular calcium mobilisation in human platelets. Human isolated (black) or PRP (red) (**A**) were incubated with a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole for 5 minutes prior to addition of CRP-XL (0.5 μg/mL) and further incubation for 20 minutes at room temperature. The level of fibrinogen binding on the platelet surface was quantified by flow cytometry. The bar graph indicates the percentage of fibrinogen binding as calculated with respect to the vehicle (0) control (considered as 100%). Similarly, the α-granule secretion in platelets was determined by quantifying the level of P-selectin exposed on the platelet surface upon activation by flow cytometry. The bar graph shows the effect of various concentrations of 1,8-cineole on α-granule secretion in human isolated platelets (black) or PRP (red) (**B**). Moreover, the effect of 1,8-cineole on dense granule secretion was quantified by measuring the level of ATP release upon activation of platelets. The human isolated

platelets were incubated with different concentrations of 1,8-cineole or a vehicle control [0.01% (v/v) ethanol] for 5 minutes and the level of ATP released upon platelet activation with 0.5 μg/mL CRP-XL was monitored using lumi-aggregometry. The traces (**C**) shown are representative of three separate experiments. The cumulative data (**D**) shown demonstrate the effect of 1,8-cineole on dense granule secretion in platelets as calculated by considering the level of ATP release observed with the vehicle control as 100%. To measure the level of intracellular calcium mobilisation, Fluo-4 AM dye labelled platelets were incubated with a vehicle control or various concentrations of 1,8-cineole for 5 minutes prior to stimulation of calcium release with 0.5 μg/mL CRP-XL. The level of calcium release was monitored for 5 minutes by spectrofluorimetry. The traces (**E**) shown are representative of three separate experiments. The cumulative data (**F**) were calculated by taking the peak calcium released in the vehicle control as 100%. Data represent mean ± SEM. (n=3). The *p* values shown (*p<0.05, **p<0.01 and ***p<0.001) are as calculated by one-way ANOVA followed by Bonferroni *post-hoc* test.

2.4 1,8-cineole inhibits intracellular calcium mobilisation in platelets

Calcium is a critical mediator of platelet activation, and its levels are largely increased in platelet cytoplasm via release from intracellular stores (dense tubular system) and influx from plasma (Varga-Szabo, Braun et al. 2009). Therefore, the impact of 1,8-cineole on the mobilisation of intracellular calcium levels was analysed using Fluo 4-calcium sensitive dye in human PRP upon activation with CRP-XL (0.5 μg/mL) by spectrofluorimetry. The pre-incubation of platelets with different concentrations of 1,8-cineole has affected the peak calcium level in platelets upon stimulation with CRP-XL (Figure 4E and 4F). The peak calcium level was reached at around 90 seconds following the addition of CRP-XL. These results demonstrate that 1,8-cineole is able to affect the intracellular calcium mobilisation which is a critical event during platelet activation and subsequent thrombus formation.

2.5 Integrin αIIbβ3-mediated outside-in signalling is affected by 1,8-cineole

Integrin αIIbβ3-mediated outside-in signalling plays critical roles to induce platelet spreading and at a later stage, clot retraction in order to facilitate wound healing (Shen, Delaney et al. 2012, Durrant, van den Bosch et al. 2017). To determine the effect of 1,8-cineole on the outside-in signalling mediated by integrin αIIbβ3, platelet spreading on fibrinogen-coated glass surface and the clot retraction assay were performed. Human isolated platelets were incubated with different concentrations (6.25 μM - 50 μM) of 1,8-cineole prior to adding them to human fibrinogen-coated glass cover slips and allowing them to spread for 45 minutes. Following permeabilisation and staining with FITC-labelled phalloidin, the platelets were visualised by confocal microscopy. The analysis of captured images demonstrates that 1,8-cineole significantly affects the number of platelets adhered with fibrinogen and their filopodia and lamellipodia formation during spreading on fibrinogen-coated surfaces (Figure 5A and 5B). At the concentration of 50 μM of 1,8-cineole, only a small number of platelets were able to adhere to fibrinogen. To determine the impact of 1,8-cineole on clot retraction, human PRP was incubated with various concentrations of 1,8-cineole (6.25 - 50 μM) prior to initiating clot formation by the addition of 1 U/ml thrombin. The rate of clot retraction was monitored over 2 hours by taking images at every 30 minutes. Finally, the impact of 1,8-cineole on clot retraction was analysed by measuring the remaining clot weight after 2 hours. As expected, the clot was completely retracted in the vehicle control, whereas the clot retraction was reduced in 1,8-cineole-treated samples with significant reduction observed at 12.5 μM and above (Figure 5C). Together, these data suggest that 1,8-cineole is able to influence integrin αIIbβ3-mediated outside-in signalling in platelets.

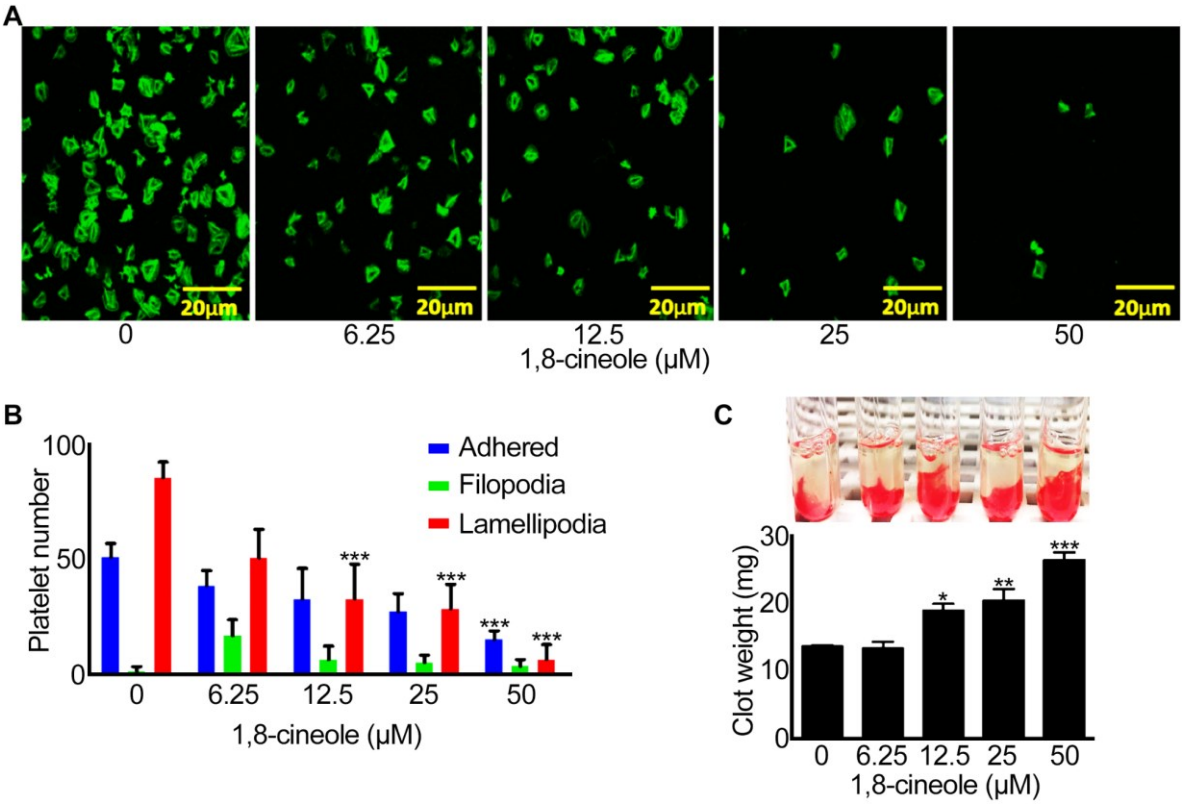

Figure 5: Effect of 1,8-cineole on integrin αIIbβ3-mediated outside-in signalling in human platelets. Human isolated platelets (at a density of $2x10^7$ cells/mL) were incubated with a vehicle control (0) or different concentrations of 1,8-cineole for 5 minutes and added onto fibrinogen- (100 µg/mL) coated coverslips and allowed them to spread for 45 minutes. Following fixation and permeabilisation, the platelets were stained with Alexa Fluor 488-conjugated phalloidin prior to analysis using a 100x oil immersion objective in a Nikon A1-R confocal microscope. The number of platelets at different stages of spreading was determined by analysing the images using ImageJ. (**A**) representative images of platelet spreading in the absence and presence of different concentrations of 1,8-cineole. (**B**) the cumulative data show the number of platelets at various stages of spreading (adhered, filopodia and lamellipodia formation). Data represent mean ± SEM (n=3 individual experiments using platelets obtained from three volunteers, and for each, 10 images were used for analysis). (**C**) to determine the impact of 1,8-cineole on clot retraction, human PRP was treated with various concentrations of 1,8-cineole prior to addition of 1 U/ml thrombin and monitoring of clot retraction for 2 hours. The images shown are representative of four separate experiments. The data shown were calculated by measuring the remaining clot weights after 2 hours of retraction. Data represent mean ± SEM (n=4). The *p* values shown (*p<0.05, **p<0.001 and ***p<0.001) are as calculated by one-way ANOVA followed by Bonferroni *post-hoc* test.

2.6 1,8-cineole reduces thrombus formation under arterial flow conditions

Platelet aggregation following vascular injury culminates in thrombus formation in order to seal the damaged area and prevent bleeding (Gibbins 2004). To determine the impact of 1,8-cineole on whole blood (in the presence of other blood cells and plasma proteins), thrombus formation on collagen-coated Vena8 biochips was analysed under arterial flow conditions. DiOC6-labelled human whole blood was incubated with various concentrations of 1,8-cineole prior to infusion over collagen-coated capillaries in Vena8 biochips and the level of thrombus formation was monitored for 10 minutes by taking images at every 30 seconds. 1,8-cineole at concentrations of 12.5 µM and 50 µM significantly inhibited the platelet adhesion, thrombus growth, volume (Figure 6A) and the fluorescence intensity (Figure 6B) of thrombi formed. Although the concentration of 6.25 µM 1,8-cineole did not reach a significant difference, it did affect the thrombus formation as shown in Figure 6A. These data suggest

that 1,8-cineole is able to affect platelet activation and subsequent thrombus formation in whole blood as similar to their inhibitory effects in isolated platelets and PRP.

2.7 1,8-cineole affects haemostasis in mice

Haemostasis is a normal physiological response of the body to prevent excessive bleeding upon vascular injury (Broos, Feys et al. 2011). In order to investigate the effect of 1,8-cineole on haemostasis, a tail-bleeding assay was performed in mice in the presence of a vehicle control or various concentrations (6.25 μM and 12.5 μM) of 1,8-cineole. Following the clipping of 3 mm tail tip, the bleeding time was monitored. Compared to the above experiments, only low concentrations of 1,8-cineole were selected for this experiment, as these can be easily achieved under physiological settings via oral administration or inhalation. The vehicle-treated mice bled for around 300 seconds, whereas the administration of 1,8-cineole extended the bleeding time to around 500 seconds at 6.25 μM and around 800 seconds with 12.5 μM (Figure 6C). These results indicate that 1,8-cineole affects the haemostasis in mice although it was only a modest effect at a low concentration of 6.25 μM.

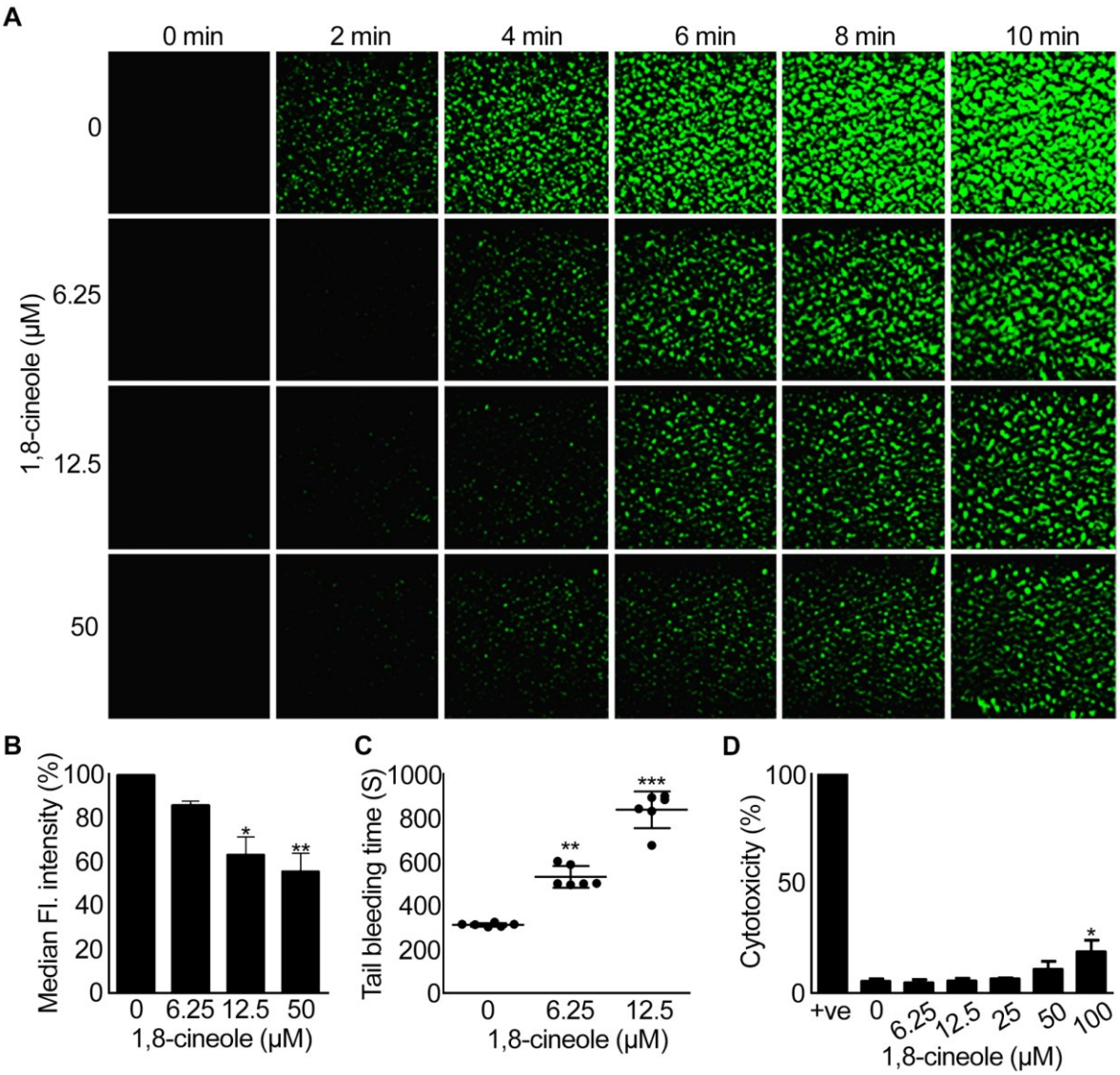

Figure 6: Impact of 1,8-cineole on thrombus formation and haemostasis. DiOC6 (a lipophilic dye) (5 μM)-labelled human whole blood was incubated with a vehicle or different concentrations of 1,8-cineole for 5 minutes and perfused through the microfluidic channels (Vena8 BioChips) coated with collagen (400 μg/mL). Thrombus formation was observed using a 20x objective on a Nikon A1-R

confocal microscope, with images captured every 30 seconds up to 10 minutes (**A**). Quantified data represent median fluorescence intensity of thrombi formed at 10 minutes in control and 1,8-cineole-treated samples as calculated using NIS elements software (Nikon) and normalised to the level of median fluorescence intensity obtained for thrombi at 10 minutes in the vehicle treated sample (**B**). Data represents mean ± SEM (n=3). The *p* values (**p*<0.05, and ***p*<0.01) shown are as calculated by one-way ANOVA with Dunnett's *post-hoc* test. (**C**) Effect of 1,8-cineole on haemostasis in mice was analysed using a tail bleeding assay. Mice (n=6 per group) were anaesthetised and a vehicle control [0.01% (v/v) ethanol] or 1,8-cineole (6.25 μM or 12.5 μM) was administered via femoral artery. After 5 minutes of incubation, tail tip was dissected, the time for cessation of bleeding was measured up to 20 minutes. Data represent mean ± SEM (n=6). The *p* values shown (***p*<0.01 and ****p*<0.001) are as calculated by non-parametric Kruskal-Wallis test**.** (**D**) To determine whether 1,8-cineole exerts any cytotoxic effects on human platelets, human PRP was exposed to a positive control, a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole for 30 minutes and the amount of LDH released was measured at 490 nm and 650 nm using spectrophotometry. The maximum LDH release obtained with the positive control was taken as 100% and the level of LDH release for 1,8-cineole treated samples was calculated accordingly. Data represent mean ± SEM (n=3). The *p* value shown (**p*<0.05) was calculated by one-way ANOVA with post-hoc Dunnett's test.

2.8 1,8-cineole is not cytotoxic to platelets at lower concentrations

Finally, to determine whether 1,8-cineole pharmacologically inhibits platelet activation or it exerts any cytotoxic effects, lactate dehydrogenase (LDH) cytotoxicity assay was performed. Human PRP was incubated with different concentrations of 1,8-cineole (6.25 μM - 100 μM) along with a positive control, and the amount of LDH released was measured as a marker for cytotoxicity using a spectrophotometer in line with the manufacturer instructions. 1,8-cineole was found to be non-toxic up to 50 μM concentration, however, a marginal level of cytotoxicity was observed at 100 μM concentration (Figure 6D). This result indicates that the inhibitory effects of 1,8-cineole up to 50 μM are due to their pharmacological effects in platelets rather than its cytotoxicity. However, caution should be taken when 1,8-cineole is used at or above 100 μM as it is likely to cause cytotoxicity at these concentrations.

2.9 1,8-cineole affects various signalling proteins in platelets

1,8-cineole has been reported to modulate various signalling pathways (e.g. cytokine production and NF-κB activity) that are involved in inflammatory responses (Juergens, Engelen et al. 2004, Greiner, Müller et al. 2013). Here, as 1,8-cineole selectively inhibited GPVI-mediated platelet activation, the effect of this molecule on the phosphorylation of key downstream proteins in GPVI signalling pathway was investigated using immunoblot analysis. While 1,8-cineole did not affect the phosphorylation of Syk (Figure 7A), it significantly inhibited the phosphorylation of LAT (Figure 7B) and dephosphorylation of Src (Figure 7C). Then, the impact of 1,8-cineole on the phosphorylation of AKT, which is the downstream effector molecule of phosphoinositide 3 kinase (PI3K) signalling was evaluated. Indeed, 1,8-cineole inhibited the phosphorylation of AKT at all the concentrations tested (Figure 7D). To determine the impact of 1,8-cineole on mitogen-activated protein kinase (MAPK) signalling pathways, the phosphorylation of p38 and ERK1/2 was analysed using immunoblots. Similar to other signalling proteins, 1,8-cineole affected the phosphorylation of both p38 (Figure 7E) and ERK1/2 (Figure 7F) at all the concentrations tested. To further explore the other targets in platelets for 1,8-cineole, the level of cAMP was measured in the absence and presence of various concentrations of this molecule. Although, 1,8-cineole did not elevate the concentration of cAMP in resting cells, it has increased the level of cAMP (Figure 7G) upon activation with CRP-XL. Similarly, the phosphorylation of VASP which is a substrate for cAMP-dependent protein kinase (PKA) was also increased by 1,8-cineole (Figure 7H). Based on these data, it is apparent that 1,8-cineole affects multiple pathways in platelets, and therefore, we cannot rule out the possibility of its impact on more signalling molecules/pathways. Further studies are required to explore its effects on other MAPK including JNK and Src phosphorylation upon stimulation with fibrinogen as well as other potential signalling pathways. We did not observe a dose-dependent effect in all cases, although this could be due to the

way the platelets were prepared and treated under non-aggregating conditions, further studies are warranted to determine the precise actions of 1,8-cineole in platelets.

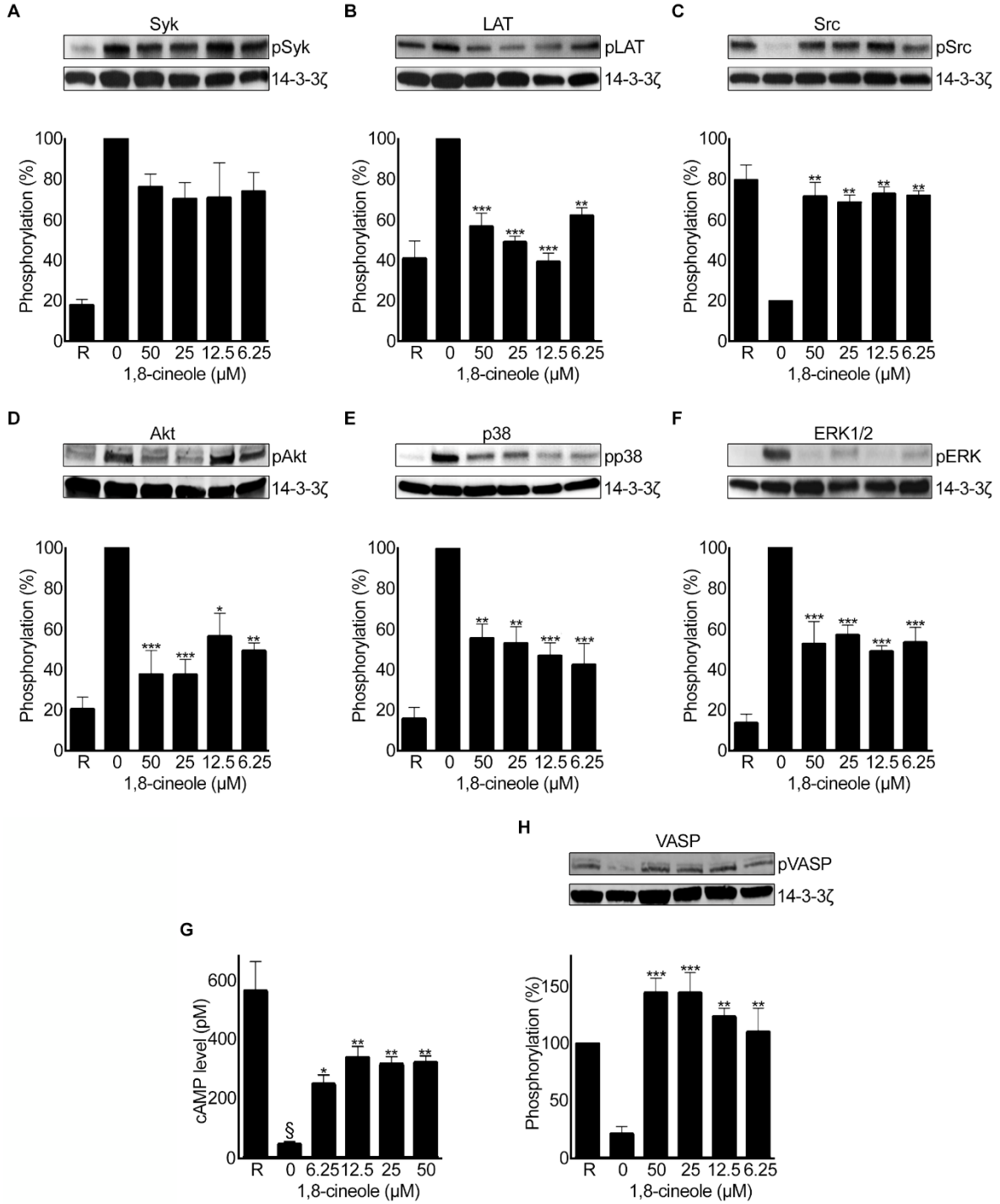

Figure 7: Effect of 1,8-cineole on specific signalling proteins in platelets. Human isolated platelets were treated with a vehicle control (0) or various concentrations of 1,8-cineole for 5 minutes before stimulation with CRP-XL (0.5 μg/mL) for 5 minutes in an aggregometer at 37°C. Then the cells were lysed using reducing sample treatment buffer and analysed in SDS-PAGE followed by immunoblots using various phospho-specific antibodies. The impact of 1,8-cineole on the phosphorylation of pSyk (Y323) (**A**), pLAT (Y200) (**B**), pSrc (Y527) (**C**), pAKT (S473) (**D**), pp38 (**E**), pERK1/2 (**F**) and pVASP (S157) (**H**) was analysed using selective phospho-specific antibodies for these proteins in immunoblots. The level of 14-3-3ζ was detected as a loading control in all these blots. The blots shown are representative of three separate experiments. Data represent mean ± SEM (n=3), normalised to loading control. (**G**) the level of cAMP in platelets that were treated with a vehicle control or various concentrations of 1,8-

cineole was measured using a cAMP ELISA kit in line with the manufacturer's instructions. Data represent mean ± SEM. (n=3). The *p* values shown (§*$p< 0.05$, **$p< 0.01$ and ***$p< 0.001$) are as calculated by one way-ANOVA followed by Bonferroni's correction for multiple comparisons. In **G**, § was when compared with resting (R) samples, and * when compared with the vehicle control (0).

3. Discussion

Over the last few decades, extensive research has been performed on medicinal plants to identify and develop new drugs with reduced side effects (Cragg and Newman 2013). Since platelets act as a powerful therapeutic target to control thrombotic diseases (Barrett, Holbrook et al. 2008), numerous plant-derived small molecules have been tested to determine their ability to inhibit platelet activation and thrombosis without any adverse effects on haemostasis. Indeed, flavonoids such as quercetin (Li, Yao et al. 2016, Patel, Mistry et al. 2018), catechin (Carnevale, Loffredo et al. 2014, Kerimi and Williamson 2015), tangeretin (Vaiyapuri, Ali et al. 2013) and nobiletin (Vaiyapuri, Roweth et al. 2015, Jayakumar, Lin et al. 2017) were extensively studied for their inhibitory effects in platelets. However, research on investigating the anti-platelet effects of essential oils that contain terpenoids is highly limited. Notably, essential oils and their chemical constituents have shown to exhibit various pharmacological effects in the cardiovascular system (Dagli, Dagli et al. 2015). For example, eugenol, from clove oil has been reported to inhibit the oxidation of low-density lipoproteins (LDL) thereby it reduces the development of atherosclerosis (Harb, Bustanji et al. 2019). α-curcumene, from turmeric essential oil exerts triglyceride-lowering activity (Yasni, Imaizumi et al. 1994). The essential oil from lavender has been reported to inhibit platelet aggregation induced by agonists such as collagen, ADP, arachidonic acid and U46619 (Ballabeni, Tognolini et al. 2004). 1,8-cineole has previously been shown to possess numerous beneficial effects including antioxidant and anti-inflammatory properties (Juergens, Dethlefsen et al. 2003, Seol and Kim 2016). However, the effects of 1,8-cineole on the modulation of platelet function have remained largely unexplored. Hence, in this study, the ability of 1,8-cineole to inhibit platelet activation and thrombus formation was investigated.

Similar to several flavonoids (Vaiyapuri, Ali et al. 2013, Vaiyapuri, Roweth et al. 2015) and eugenol (Chen, Wang et al. 1996), 1,8-cineole inhibits platelet activation induced by agonists such as collagen and CRP-XL in human isolated platelets and PRP (although a small reduction in their inhibitory effects were observed). The binding of small molecules to plasma proteins was previously reported for various plant-derived compounds (Howard, Hill et al. 2010, Vaiyapuri, Ali et al. 2013). For example, tangeretin a flavonoid rich in lemon peel (Vaiyapuri, Ali et al. 2013) and quercetin which is rich in red onions (Wright, Gibson et al. 2010) were shown to bind plasma proteins to an extent. Therefore, the binding of 1,8-cineole to plasma proteins may reduce their bioavailability in order to inhibit platelet function. 1,8-cineole marginally inhibited thrombin- or ADP-induced platelet aggregation at a higher concentration of 100 μM. A previous study on rabbit isolated platelets investigated the anti-platelet aggregating activity of nutmeg oil, in which eugenol was the most active component (Janssens, Laekeman et al. 1990). Similar to our findings with 1,8-cineole, eugenol was found to inhibit collagen-induced platelet aggregation with reduced effects on ADP- and thrombin-induced aggregation (Chen, Wang et al. 1996). Another study has demonstrated that the essential oil of cloves in which eugenol was a major component, inhibits collagen-induced platelet aggregation but was not able to cause a significant inhibition on ADP-induced platelet aggregation (Srivastava and Justesen 1987). The essential oil of lavender has been reported to inhibit platelet aggregation induced by collagen, thromboxane receptor agonist (U46619), arachidonic acid and ADP on PRP (Ballabeni, Tognolini et al. 2004). In the same study, the antiplatelet effect of the main components of lavender essential oil [linalyl acetate (36.2%), linalool (33.4%), camphor (7.6%) and 1,8-cineole (5.8%)] were also investigated (Ballabeni, Tognolini et al. 2004). These observations strongly indicate that 1,8-cineole may selectively affect GPVI-induced platelet activation pathway (Vaiyapuri, Roweth et al. 2015). Depend on the nature of small molecule and its ability to bind various target proteins, they may preferentially inhibit specific receptor-mediated

platelet activation. However, even lower concentrations of 1,8-cineole over a long period of exposure may affect platelet activation under physiological conditions.

The stimulation of platelets by agonists induce inside-out signalling that transforms the conformation of the extracellular domain of integrin αIIbβ3 resulting in an increase in its binding affinity for plasma fibrinogen (Shattil, Kashiwagi et al. 1998). The fibrinogen bound on the platelet surface acts as a bridge to bind other platelets and thereby to facilitate platelet aggregation. As similar to its effects on aggregation, 1,8-cineole inhibits the level of fibrinogen binding on platelet surface induced by CRP-XL. Moreover, 1,8-cineole inhibited both α- and dense granule secretion in platelets upon stimulation with CRP-XL. As components released from dense granules (e.g. ADP) and α-granules (e.g. vWF and fibrinogen) are essential regulators of secondary activation of platelets and thrombus formation (Whiteheart 2011), the inhibition of granule secretion by 1,8-cineole suggests its ability to supress the positive feedback cascades that lead to a rapid and large activation of platelets during thrombus formation. Similar to 1,8-cineole, elemicin and eugenol isolated from essential oils of *Cymbopogon ambiguus* are volatile monoterpenoids with potent anti-inflammatory effects (Grice, Rogers et al. 2011), and they have been reported to possess anti-platelet effects by inhibiting ADP-induced secretion of serotonin. Eugenol exhibited potent inhibitory activity on ADP-induced platelet aggregation (Grice, Rogers et al. 2011). Another study has demonstrated inhibitory effects of eugenol on human PRP aggregation induced by arachidonic acid, ADP and collagen with prominent inhibitory effects on arachidonic acid-induced platelet aggregation (Raghavendra and Naidu 2009). In addition, 1,8-cineole has affected intracellular calcium mobilisation in platelets. The elevation of calcium levels through release from intracellular stores and entry from outside via influx mechanisms is critical during platelet activation (Varga-Szabo, Braun et al. 2009).

Integrin αIIbβ3-mediated outside-in signalling amplifies a range of cellular events that are essential for platelet functions such as spreading and clot retraction (Shen, Delaney et al. 2012). 1,8-cineole significantly inhibited both platelet spreading on fibrinogen-coated surface and clot retraction. Platelet spreading is critical to allow platelet adhesion at the injury site and to provide surface for clotting cascades to take place (Aslan, Itakura et al. 2012). The effect of 1,8-cineole on platelet spreading is similar to some flavonoids including tangeretin (Vaiyapuri, Ali et al. 2013), nobiletin (Vaiyapuri, Roweth et al. 2015) and chrysin (Ravishankar, Salamah et al. 2017). The clot retraction is another assay where the significance of integrin αIIbβ3-mediated outside-in signalling can be assessed (Tucker, Sage et al. 2012). Our result shows that 1,8-cineole inhibited clot retraction at concentrations of 25 and 50 μM. Likewise, essential oil of lavender inhibited the clot retraction induced by thrombin in PRP. The same study reported that 1,8-cineole partially inhibited clot retraction in PRP (Ballabeni, Tognolini et al. 2004). Similar to 1,8-cineole, other essential oils such as oils of *Ocotea quixos* (Ballabeni, Tognolini et al. 2007) and *Foeniculum vulgare* (Tognolini, Ballabeni et al. 2007) reduced the clot retraction rate indicating their significance in integrin αIIbβ3-mediated outside-in signalling.

The impact of 1,8-cineole on whole human blood was investigated by an *in vitro* thrombus formation assay under arterial flow conditions. Indeed, 1,8-cineole reduced thrombus formation significantly by inhibiting platelet adhesion, thrombi number and volume over time. In contrast to other assays where isolated platelets or PRP were used, here the whole blood was used. Hence, this demonstrates the ability of 1,8-cineole to inhibit platelet function in the presence of plasma proteins and other blood cells. Although the low concentration of 6.25 μM did not show significant effect on thrombus formation, the prolonged exposure of this compound to platelets in the circulation may cause modest inhibition over time in order to prevent the unwarranted activation of platelets. Finally, 1,8-cineole (at 12.5 μM and 6.25 μM) has shown to moderately extend the bleeding time in mice, which reflects its actions under physiological settings. The effect of 1,8-cineole on bleeding time could also be due to its vasodilation effects as reported previously (Pinto, Assreuy et al. 2009). However, the impact of 1,8-cineole on the modulation of haemostasis in humans under diverse pathophysiological scenarios should be investigated. Interestingly, 1,8-cineole was found to be non-cytotoxic to platelets up to 50

μM, and only a concentration of 100 μM has caused a mild (significant) toxic effect although this is a supraphysiological concentration which is unlikely to be achieved therapeutically.

The molecular mechanistic studies indicated that 1,8-cineole may have multiple targets in platelets as similar to several other plant-derived small molecules. 1,8-cineole inhibits the phosphorylation of LAT and dephosphorylation of Src which are mainly involved in GPVI signalling pathway (Rayes, Watson et al. 2019). This may reflect on the inhibitory effects of 1,8-cineole selectively on collagen and CRP-XL-induced platelet activation. During clot retraction process, the initial binding of cytoskeletal myosin depends on the phosphorylation of integrin β3 subunit with important downstream roles of Src and PLCγ2 (Suzuki-Inoue, Hughes et al. 2007). The inhibitory effects of 1,8-cineole on clot retraction can be through the decrease of the activity of Src-family kinase as 1,8-cineole is able to attenuate this activity. However, the phosphorylation of Src upon stimulation with fibrinogen was not analysed in this study. Furthermore, it inhibits the phosphorylation of AKT at Serine 473, which is a downstream effector molecule of PI3K that plays a key role in platelet activation (Kim, Jin et al. 2004). A previous study has shown that 1,8-cineole inactivates AKT in mice that exhibit hepatic lesions where the levels of AKT are upregulated (Murata, Ogawa et al. 2015). Other studies have indicated that 1,8-cineole inactivates AKT and survivin in human colon cancer cell lines (Murata, Shiragami et al. 2013) and it inhibits GSK-3 in nasal polyps in patients with chronic rhinosinusitis (Bruchhage, Koennecke et al. 2018). Previous studies that were aimed to investigate the anti-inflammatory activity of 1,8-cineole have also indicated that 1,8-cineole interferes with various signalling pathways (Juergens, Engelen et al. 2004, Greiner, Müller et al. 2013). For example, in human umbilical vein endothelial cells (HUVECs), it has reported that 1,8-cineole mainly mediates its inhibitory effects via the NF-κB signalling pathway resulting in suppression of pro-inflammatory cytokine release induced by bacterial LPS. Moreover, 1,8-cineole has shown to inhibit MAPKs including p38 and ERK1/2 (although it's likely affect others too) which are also critically important to regulate various cellular events in platelets (Kim, Lee et al. 2015). Interestingly, 1,8-cineole increased the level of cAMP in platelets following agonist-induced activation. The increased level of cAMP was also observed in platelets when treated with different flavonoids (Vaiyapuri, Ali et al. 2013). Together, these data demonstrate that 1,8-cineole inhibits platelet activation via multiple signalling pathways or targets. Further studies are required to establish the complete landscape of the mechanisms through which 1,8-cineole modulate platelet activation.

1,8-cineole was reported to quickly absorb from the gastrointestinal tract, although its absorption is enhanced in the presence of milk (Bhowal and Gopal 2015). The absorption via lungs upon inhalation of essential oils was also reported although it's likely to be toxic to the lungs (Jäger, Nasel et al. 1996). 1,8-cineole gets metabolised into several derivatives and excreted via urine. Oral administration of 200 mg/kg of 1,8-cineole in rabbits displayed peak plasma concentrations of this molecule along with its unconjugated metabolites within 30 minutes to 1 hour. While the original compound reached a maximum of 8.4 μg/mL in 30 minutes, its primary unconjugated metabolite reached 24 μg/mL within 1 hour, and decreased between 2 to 6 hours. It was suggested that up to 200 mg/kg, 1,8-cineole is likely to go through a rapid absorption, metabolism and conjugation of metabolites, although at higher concentrations, the metabolism appeared to be slow (Bhowal and Gopal 2015). Overall, due to numerous beneficial effects of 1,8-cineole in humans along with its excellent pharmacological and physicochemical properties, further clinical studies to establish its therapeutic values as an anti-thrombotic agent will be warranted.

4. Materials and Methods

Platelet preparation

The preparation of human platelets was performed using standard protocols as described previously (Ravishankar, Salamah et al. 2017, Salamah, Ravishankar et al. 2018). All the experiments were performed in accordance with relevant institutional and national guidelines. A written informed consent was obtained from human volunteers according to the procedures approved by the University of Reading Research Ethics Committee (Reference no: UREC 17/17, approved on the 10th May 2017).

The blood was drawn from healthy, aspirin-free human volunteers via venepuncture into vacutainers containing 3.2% (w/v) sodium citrate as an anticoagulant.

Platelet-rich plasma (PRP) preparation: Blood samples were centrifuged at 102 g for 20 minutes at 20°C. The resultant supernatant (PRP) was collected and rested for 30 minutes at 30°C prior to using them in aggregation, flow cytometry and clot retraction assays.

Preparation of isolated platelets: 50 mL of whole blood was mixed with 7.5 mL of ACD [acid citrate dextrose; 20 g/L glucose, 25 g/L sodium citrate and 15 g/L citric acid] and centrifuged at 102 g for 20 minutes at 20°C. The supernatant (PRP) was aspirated and to this 3 mL of ACD and 10 μL of prostaglandin I_2 (PGI_2) (125 μg/mL) were added prior to centrifuging at 1413 g for 10 minutes at 20°C. The resulting platelet pellet was washed by resuspending in modified Tyrodes-HEPES buffer (25 mL) [2.9 mM KCl, 134 mM NaCl, 0.34 mM $Na_2HPO_4.12H_2O$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 20 mM HEPES, pH 7.3] in the presence of 10 μL of PGI_2 (125 μg/mL) and centrifuging at 1413 g for 10 minutes. The resulting platelet pellet was finally resuspended in modified Tyrodes-HEPES buffer at a density of 4×10^8 cells/mL and rested for 30 minutes before use in aggregation and immunoblot assays.

Preparation of 1,8-cineole

Clinical grade 1,8-cineole (Sigma-Aldrich, UK) dissolved in 100% ethanol was prepared as 1 mM stock. This was further diluted in modified Tyrodes-HEPES buffer to the desirable concentrations for assays and the final concentration of ethanol in platelets was maintained at 0.01% (v/v). A vehicle control [ethanol at a concentration of 0.01% (v/v)] was included in all the experiments and this concentration did not affect the platelet function.

Aggregation and ATP release assays

Platelet aggregation assays were performed using optical aggregometer (Chrono-Log, USA) as described by us previously (Jones, Sage et al. 2014, Salamah, Ravishankar et al. 2018). Platelets (445 μL) were incubated with different concentrations of 1,8-cineole or a vehicle control [0.01% (v/v) ethanol] (5 μL) for 5 minutes at 37°C. The samples were then activated with 50 μL of different concentrations of ADP (Sigma-Aldrich, UK) or collagen (Horm collagen, Nycomed, Austria) or CRP-XL (obtained from Professor Richard Farndale at the University of Cambridge) or thrombin (Sigma-Aldrich, UK) and the level of platelet aggregation was monitored for 5 minutes.

ATP release was determined as a measure for dense granule secretion in platelets using the luciferin-luciferase reagent by lumi-aggregometry (Chrono-Log, USA). Briefly, platelets (395 μL) were incubated with Chrono-Lume reagent (50 μL) for 2 minutes at 37°C. After this, 5 μL of various concentrations of 1,8-cineole were added and incubated for 5 minutes prior to activation with 50 μL of agonist as stated above.

Flow cytometry-based assays

The human isolated platelets or PRP were incubated with different concentrations of 1,8-cineole or a vehicle control for 5 minutes in the presence of FITC-labelled anti-human fibrinogen antibodies (Dako, UK) and PECy5-labelled CD62P (P-selectin) antibodies (BD Biosciences, UK). Platelets were then activated with CRP-XL (0.5 μg/mL) for 20 minutes at room temperature. Following this, 0.2% (v/v) formyl saline was added to fix the platelets and the levels of fibrinogen binding (a marker for inside-out signalling to integrin αIIbβ3) and P-selectin exposure (a marker for α-granule secretion) were measured by flow cytometry (Accuri C6, BD Biosciences, UK). The median fluorescence intensity was used to assess the levels of fibrinogen binding and P-selectin exposure on the platelet surface. The level of fluorescence obtained with the vehicle control was taken as 100% to calculate the level of fibrinogen binding and P-selectin exposure in 1,8-cineole treated samples.

Calcium mobilisation

The intracellular calcium levels in platelets were measured using Fluo-4 AM calcium-sensitive dye (ThermoFisher Scientific, UK), which binds free intracellular calcium. 2 mL of human PRP were loaded with 2 μl (2 μM final concentration) of Fluo-4 AM and incubated for 45 minutes at 30°C in the dark. After this, the PRP was centrifuged at 350 g for 15 minutes and, the resulting platelet pellet was resuspended in 500 μl of modified Tyrodes-HEPES buffer. The isolated platelets loaded with Fluo-4 AM were incubated with a vehicle control [(0.01% (v/v) ethanol] or different concentrations (6.25, 12.5, 25, and 50 μM) of 1,8-cineole before activating with 0.5 μg/ml CRP-XL. The level of fluorescence intensity was measured by a NOVOstar plate reader (BMG Labtech, Germany) at 37°C for 5 minutes using an excitation wavelength of 480 nm, and emission at 520 nm. The data were analysed by measuring the percentage of the maximum level of calcium was released in all the samples.

Platelet spreading

Fibrinogen (100 μg/ml) was coated on cover glasses for one hour and then blocked with 1 % (w/v) bovine serum albumin (BSA) for another one hour. Following washing using PBS, human isolated platelets at a density of 2×10^7 cells/mL pre-treated with various concentrations of 1,8-cineole for five minutes were allowed to spread on fibrinogen-coated cover glasses for 45 minutes at 37°C. Following fixation using 0.2% (v/v) formyl saline, and the cells were permeabilised using 0.1% (v/v) Triton X-100 for five minutes. Following washing, the cells were stained with Alexa Fluor 488-labelled phalloidin by incubating in the dark for 30 minutes. Then the cover glasses were mounted on to slides, and visualised using a Nikon A1 confocal microscope.

Clot retraction assay

Human PRP (200 μL) and red blood cells (5 μL) were mixed with modified Tyrodes-HEPES buffer in the presence and absence of various concentrations of 1,8-cineole to a final volume of 950 μL and incubated for 5 minutes. Then 50 μL thrombin (1 U/mL) was added to initiate clot formation. A blunt glass capillary was placed inside the tube around which the clot was formed and the clot retraction was monitored over a period of 2 hours at room temperature. After 2 hours, the remaining clot weight was measured as a marker for clot retraction.

In vitro thrombus formation

Human whole blood was incubated with 5 μM of a lipophilic dye, DiOC6 (3,3'-Dihexyloxacarbocyanine Iodide) (Sigma Aldrich, UK) at 30°C for 30 minutes. Vena8 BioChip (Cellix Ltd, Ireland) microfluidic channels were coated with collagen (400 μg/ml) for one hour. Following blocking with 1% (w/v) BSA for one-hour, human whole blood pre-incubated with a vehicle control or various concentrations (6.25, 12.5 and 50μM) of 1,8-cineole for 5 minutes was perfused through the collagen-coated microfluidic channels at a shear stress of 20 dynes/cm^2 for 10 minutes. The level of thrombus formation was observed using a Nikon A1-R confocal microscope using 20x objective. Fluorescence images of thrombi were captured every 30 seconds continuously for 10 minutes. The median thrombus fluorescence intensity was calculated using NIS Elements software (Nikon, Japan) and the images were analysed using ImageJ software (National Institute of Health, USA).

Tail bleeding assay

This experiment has been approved by the University of Reading Research Ethics Committee and the British Home Office. Briefly, 12 weeks old C57BL/6 mice (Envigo, UK) were anaesthetised [using ketamine (80 mg/kg) and xylazine (5 mg/kg)] via intraperitoneal route and the mice were placed on a heated pad (37°C). After 20 minutes of anesthetisation, a vehicle control [0.01% (v/v) ethanol] or 1,8-cineole (12.5 μM and 6.25 μM - final concentration was determined based on the estimated volume of blood in each mouse in line with their weight) was administered via femoral artery and incubated for 5 minutes. Then the distal 3 mm segment of the tail tip was dissected using a scalpel blade and the tail tip was placed in sterile saline at 37°C and the time taken to cessation of bleeding was measured up to 20 minutes at which point the assay was terminated.

LDH cytotoxicity assay

LDH Cytotoxicity assay was performed using Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher, UK) according to manufacturer's instructions. Human PRP was incubated with various concentrations of 1,8-cineole or a positive control (a detergent provided in the kit) for 5 minutes. To this, the reaction mixture (provided in the kit) was added and incubated for 30 minutes. Following incubation, a stop solution (also provided in the kit) was added to terminate the reaction and the absorbance of this mixture was read at 490 nm and 650 nm using spectrophotometer (Molecular devices, UK).

Immunoblotting analysis

SDS-PAGE and immunoblotting analysis were performed using standard protocols (Elashry, Collins-Hooper et al. 2012, Jones, Sage et al. 2014, Vaiyapuri, Sage et al. 2015). Human isolated platelets were treated with different concentrations of 1,8-cineole and a vehicle control [0.01% (v/v) ethanol] for 5 minutes and 0.5 μg/mL CRP-XL was added to trigger platelet activation. After 5 minutes, the activation was stopped by adding reducing sample treatment buffer and the obtained platelet lysates were used for immunoblotting experiments with various antibodies. The phospho-specific antibodies used in this study were obtained from Abcam, UK. The mouse anti-human 14-3-3ζ antibody (Santa Cruz Biotechnology, USA) was used to detect the protein, 14-3-3ζ as a loading control in immunoblots. The Cy5-conjugated goat anti-rabbit and anti-mouse IgG (ThermoFisher Scientific, UK) were used as secondary antibodies in these experiments.

Quantification of cAMP levels in platelets

The cAMP levels in platelets were quantified using cAMP ELISA quantification kit (Enzo Life sciences, UK) according to the manufacturer's instructions. Human isolated platelets were treated with a vehicle control or different concentrations of 1,8-cineole prior to activation with 0.5 μg/ml CRP-XL and measuring the level of cAMP using a cAMP ELISA kit. The amount of cAMP was quantified using the standard curve which was plotted using the control samples provided in the kit.

Statistical analysis

All the data are represented as mean ± SEM. The statistical significance was determined using one-way ANOVA except for the tail bleeding assay where the data were analysed using a non-parametric Kruskal-Wallis test. All the statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc., USA).

5. Conclusions

In conclusion, essential oils extracted from medicinal plants have been extensively used for the treatment of various diseases and they are becoming as alternative therapeutics worldwide (Burt 2004, Dagli, Dagli et al. 2015). 1,8-cineole has been used for many years for it is anti-inflammatory and antioxidant effects (Seol and Kim 2016). Several studies have shown that 1,8-cineole is effective and safe in treatment of several diseases such bronchitis and inflammatory conditions (Seol and Kim 2016). Indeed, the clinical grade 1,8-cineole has been approved as a drug to treat some of these conditions. The results obtained in this study demonstrate that 1,8-cineole has potent inhibitory effects on human platelet function in isolated platelets, PRP (in the presence of plasma proteins) and in whole blood. As low as 6.25 μM may be sufficient to reduce unwarranted platelet activation in the circulation. Therefore 1,8-cineole could be beneficial in reducing thrombotic diseases. Further studies to investigate the therapeutic potential and safety profile of 1,8-cineole in humans may aid in the design and development of novel anti-thrombotic strategies using this compound as a source or template. Due to its numerous beneficial effects and pharmacological properties, this may be used as a safe and effective anti-thrombotic agent to treat and prevent thrombotic diseases.

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Chapter-1
Introduction

1. INTRODUCTION

Cardiovascular diseases (CVDs) describe a range of pathological conditions that affect the heart and blood vessels. CVDs include coronary heart disease (CHD), angina, ischemic stroke, peripheral arterial disease and deep vein thrombosis. CVD was first characterised when a German physician, Fredrich Hoffmann (1660–1742) who recognised that coronary heart disease resulted from a 'reduced passage of the blood flow within the coronary arteries' (Feinleib 1984).

Currently, CVD is the most common cause of mortality and morbidity worldwide particularly in the western world (Loucks, Lynch et al. 2009). Between 2016-2018, approximately 7.4 million people are living with heart and circulatory diseases in the UK and cause nearly 170,000 deaths each year (British Heart foundation, UK). Furthermore, it has been projected that, by 2030, around 23 million people worldwide will die annually from CVD (Mathers and Loncar 2006). Additionally, the total cost for the treatment and prevention of CVD was estimated to cost UK economy over €26 billion, in 2015 (Group, ESC Atlas of Cardiology is a compendium of cardiovascular statistics compiled by the European Heart Agency et al. 2020).

In the 1950s, acute myocardial infarction (MI) (usually called as acute 'heart attack') was recognised as one of the major causes of mortality in the developing world (Friedberg 1950). Heart attacks occur due to the blocked blood supply to the heart, and this block is caused mainly by a built up of fat and cholesterol which form a plaque within arteries that supply the heart with blood. The rupture of formed plaque in arteries results in the formation of a clot that blocks blood flow to the heart. Heart attacks have a range of symptoms, including angina (chest pain) and irregular heartbeats, which can result in sudden death. The primary cause of a heart attack is

atherosclerosis, which causes infarction and, potentially, a sudden disruption to blood flow to the heart.

It is well-known that ischemic heart disease [also called as coronary heart disease (CHD)] and strokes are the primary CVDs that lead to majority of deaths and disabilities around the world (Braunwald 1988, Loucks, Lynch et al. 2009). CHD also known as coronary artery disease, is a result of accumulation of atherosclerotic plaques within the blood vessels that supply blood to the heart muscle (Mackay and Mensah 2004). The clinical symptoms of CHD are usually diagnosed at a later stage of the disease, as the disease progression happens over a long period of time (Fleiner, Kummer et al. 2004). After years of progression, the atherosclerotic plaques tend to rupture and as a result leads to the formation of blood clots (thrombi) and subsequently block the blood flow to the heart muscle. This ultimately results in sudden death due to cardiac arrest (Fuller, Garland et al. 2017).

Moreover, thrombotic (ischemic) stroke caused by a blood clot (thrombus), usually arises in an artery that is affected by atherosclerosis as a result of an accumulation of fatty deposits (plaques) inside the blood vessels (such as carotid/cerebral arteries) that supply blood to the brain (Tsompanidi, Brinkmeier et al. 2010). This clot can lead to a complete occlusion of blood flow to a specific region of the brain, causing an ischemic stroke. This will lead to the dysfunction of brain cells in the affected area, which will result in death of these cells due to insufficient blood supply and therefore lack of glucose and oxygen (called as 'ischemia'). Based on the major causes, stroke can be divided into two main categories: ischemic and haemorrhagic. While a majority (around 85%) of strokes are ischemic, approximately 15% strokes are haemorrhagic (Caplan 1992, Qureshi, Tuhrim et al. 2001). Haemorrhagic strokes are caused by the leakage of blood within the brain. This is due

to the rupture of blood vessels due to trauma or damage to blood vessels, either within the brain or on the brain's surface (mostly in the subarachnoid space). Haemorrhagic stroke has a higher rate of mortality than that of ischemic stroke due a significantly large haematoma forming from the leaked blood which will compress the brain tissues (Vermeer, Algra et al. 2002).

In the circulatory system, platelets normally circulate as individual discoid bodies, although they possess a remarkable ability to adhere to the damaged site and form a thrombus to maintain haemostasis via support of integrin and other adhesion proteins. Haemostasis is a mechanism which is a normal physiological response aimed to protect blood loss and prevent bleeding from damaged blood vessels. As highlighted above, the rupture of atherosclerotic plaque within a blood vessel triggers platelet activation, aggregation and thrombus formation on the damaged vascular site. This will also result in activation of clotting cascades leading to the formation of a firm blood clot resulting in vessel occlusion leading to heart attack or stroke (Furie and Furie 2007). The activated platelet surface acts as a platform for the clotting cascades to take place which convert the inactive form of clotting factors into active clotting factors. Hence, in addition to their essential roles in the maintenance of haemostasis, platelets are recognised as major contributors to thrombosis (Jennings 2009). Consequently, thrombotic diseases such arterial thrombosis is treated by anti-platelet drugs that affect platelet activation and aggregation. Whereas, in venous thrombosis coagulation plays a significant role, so, anti-coagulant drugs are used to treat venous thrombosis.

Aspirin, also known as acetylsalicylic acid, was first marketed in 1899, and this is a synthetic prodrug of salicylic acid, which remains the cornerstone of anti-platelet

therapy. This is the leading pharmacological drug in the treatment and secondary prevention of CVD such as thrombotic complications (Patrono, Baigent et al. 2008). It is a non-steroidal anti-inflammatory drug, used to reduce pain and fever or inflammation, that acts by blocking the cyclooxygenase-1 (COX1) enzyme in platelets and thereby, inhibiting the synthesis of thromboxane A2 (TxA2) (a platelet activator molecule) from arachidonic acid. Aspirin still helping thousands of lives, but it has been linked with some of serious side effects. The side effects of aspirin include gastrointestinal bleeding and a development of aspirin-resistance in some patients. These side effects make it undesirable for long-term treatment for a majority of patients.

In addition to Aspirin, oral inhibitors of ADP receptor, P2Y12 such as thienopyridines including clopidogrel, ticlopidine, prasugrel and ticagrelor are other widely used anti-platelet drugs, which work by blocking P2Y12 receptor [which binds to adenosine diphosphate (ADP) to trigger platelet activation] and consequently inhibiting platelet activation. Clopidogrel is widely used as in combination therapy with aspirin and also as an alternative medication for aspirin-resistant individuals (Grise and Verma 2009). However, clopidogrel can trigger excessive bleeding in some patients, therefore it is not recommended as a long-term treatment.

These antiplatelet drugs are used to reduce the risk of myocardial infarction and stroke in only about one quarter of events (Hankey and Eikelboom 2003), which means the efficacy of available antiplatelet drugs is limited indicating the need for new approaches. As platelets play a key role in the progression of atherosclerosis and thrombotic complications, they act as a predominant target for the development of therapeutic strategies to treat and/or prevent CVD. Recently, there is a trend for the

development of natural compounds from traditionally used plants as antiplatelet agents (Ain, Khan et al. 2016).

The aim of this PhD work was mainly focussed on determining the impact of a potentially interesting molecule's antiplatelet effects by using a range of functional platelet responses. Before describing the significance and background of this molecule, here a brief background for the biology and functions of platelets is provided.

1.1 Platelet production

In the late 19th century, platelets (also called thrombocytes) were identified for the first time by an Italian Professor, Giulio Bizzozero, who described them as 'blood particles' in addition to white and red blood cells in the blood (Mazzarello, Calligaro et al. 2001). These particles, which later became known as platelets, have a discoid shape with a size between 2 - 4 μm in diameter. Platelets are the smallest circulating blood cells that have a lifespan of 6 - 9 days in humans and 4 - 5 days in mice (Aster 1967, Schmitt, Guichard et al. 2001). Their levels are maintained between 150 and 400×103/μL of whole blood. Indeed, approximately 100 billion new platelets are produced daily to maintain the haemostasis in the body (Larson and Watson 2006).

Platelets, like other blood cells, are produced from haematopoietic stem cells in the bone marrow during the process of haematopoiesis. Platelets are specifically produced by their parental cells, megakaryocytes in the bone marrow and released directly into circulation via sinusoids. This process is regulated by the production of a cytokine, thrombopoietin from the liver and kidneys (Kaushansky 2006). Megakaryocytes are derived from pluripotent stem cells, and undergo multiple stages of DNA replication, which are stimulated by thrombopoietin via a complex process called endomitosis. Mature megakaryocytes increase in size to around 100 μm, and they contain a high concentration of ribosomes that are needed to synthesise the proteins in order to produce thousands of new platelets (Long, Williams et al. 1982). Upon maturity, megakaryocytes extend their membrane into long branching containing proplatelets and protrude through junctions into blood sinusoids in order to release the platelets into the circulation (Figure 1-1).

Approximately 1,000 to 3,000 platelets are produced from each megakaryocyte (KAUFMAN, AIRO et al. 1965, Trowbridge, Martin et al. 1984), and they are capable

of contributing to the maintenance of haemostasis (Italiano, Patel-Hett et al. 2007). Notably, it was recently demonstrated that megakaryocytes can be found in the lungs where platelet production was observed in mice under specific thrombocytopenic conditions (Lefrançais, Ortiz-Muñoz et al. 2017). However, its relevance to humans has not yet been established.

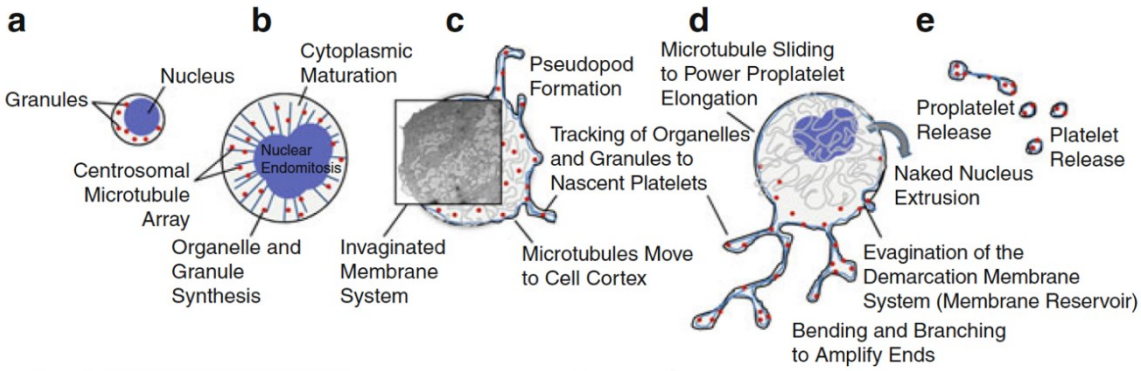

Figure 1-1: Different stages of platelet production. (a) Immature megakaryocytes are released. (b) As the megakaryocyte matures, it undergoes nuclear endomitosis, organelle synthesis, cytoplasmic maturation and expansion. (c) Before proplatelet formation, microtubules translocate to the cell cortex. (d) Proplatelet production begins as the megakaryocyte cytoplasm starts to increase at one pole. (e) The megakaryocyte cytoplasm is transformed into a form of proplatelets. This figure was adapted from (Thon and Italiano 2012).

1.2 Platelet structure and their important organelles

The ultrastructure structure of platelets, like many other cell types, mainly includes a plasma membrane, endoplasmic reticulum (also called as dense tubular system), mitochondria, and cytoskeleton (Figure 1-2). The cytoplasmic components of platelets include organelles and enzymatic proteins as well as a cytoskeleton network. Resting platelets have three structures; the peripheral structure which is composed of a glycocalyx coating, a cytoskeleton and a plasma membrane (Cimmino and Golino 2013). The glycocalyx contains glycoproteins which are important for platelet-endothelial interactions, for prompt activation and adhesion at the damaged site. The plasma membrane of platelets contains various receptors, including G protein-coupled receptors (GPCRs), adhesion receptors, and integrins, which are important for platelet function such as adhesion, aggregation, granule secretion and finally, thrombus formation. Here, I briefly describe the significance of important platelet organelles.

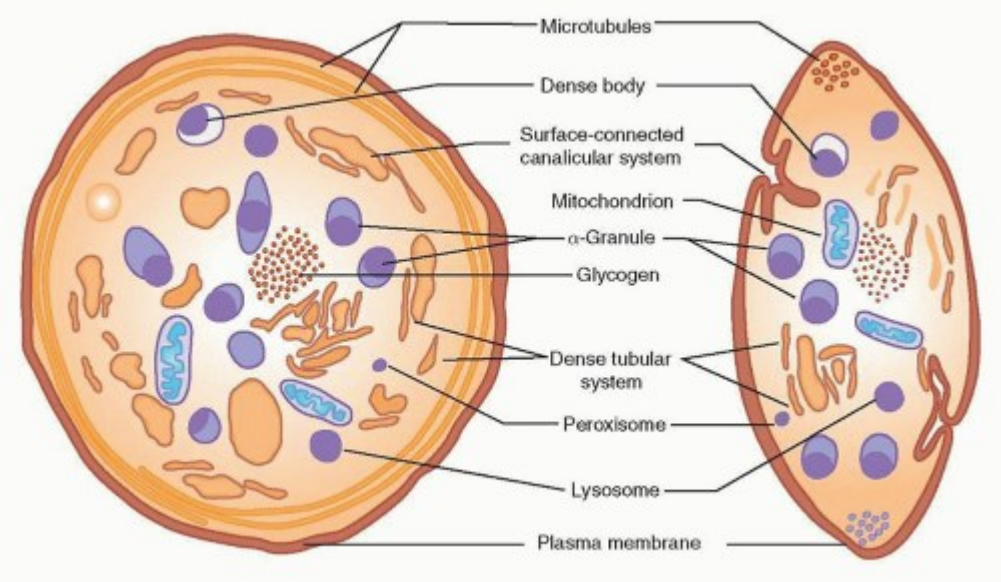

Figure 1-2: A schematic diagram depicts the structure of a human platelet. The plasma membrane of platelets is surrounded by an external coat of glycoproteins and glycocalyx. The membrane is rich in actin, microfilaments and microtubules that maintain the discoid shape of the resting platelets. The cytosol region contains α- and dense granules, lysosomes, mitochondria and glycogen storage granules. The open canalicular system is a distinct membrane system, which maintains the movement of molecules in and out of the platelet. The dense tubular system in the cytosolic region provides a store of calcium in the platelets. This diagram was adapted from (Bentfeld-Barker and Bainton 1982)

1.2.1 Plasma membrane

The structure of the plasma membrane in platelets is a standard phospholipid bilayer with phospholipids, cholesterol and membrane proteins. The membrane is selectively permeable to phospholipids that support platelet activation (internally) and blood coagulation (externally) (Bevers, Comfurius et al. 1983, Rumbaut and Thiagarajan 2010). The plasma membrane of platelets is supported by cytoskeleton that made up of actin, filamin, tubulin and spectrin. Platelets in resting state have discoid shape which is provided by microtubules that exist in the form of coils in sub-membranous part of the plasma membrane (Smyth, Whiteheart et al. 2010). Actin filaments in cytoplasm play important roles to maintain a dynamic equilibrium between a monomeric globular form and polymeric form, and it is polymerisation maintains platelet shape change upon activation (Bearer, Prakash et al. 2002). Moreover, platelet plasma membrane forms open canalicular system (invaginated membrane folds) that primary facilitates the release of platelet granules and influx of external substances into the cells upon activation (Reed 2002).

1.2.2 Dense tubular system (DTS)

Dense tubular system, in platelet cytoplasm acts as the main store for Ca2+ that controls platelet activation (Gremmel, Frelinger III et al. 2016). Upon platelet activation, DTS transforms to a vesicular form along with Ca^{2+} release, causing a rapid increase in cytosolic Ca^{2+} in order to support platelet activation (Ebbeling, Robertson et al. 1992, Jardín, López et al. 2008). In addition, DTS is also a site where TxA2 is produced by cyclooxygenase-1 from arachidonic acid (Ebbeling, Robertson et al. 1992). The synthesis and release of TxA2 provides a positive feedback activation for platelets during blood clotting or thrombosis (Rendu and Brohard-Bohn 2001).

1.2.3 Granules

In addition to open canalicular system (OCS), platelets contain major types of storage granules such as lysosomes, alpha (α) and dense granules, where the α-granules is the most abundant (Rivera, Lozano et al. 2009).

a- α-granules

α-granules contain various proteins such as von Willebrand factor (vWF), platelet (P)-selectin, platelet-derived growth factor (PDGF), fibrinogen, fibronectin, CD40 ligand (CD40L), platelet endothelial cell adhesion molecule (PECAM-1), and Factor V along with several other minor components. These molecules participate in numerous functions of platelets including activation, adhesion, inflammation, angiogenesis and wound healing. α-granules are around 0.2 - 0.4 μm in size, which represents around 10% (around 50 to 60 granules in each platelet) of a platelet's total volume (Blair and Flaumenhaft 2009).

b- Dense granules

Dense granules are smaller in size with around 0.15 μm, and they contain non-proteineous substances such ADP, serotonin, calcium and histamine (Gibbins 2004, Rivera, Lozano et al. 2009). These granules also contain a significant amount of calcium phosphate and magnesium ions (Ruiz, Lea et al. 2004). Number of dense granules in each platelet accounts between 3 to 5, however, they store important nucleotides such as ADP which upon release stimulate further platelet activation (McNicol and Israels 1999).

c- Lysosomes

Around 1 to 3 lysosomes are present in a single platelet. These lysosomes contain a high amount of hydrolytic enzymes that include acid phosphatase, cathepsins, hexosaminidase and β-galactosidase (Heijnen and Van der Sluijs 2015). There is a number of proteins present on lysosome surface such as the lysosomal associated membrane proteins (LAMP) ; LAMP-1, 2 and 3 (CD63) (Schwake, Schröder et al. 2013). The main functions of these lysosomes in platelets are still poorly understood, but they are considered to be involved in digestion of external substances and resolution of thrombi (Rendu and Brohard-Bohn 2001).

1.3 Platelet receptors

Platelets play a critical role in the maintenance of haemostasis, as well as the regulation of thrombosis, inflammation and vascular repair. Therefore, they possess numerous types of receptors on their surface, including glycoproteins, integrins, immunoglobulin and seven-transmembrane region-containing receptors to perform a range of different physiological responses. Signal transduction downstream of these receptors has been extensively studied, due to their critical roles in the regulation of haemostatic functions. The main role of some of these key receptors in platelets is to mediate platelet adhesion and subsequent activation. Platelets are activated when they are exposed to their range of different agonists, which are released either from activated platelets due to vessel wall damage during vascular injury or inflammatory conditions.

As the activation of platelets is a rapid process, many proteins/receptors play important roles in an orchestrated manner via different pathways to regulate platelet activation. The receptors present on the platelet plasma membrane can be divided into three major classes. First, receptors that bind to matrix proteins such as collagen and these include glycoprotein VI (GPVI), integrin $\alpha_2\beta_1$ and GPIb-V-IX complex. Second type of receptors are G-protein coupled receptors (GPCRs) that stimulate platelets via soluble mediators that are released from activated platelets such as ADP and TXA_2 or synthesised on the surface of platelets as a result of coagulation cascade such as thrombin. Thirdly, receptors that enable platelets to aggregate and form a thrombus such as integrin $\alpha IIb\beta_3$ which acts as a bridge between platelets via binding to plasma fibrinogen. All these classes of receptors are important in platelets in order to regulate haemostasis and other physiological responses. Some of the major platelet receptors are shown in Figure 1-3.

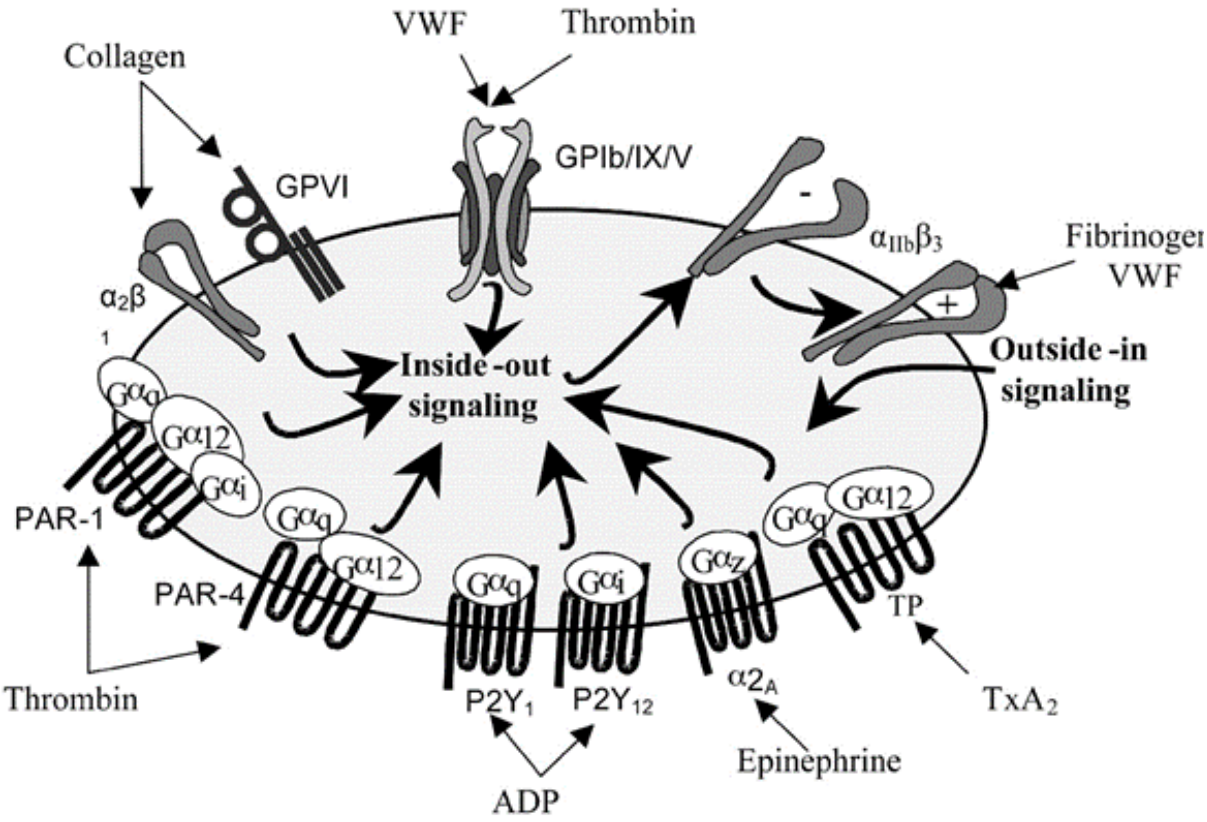

Figure 1-3: A schematic diagram to show major platelet receptors along with their ligands. The binding of vWF to GPIb/IX/V complex facilitates the subsequent binding of platelets to the subendothelial collagen, followed by the activation of platelets via GPVI and integrin $\alpha_2\beta_1$. GPCRs such as ADP receptors ($P2Y_1$ and $P2Y_{12}$), thrombin receptors (PAR1 and PAR4) , TXA2 receptor (TP) are activated by diverse ligand molecules as shown in the figure. Integrin αIIbβ3 provides a primary link between the outside of the platelets and the cytoskeleton. The activation of these receptors drives a cascade of signalling events leading to the elevation of intracellular Ca^{2+} levels, resulting in granule secretion and further augmentation of platelet activation. This figure was adapted from (Offermanns 2006).

1.3.1 GPVI

Collagen is the main insoluble fibrous and the most abundant protein in the body due to its role in supporting connective tissue to be strong in various parts of the body. Collagen fibres are types of biological molecules and there are around 20 types of collagen, where type I and III are the most common types of collagen in blood vessels (Nieswandt and Watson 2003). As vessel wall injury triggers platelet activation and clot formation, the first step in haemostatic cascade response is the platelet interaction with extracellular matrix, where collagen play a major role (Baumgartner 1977). The role of platelets is to initiate the formation of platelet plug at the site of damaged vessel to stop bleeding and to permit wound healing at a later stage. During vascular injury, subendothelial collagen becomes exposed, where plasma vWF can bind to exposed collagen. Circulating platelets start rolling at the damaged site and get activated by binding to exposed collagen through two major collagen receptors on platelet surface, GPVI and integrin $\alpha_2\beta_1$ (Gibbins, Okuma et al. 1997).

GPVI is a 62 kDa type I transmembrane glycoprotein of the immunoglobulin superfamily (Ig) that was identified as a second collagen receptor on the platelet membrane, and it serves as a major platelet activating receptor for collagen. GPVI consists of two extracellular Ig domains, extracellular mucin-like domain which is followed by transmembrane domain and a cytoplasmic tail with 51 amino acids. The cytoplasmic tail contains a proline-rich sequence which binds Src kinases, Lyn and Fyn that transmit collagen-mediated signals. GPVI binds collagen through specific Gly-Pro-Hypo (GPO) sequences (Munnix, Gilio et al. 2008). GPVI is also associated with the Fc receptor γ-chain homodimer (FcRγ), which is a transmembrane protein that is important for the dimerisation of GPVI on platelet surface and signalling in platelets (Gibbins, Okuma et al. 1997).

FcRγ contains the immunoreceptor tyrosine activation motif (ITAM) in the cytoplasmic tail, which clusters upon phosphorylation by Src family kinases (Lyn and Fyn) as a result of collagen binding to GPVI (Ezumi, Shindoh et al. 1998). In addition, the phosphorylation of ITAM causes the recruitment and binding of SYK via Src-homology2 (SH2) domains, which leads to the autophosphorylation and activation of SYK (Li, Delaney et al. 2010). The activation of SYK is followed by further phosphorylation of an adapter protein, SLP-76 and phosphorylation of the LAT. This results in the formation of a protein complex, namely the 'LAT signallosome', which has a critical role in the recruitment of phospholipase Cγ2 (PLCγ2) and phosphatidylinositol 3 kinase (PI3K) (Li, Delaney et al. 2010). PI3K plays an important role in the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3). The formation of PIP3 enables PLCγ2 activation result in generate secondary messengers, IP3 and DAG. These activatory processes induced by collagen lead to functional events, including intracellular calcium mobilisation, granule secretion, integrin αIIbβ3 activation and finally culminates in platelet aggregation or thrombus formation.

In a number of studies, platelets lacking GPVI have been reported to show a weak response to collagen, resulting in a resistance to thromboembolism induced by collagen infusion (Nieswandt, Schulte et al. 2001, Kato, Kanaji et al. 2003). This observation suggests that the main roles of GPVI in platelet signalling includes platelet activation and aggregation. In addition, integrin $\alpha_2\beta_1$ and the GPIb-IX-V complex mainly have adhesive roles in platelets (Nieswandt and Watson 2003, Gibbins 2004). Figure 1-4 summarises the signalling molecules involved GPVI-mediated signalling pathway.

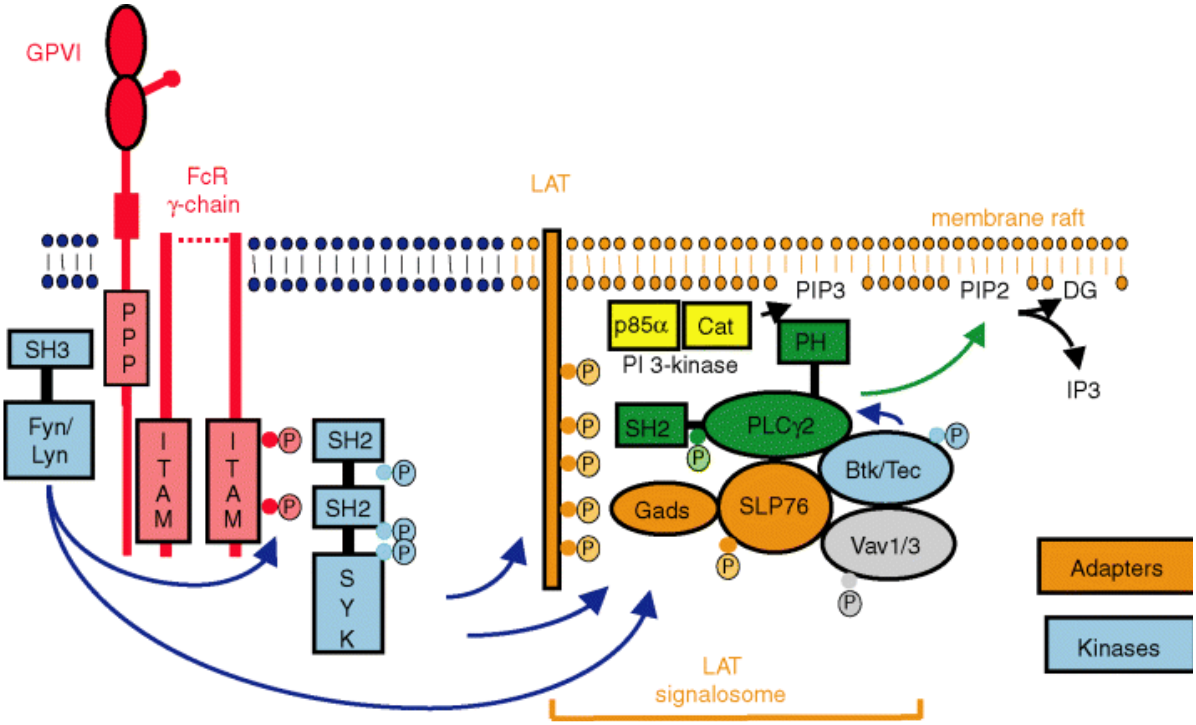

Figure 1-4: The GPVI signalling cascade and its components during platelet activation. The binding of an agonist such as collagen to GPVI triggers receptor clustering and tyrosine phosphorylation of the ITAM in FcRy-chain by Src family kinases such as Fyn and Lyn. This will lead to the phosphorylation of SYK. The phosphorylation of SYK leads to the phosphorylation of LAT, resulting in the formation of LAT signallosome. Then the activation of PLCy2, is associated directly with LAT through adapter proteins such as Gads and SLP-76. The recruitment of phosphatidylinositol-3 kinase (PI3K) to the signallosome leads to the conversion of phosphatidylinositol-3,4,5-trisphosphate (PIP3) into phosphatidylinositol-4,5-bisphosphate (PIP2). PIP3, via pleckstrin homology (PH) domains, causes recruitment and phosphorylation of phospholipase Cγ2 (PLCy2). The activation of PLCy2 cleaves PIP2 into inositol (1,4,5)-trisphosphate (IP3) and diacylglycerol (DAG). IP3 stimulates calcium mobilisation from endoplasmic reticulum, and DAG leads to the activation of PKC. This figure was adapted from (Watson, Auger et al. 2005)

1.3.2 Integrins

Platelets express different types of integrins that have important roles in adhesive and intercellular signalling activities. Integrins are a family of transmembrane adhesion receptors, and are heterodimers that contain non-covalently associated α and β subunits (Campbell and Humphries 2011). The structure of integrins, similar to that of most other receptors, extends to the extracellular region with a single transmembrane domain, with end in a cytoplasmic domain. This structure allows the integrin to interact with both the extracellular matrix and cytoplasmic proteins (Shattil and Newman 2004). In humans, there are approximately 18 α subunits and 8 β subunits (Ginsberg, Xiaoping et al. 1993). The most extensively investigated integrins in platelets are the integrin αIIbβ3 (GPIIb-IIIa) and integrin α2β1, due to their critical roles in binding fibrinogen and collagen, respectively during platelet activation. Around 2,000 to 4,000 copies of integrin α2β1 are present on each platelet surface compared to 40,000 - 80,000 copies of integrin αIIbβ3.

1.3.2.1 Integrin $\alpha_2\beta_1$

Integrin $\alpha_2\beta_1$ is known to be a major adhesion receptor that is responsible for collagen binding to platelets (He, Pappan et al. 2003). Integrin $\alpha_2\beta_1$ is able to bind different types of collagen including type I, II, III, IV and XI (Kunicki, Nugent et al. 1988). This binding occurs at the α_2 subunit via binding to the I domain containing Mg^{+2} that acts as a site for collagen binding (Jokinen, Dadu et al. 2004). In a resting condition, integrin $\alpha_2\beta_1$ is present in a low-affinity state with the extracellular domain set in a closed conformation. The activation of GPVI results in a rapid shift of this integrin into a high-affinity state, including conformational changes to the extracellular domains from a closed to an open conformation to bind collagen (Nuyttens, Thijs et al. 2011).

These changes allow integrin $\alpha_2\beta_1$ to bind to collagen, resulting in platelet adhesion and binding to the extracellular matrix of the damaged blood vessel walls. Whilst the interaction between GPIb-V-IX and vWF is important under high shear conditions, the interaction between integrin $\alpha_2\beta_1$ and collagen is critical under low shear conditions (Ginsberg, Xiaoping et al. 1993, Cruz, Chen et al. 2005, Farndale, Slatter et al. 2007).

In addition to the supporting role of integrin $\alpha_2\beta_1$ in platelet adhesion, it has been demonstrated that integrin $\alpha_2\beta_1$ also triggers signalling into the platelets by stimulating a number of signalling molecules such as Src, SYK and PLCγ_2 upon binding to collagen. This leads to changes in platelet morphology, including the formation of filopodia and lamellipodia upon adhesion to collagen (Inoue, Suzuki-Inoue et al. 2003).

1.3.2.2 Integrin $\alpha_{IIb}\beta_3$

Integrin $\alpha_{IIb}\beta_3$ (with a molecular weight of around 230 kDa) also called glycoprotein IIb-IIIa (GPIIb-IIIa) or CD41/CD61, is present on the surface of platelets in large number (i.e. between 40,000 to 80,000 copies per platelet) (Kasirer-Friede, Kahn et al. 2007). Integrin $\alpha_{IIb}\beta_3$ plays an important role in facilitating platelet-platelet interactions and subsequent aggregation. This is due to the activation of integrin $\alpha_{IIb}\beta_3$ and subsequent conformational changes to enable binding to fibrinogen, fibrin and vWF (Bennett and Vilaire 1979, Ruggeri, Lombardi et al. 1982, Hantgan 1988).

The initial platelet activation induces inside-out signalling to integrin $\alpha_{IIb}\beta_3$ to enable conformational changes from its low-affinity state in resting platelets to a high-affinity state in order to efficiently bind fibrinogen. The fibrinogen bound to the integrin $\alpha_{IIb}\beta_3$ acts as a bridge to facilitate the interactions between numerous platelets in order

to form platelet aggregates (Garraud, Hamzeh-Cognasse et al. 2013). Notably, integrin $\alpha_{II}b\beta_3$ provides a physical link between the outside of the platelet and the cytoskeleton.

The activation of integrin $\alpha_{II}b\beta_3$ drives a cascade of signalling events leading to the elevation of intracellular Ca^{2+} levels, resulting in granule secretion and further augmentation of platelet activation (Rivera, Lozano et al. 2009). The ligand binding to integrin $\alpha_{II}b\beta_3$ plays an important role in mediating various platelet functions including adhesion, aggregation, spreading and clot retraction (Li, Delaney et al. 2010).

Due to its prominent roles in the regulation of platelet function, integrin $\alpha_{II}b\beta_3$ has become a main target to develop novel therapeutic agents to treat and/or prevent thrombotic diseases (Offermanns 2006). A number of integrin $\alpha_{II}b\beta_3$ inhibitors such as abciximab, eptifibatide and tirofiban are its antagonists used as antiplatelet agents to treat/prevent thrombotic diseases (Topol, Moliterno et al. 2001, Subban and Chandra 2013).

1.3.3 GPCRs and their signalling in platelets

A number of platelet agonists such as ADP, TXA_2 and thrombin act through GPCRs. These agonists and their receptors play a critical role in secondary platelet activation and their recruitment in order to form a platelet plug to seal the damaged area. GPCRs are a family of membrane receptors comprised of seven transmembrane domains that contain an extracellular N-terminus and intracellular cytoplasmic tail. Interestingly, more than 800 GPCRs have been found in the human genome (Fredriksson and Schiöth 2005). The binding of agonists to GPCRs causes conformational changes in the transmembrane as well as intracellular regions. This

results in the activation of G proteins, which are heterotrimeric proteins containing three subunits (Gα, Gβ and Gγ). In the resting state, Gα binds to guanosine diphosphate (GDP) and is maintained in an inactive status. Upon platelet activation, GDP gets released and replaced by guanosine triphosphate (GTP). The binding of GTP to Gα causes the dissociation of the Gα from the Gβ/γ dimer (Oldham and Hamm 2008). Activated Gα induces platelet activation via the stimulation of intracellular signalling through several mechanisms. Platelets also express another group of GPCRs called protease-activated receptors (PARs), which are activated by cleavage of their N-terminal regions by thrombin (Clemetson 2012).

1.3.3.1 PARs

PARs play a critical role in normal and pathological state and have been targeted to treat cardiovascular disease. Around (1000-2000) PAR-1 receptors are present in each platelet. PAR-1 that contains 425 amino acids is the main receptor for thrombin with high affinity and, it is fast and induce transient downstream signalling.

Thrombin, a member of the serine protease family is produced at the site of vascular damage as a result of coagulation cascades. Thrombin contributes to haemostasis via two different mechanisms: (I) In the coagulation cascade, it converts fibrinogen into fibrin monomers, which polymerise and trap blood cells at the vessel injury site, leading to the development of a fibrin clot (Martin, Robertson et al. 1992); and (II) it induces platelet activation via PARs simultaneously to augment the coagulation process (Rivera, Lozano et al. 2009).

Thrombin-induced platelet activation occurs via PARs, PAR1 and PAR4. Thrombin binds to PAR-1 through hirudin-like sequence of the N-terminal exodomain and cleaves the extracellular domain, thus exposing the tethered ligand, which binds

to the receptor's extracellular loop. This binding results in conformational changes in PAR-1 receptor which leads to activation of $G\alpha_q$ and $G\alpha_{12/13}$ (Nanevicz, Ishii et al. 1995). The ligand binding initiates intracellular signalling leading to subsequent platelet activation, granule secretion and platelet aggregation through guanine nucleotide exchange factor (Rho-GEF) and activation of phospholipase C-β (PLCβ). The activation of PLCβ mediates the cleavage of PIP2 into IP3 and DAG, resulting in release of Ca^{2+} from intracellular stores and PKC activation, respectively (Brass 2003). Ca^{2+} release and activation PKC lead to granule secretion and integrin αIIbβ3 activation resulting in platelet activation and aggregation.

1.3.3.2 P2Y receptors

In 1961, ADP released from red blood cells was observed as an agonist of platelet activation by Gaarder et al. In 1962, Born observed that ADP is an agonist and can stimulate platelet activation (Fogelson and Wang 1996). In addition, it was found that dense granule secretion leads to the release of ADP, which has an important role in augmenting platelet aggregation and thrombus formation (Gachet 2006). ADP interacts with two different purinergic receptors: $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ (linked with $G\alpha_q$) is known to play a role in controlling the activation of PLCβ, which stimulates changes in the intracellular calcium concentration (Ohlmann, Laugwitz et al. 1995, Kauffenstein, Bergmeier et al. 2001). Such changes indirectly cause phosphorylation and activation of several signalling proteins.

In contrast, ADP binding to P2Y12 receptors is critical for $G\alpha_i$ activation, which binds to adenylyl cyclase and block the production of adenosine monophosphate (cAMP) (Yang, Wu et al. 2002). cAMP is important for the stimulation of protein kinase A (PKA), that play critical role in keeping platelets in resting state (Siess and Lapetina 1990, Raslan and Naseem 2014). P2Y12 receptor mediates signalling via binding to

Gβy subunit, result in the activation of phosphoinositide 3 kinase (PI3K), which is important signalling molecules for P2Y12 to mediate dense granule secretion (Vanhaesebroeck and Waterfield 1999, Lian, Wang et al. 2005). This activation of PI3K plays a critical role in platelet functional responses to ADP (Garcia, Kim et al. 2010). PI3K activation results in the production of PIP3 leading to activation of two main downstream targets, protein kinase B (AKT) and GTPase Rap1 B, that contribute to the activation of integrin αIIbβ3 (Canobbio, Stefanini et al. 2009). It has been reported that, in PI3K deficient platelets the aggregation response to ADP was reduced, demonstrating that PI3K is important for platelet activation by ADP (Hirsch, Bosco et al. 2001). In addition, *in vivo* studies using PI3K selective inhibitor (TGX-221), decrease arterial thrombus formation without significant bleeding effect, demonstrating that PI3K inhibition provides protective mechanism to treat arterial thrombosis (Gratacap, Guillermet-Guibert et al. 2010, Martin, Guillermet-Guibert et al. 2010, Laurent, Séverin et al. 2015).

Moreover, it has been observed that activation of both these receptors (P2Y1 and P2Y12) may be required for ADP-induced platelet activation (Pulcinelli, Ciampa et al. 1999). It has also been reported that blocking one of these receptors is sufficient to prevent ADP-induced platelet aggregation (Jin, Daniel et al. 1998). In antithrombotic therapy, purinergic receptors (mainly P2Y12) are important targets for thienopyridine class of drugs such as clopidogrel, due to their significant role in amplifying platelet activation (Jarvis and Simpson 2000). Clopidogrel is a pro-drug of an ADP receptor antagonist selectively inhibiting P2Y12 signalling pathway; include adenylyl cyclase down regulation and PI3K activation, which will reproduce platelet function defects to ADP (Cattaneo and Gachet 1999). Moreover, this pharmacological effect of

clopidogrel on platelet function was similar to the effect obtained P2Y12 knock-out mice and patients with deficient P2Y12 (Cattaneo 2005, Cattaneo 2011).

1.3.3.3 Thromboxane A_2 (TXA_2) receptor

TXA_2 is a prothrombotic mediator which is synthesised and released from platelets upon activation to augment further platelet activation. Initial platelet stimulation by an agonist such as collagen (or thrombin and ADP at later stages) causes a conversion of membrane phospholipids into arachidonic acid, which gets metabolised by cyclooxygenase-1 (COX-1) to produce TXA_2. TPα and TPβ are TXA_2 receptors in platelet plasma membrane that coupled to Gα and $G_{12/13}$, respectively. Similar to the mechanisms driven by thrombin and ADP, these two receptors induce platelet shape changes, regulation of integrin αIIbβ3 actvation and subsequent platelet aggregation and thrombus formation (Huang, Ramamurthy et al. 2004, Offermanns 2006). It has been reported that in mice which are deficient for TP receptors, a reduced platelet aggregation stimulated by collagen, prolonged bleeding time and weak platelet responses were observed (Thomas, Mannon et al. 1998).

1.4 The physiological role of platelets

Under normal conditions, platelets move through the circulatory system in a resting state. This resting state is maintained by certain substances, such as nitric oxides (NO) and prostaglandin (PGI_2), which are released from healthy endothelial cells. During vascular injury, platelets play an important role as the first line of defence. Platelet activation is vital for the maintenance of haemostasis. Their primary physiological function is to detect vascular damage and initiate coagulation by rapidly

aggregating together to seal the site of damaged tissue and prevent bleeding (Figure 1-5). Platelet activation produces thrombin, which in turn plays a role in activating the coagulation cascade and more platelets. The coagulation cascade leads to the formation of a fibrin-rich haemostatic plug, which is produced to prevent the blood loss from injured vessels. The haemostatic role of platelets begins upon vascular injury and can be classified into three stages: adhesion, activation and aggregation. Platelets are also involved in the coagulation cascade (known as the second wave of haemostasis), which leads to the formation of a stable thrombus.

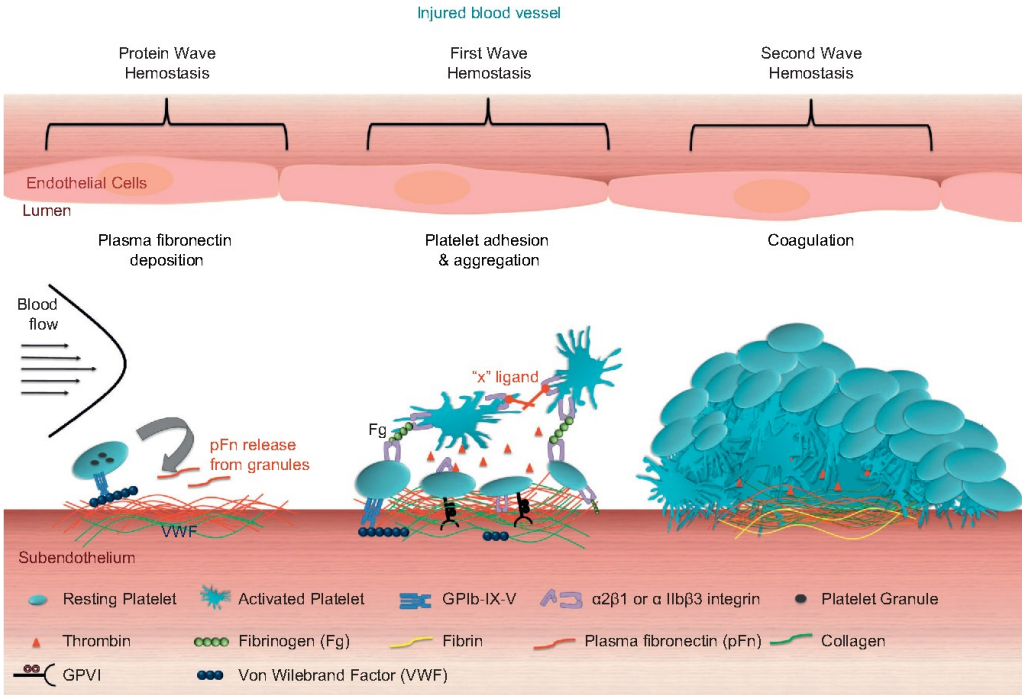

Figure 1-5 Platelet activation in response to a vascular injury. Platelets circulate in circulation in resting state due to the release of NO and PGI_2 from healthy endothelial cells. Injuru in a blood vessel exposes collagen which allow the binding of platelets due to platelet at high shear rate. Platelets aggregate on subendothelial collagen via GPVI and vWF through GPIb-V-IX, followed by platelet adhering along the subendothelial matrix. This followed by platelet activation due to binding of collagen to GPVI receptors, which initiates signalling events that lead to platelet shape change, granule secretion, integrin activation and fibrinogen binding. Release of TXA_2 and ADP causes further platelets to adhere to the site of injury and contributes to the coagulation cascade. In the haemostatic plug, fibrin and fibronectin form bridges between platelets to form a platelet plug. This figure was adapted from (Hou, Carrim et al. 2015)

1.5 Thrombosis

Platelets and coagulation factors together with endothelial cells are important mediators in haemostasis and thrombus formation. Inappropriate platelet activation upon the rupture of atherosclerotic plaque results in the formation of thrombi within the circulation. Thrombus formation results in occlusion or obstruction in the blood vessel.

Thrombosis can be classified into two main types; venous and arterial thrombosis, defining according to their site, whether it is in a vein or an artery. Arterial thrombosis is the main cause of myocardial infarction and stroke, and it results from the rupture of atherosclerotic plaque. This type of thrombosis is mainly activated by platelets and it primarily occurs at the site of high shear flow in arteries. Due to prominent roles of platelets, arterial thrombosis is treated with drugs that inhibit platelet activation and aggregation (e.g., Aspirin).

Venous thrombosis is a thrombotic vascular disease which affects deep and superficial veins of lower limbs. The main pathological mechanism which causes venous thrombosis is thrombud formation due to hypercoagulability and blood stasis (Pomero, Di Minno et al. 2015, Gaiz, Mosawy et al. 2017). The most common venous thrombosis is called a deep vein thrombosis constituting of fibrin, red blood cells and platelets. A thrombus that formed in the vein occurs due to stasis of blood in veins leading to activation of coagulation cascades and minor activation of platelets. Therefore, venous thrombosis is mainly treated with anti-coagulant therapies targeting proteins mediating coagulation [e.g. rivaroxaban (FXa inhibitor)] (Cines, Lebedeva et al. 2014).

1.6 Platelet activation

Initial platelet activation is triggered by collagen exposed from the sub-endothelial matrix at the damaged blood vessels. During vascular injury, platelets adhere to immobilised adhesive proteins, such as vWF and collagen, which are exposed in the subendothelial matrix (Nuyttens, Thijs et al. 2011). The vWF binds to glycoprotein receptors (GPIb-V-IX) through its A1 domain on the platelet's surface, and this binding causes the platelets to roll on the endothelium in the area of the damaged vessel (López and Dong 1997). The binding of platelets to vWF enables the interaction between platelets and collagen via GPVI and integrin $\alpha_2\beta_1$, allowing the formation of a platelet monolayer at the injury site.

This interaction between collagen and GPVI, is followed by signalling events leading to the secretion of pro-thrombotic agents such as ADP and release of TXA_2 (produced by oxygenation of arachidonic acid by cyclooxygenase-1 and TXA_2 synthase). These molecules act via specific platelet surface receptors, and activate more circulating platelets near the injury site, to form platelet plug. Following the formation of platelet plug, intracellular signalling occurs in activated platelets to stabilise the thrombus. This initiated by outside-in signalling due to the attachment of fibrinogen to αIIbβ3 as intracellular signalling stimulates the transformation of integrin αIIbβ3 from a low affinity state to a high affinity state (Rumbaut and Thiagarajan 2010). The activation of integrin αIIbβ3 in turn activates the fibrinogen receptor, which increases fibrinogen affinity/binding and facilitates the formation of cross links of fibrinogen between platelets to create a stable platelet aggregate characterised by cytoskeletal reorganisation and clot retraction, which contributes to haemostatic plug (Sánchez-Cortés and Mrksich 2009).

Moreover, Platelet activation produces thrombin, which in turn plays a role in activating the coagulation cascade. The coagulation cascade involves the conversion of blood from the liquid phase to the thick structure phase to prevent blood loss. This process involves more than 30 proteins that are known as coagulation factors; these proteins act through different pathways, however, both intrinsic and extrinsic pathways generate activated factor X (Spronk, Govers-Riemslag et al. 2003), which leads to the cleavage of prothrombin (Factor II) to thrombin (Factor IIa). Factor IIa acts as a catalyst for the conversion of soluble fibrinogen (Factor I) to insoluble fibrinogen (Factor Ia), which, along with platelet activation, results in a mesh-like network of aggregated platelets (Chu 2011). This cascade leads to the formation of a fibrin-rich haemostatic plug, which is produced to prevent blood loss from injured vessels (Hoffman 2003).

Finally, fibrinolysis is a regulated enzymatic mechanism that prevents the unnecessary growth of thrombi by preventing the extra accumulation of fibrin, via inducing fibrinolysis (Chapin and Hajjar 2015). Plasmin is formed from plasminogen through the action of tissue plasminogen activator on the surface of the fibrin clot, and it plays an important role in dissolving the clot (Chapin and Hajjar 2015). This process limits the development of the thrombus, and also dissolves the clot to rescue the blood supply and induce wound healing.

1.7 Common platelet activation processes

Changes in the cytosolic Ca^{2+} level are a primary mechanism of action that is shared by all platelet agonists/receptors to activate downstream pathways (Varga-Szabo, Braun et al. 2009). Ca^{2+} signalling is well known to play a critical role in the platelet activation process via regulating the cytoskeletal rearrangement and degranulation, as well as raising the affinity of integrin αIIbβ3 to fibrinogen (Rink and Sage 1990, Harper and Sage 2017). The main mechanisms to increase calcium concentration are via; (I) production of IP3 that causes the release of Ca^{2+} from endoplasmic reticulum and (II) calcium channels in the plasma membrane via increase in the influx of extracellular Ca^{2+} from plasma (Bergmeier and Stefanini 2009).

As a result of increased Ca^{2+} ions in the cytosol, many activation events occur in platelets such as activation of integrin αIIbβ3, granule secretion and synthesis of TXA_2 (Stefanini, Roden et al. 2009). PLC activation plays a fundamental role in platelet activation and it can be activated by various platelet agonists. The activation of PLC causes downstream events; PLC catalyses the production of PIP2 leading to formation of IP3 and release as a soluble molecule into the cytosol. IP3 then binds to IP3 receptors, main calcium channels in the DTS, causing an increase in cytosol calcium concentration (Brass and Joseph 1985, Varga-Szabo, Braun et al. 2009). The DTS is the main store of intracellular Ca^{2+}. The secretion of Ca^{2+} from the endoplasmic reticulum into the cytosol facilitates further extracellular influx of Ca^{2+} across the plasma membrane from plasma (Stathopulos, Zheng et al. 2008).

The extracellular influx of Ca^{2+} mainly mediated via store-operated calcium entry (SOCE). The interaction of activated STIM1 with Orai1 allows Ca^{2+} to enter through this channel (Grosse, Braun et al. 2007). Ca^{2+} can also enter the platelets through a mechanism independent of SOCE. It has been suggested that synthesis of

DAG leads to activation of the transient receptor potential channel sub-family C (TRPC6), a non-selective cation channel that controls the influx of Ca^{2+} into the platelets in a SOCE-independent mechanism (Hassock, Zhu et al. 2002, Authi 2007).

Apart from this, P2X receptors (P2X1-P2X7), which are ATP-gated ion channels, are recognised as highly permeable channels for Ca^{2+} entry (Mahaut-Smith, Jones et al. 2011, M Waszkielewicz, Gunia et al. 2013). A purinergic P2X1 receptor activated by ATP binding and it facilitates Ca^{2+} entry via non-selective cation channels (AVDONIN, CHEGLAKOV et al. 1991). Indeed, ATP has been suggested as the main physiological agonist for P2X1 in platelets (Mahaut-Smith, Tolhurst et al. 2004, FUNG, CENDANA et al. 2007).

1.8 Negative regulation of platelet activation

As detailed above, platelets play an essential role in maintaining the normal haemostatic process. However, any impairment to this process can lead to inappropriate activation of platelets, which can lead to pathological conditions such as thrombosis. Therefore, in the absence of injury, the balance is maintained in platelets by keeping them in an inactive state via the action of inhibitory agents that secreted via healthy endothelial cells. These cells express and release molecules such as nitric oxide (NO) and prostacyclin (PGI_2) to control inappropriate activation of platelets (Mitchell, Ali et al. 2008). Therefore, endothelial dysfunction can result in an imbalance between procoagulant and anticoagulant molecules leading to pathological conditions such as atherosclerosis and venous thrombosis (Widmer and Lerman 2014). The most common mechanism that causes endothelial dysfunction is increased oxidative stress and thereby a decreased level in NO bioavailability (Lavi, Yang et al. 2008).

1.8.1 PGI_2

PGI_2 is produced from endothelial cells by the metabolism of arachidonic acid and secreted into the circulation (Mitchell and Warner 1999). PGI_2 functions by binding to the prostaglandin receptor that is coupled to the $G_{\alpha s}$ in the platelet membrane (Dutta-Roy and Sinha 1987). The activation of $G_{\alpha s}$ results in the activation of adenylyl cyclase, which in turn induces the production of cAMP by catalysing ATP (Gorman, Bunting et al. 1977). cAMP is responsible for phosphorylation of several substrates, such as Rap1B. Rap1B is important in integrin $\alpha IIb\beta_3$ activation and is either directly phosphorylated or phosphorylated in a CalDAG-GEFI-dependent manner via IP3, which is critical for intracellular calcium mobilisation. Thus, low levels of cAMP leads to the inhibition of PKA (Bye, Unsworth et al. 2016). Moreover, vasodilator stimulated

phosphoprotein (VASP), which is a substrate of PKA, is involved in remodelling the platelet cytoskeleton via actin polymerisation. The phosphorylation of VASP downregulates platelet shape changes (Harbeck, Hüttelmaier et al. 2000, Jensen, Selheim et al. 2004).

1.8.2 NO

NO is a small signalling molecule that is synthesised and released into the circulation from endothelial cells using an amino acid, L-arginine via the action of an enzyme, endothelial nitric oxide synthase (eNOS) (Tousoulis, Kampoli et al. 2012). The NO rapidly diffuses across the platelets through the plasma membrane and binds to soluble guanylyl cyclase (sGC). This results in the formation of cyclic guanosine monophosphate (cGMP), leading to the activation of protein kinase G (PKG), which is a cGMP-dependent protein kinase (Du 2007). Similar to PKA, PKG can also affect platelet activation via controlling the inositol triphosphate receptor (IP3R), which mediates calcium release from intracellular stores (Schlossmann, Ammendola et al. 2000). cGMP/PKG also causes inhibition of TXA_2 function via phosphorylation of its receptor. Similar to TXA_2, VASP phosphorylation is activated by PKG activity (Low and Bruckdorfer 2004). In addition, cGMP can downregulate the activity of phosphodiesterase-3, the enzyme that is responsible for cAMP degradation, as well as negatively regulate the activity of granule secretion, integrin activation and aggregation (Pigazzi, Heydrick et al. 1999, SMOLENSKI 2012).

1.9 Pathological roles of platelets

1.9.1 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease that plays a significant in the progression of CVD specifically thrombotic diseases. It mostly occurs in the arteries at sites, such as in the branches of blood vessels (Tsompanidi, Brinkmeier et al. 2010). Atherosclerosis could be defined as a collection of fatty deposits in a form of an “atheroma” within arteries. The presence of an atheroma in coronary arteries will cause a partial occlusion of blood flow, leading to angina, and if a clot is formed (thrombosis) upon rupture of the plaque, it could cause a complete blockage resulting in myocardial infarction (heart attack).

The sub-endothelial deposition of low-density lipoprotein (LDL) rich in free cholesterol, is the first stage of the development of vascular endothelial inflammation that leads to the progression of atherosclerotic plaques. LDL is subsequently modified to oxidized LDL (oxLDL), a process that is influenced by cardiovascular risk factors such as hypertension, diabetes, or smoking that all increase oxidative stress in the vascular wall (Witztum and Steinberg 1991, Stocker and Keaney Jr 2004).

Oxidized LDL leads to the expression a number of adhesion molecules on the vascular surface of endothelial cells including vascular adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), that leads to the recruitment of monocytes to the vascular wall (Huo, Hafezi-Moghadam et al. 2000) and their subsequent migration to the intima. The local inflammation leads to further activation monocytes to transform into macrophages to initiate inflammatory response by secreting a number of cytokines such as IL-1β, and TNF-α (Moore, Sheedy et al. 2013). The monocytes continue to recruit to form more macrophages which engulf the

excessive oxLDL and form the fatty steak in vascular wall. More cells such as vascular smooth muscle cells (VSMC) proliferate and contribute to the growth of plaque by covering the damaged area. This growth of plaque will result in local tissue hypoxia, which in turn helps neovascularisation in the atherosclerotic lesions (Falk, Shah et al. 1995). The inflammatory response of endothelium also generates a pro-thrombotic response (Theilmeier, Michiels et al. 2002, Steffel, Hermann et al. 2005). Over time, this developing lipid deposit will narrow the lumen of blood vessels (Tsompanidi, Brinkmeier et al. 2010). The plaque will gradually increase in size, leading to damage of the arterial endothelium and expose subendothelial matrix causing platelet activation, which in turn initiate thrombosis (Figure 1-6).

Huo et al. (2003) demonstrated that the activation of platelets aids in the progression of atherosclerosis in a paracrine manner by the formation of platelet–leukocyte aggregates and via the accumulation of pro-inflammatory mediators (Huo, Schober et al. 2003). Moreover, *in vivo* studies examining receptor–ligand interactions at the cellular level have demonstrated that the adhesion of platelets to the endothelium is important for the early stages of atherosclerotic lesion formation (Massberg, Brand et al. 2002). By contrast, a normal, healthy endothelium is known to prevent platelet adhesion to the vascular wall. Moreover, *in vitro* studies have demonstrated that the adhesion of platelets to endothelial monolayer results in platelets producing pro-inflammatory cytokines, such as CD40L and IL-1β. These molecules augment endothelial inflammation, and stimulate the pro-atherosclerotic properties of endothelial cells (Henn, Slupsky et al. 1998).

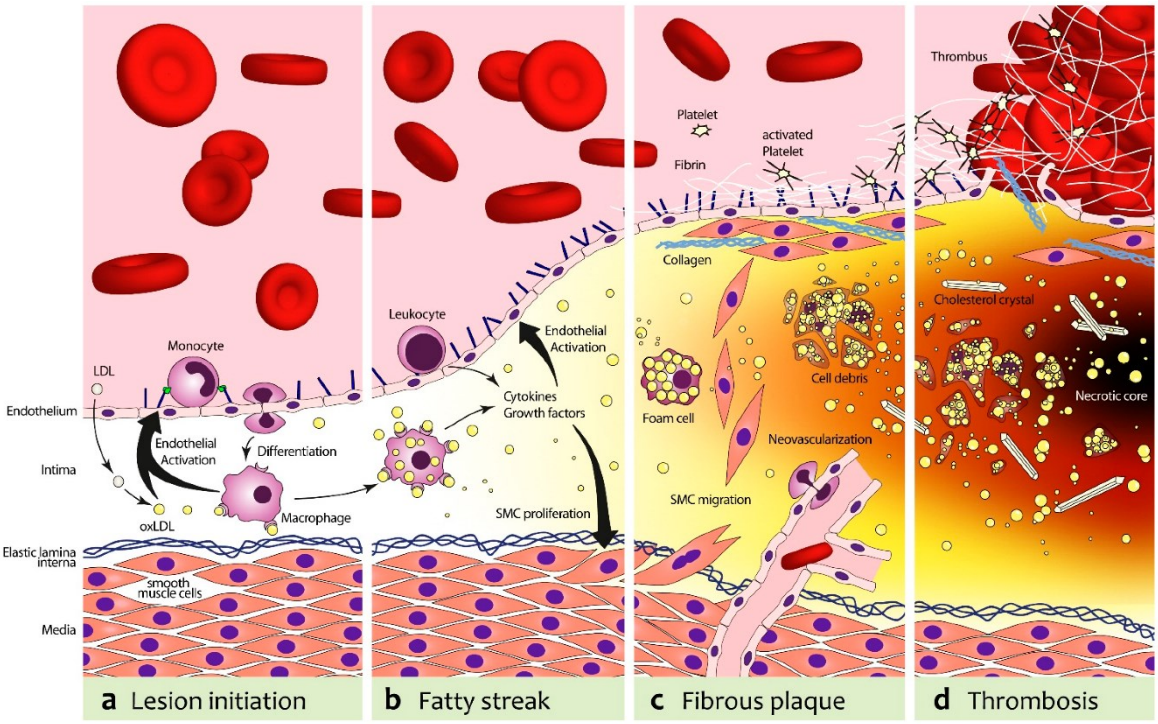

Figure 1-6. Pathogenesis of atherosclerosis development. (**a**) In the first step, low density lipoprotein (LDL) rich in free cholesterol enters in the endothelial space in large quantity and undergoes oxidation to form oxidised LDL (oxLDL). OxLDL stimulates endothelial cells to express VCAM-1, and various of pro-inflammatory molecules. This leads to adherence and recruitment of monocytes into the affected region, and they transform into macrophages; (**b**) Macrophages harvest residual oxLDL leads to further endothelial activation and, subsequently, immune cells recruitment. Macrophages that engulfed a large amount of oxLDL appear to be foam cells. (**c**) The increasing plaque size promotes neovascularization. Proliferating smooth muscle cells (SMCs) to stabilise the nascent fibrous plaque. The deposition of fibrin and activated platelets on damaged endothelium expresses tissue factor and vWF; (**d**) Macrophages (foam cells) undergo apoptosis and release lipid core, which will result in the formation of a necrotic core. In addition, proteases secreted from foam cells can destabilize the plaque and can lead to plaque rupture, which can cause thrombotic events. This figure was adapted from (Steinl and Kaufmann 2015).

1.10 Inflammation

Inflammation is a biological response that protects the human body from pathogens and repairs damaged tissues. Inflammatory responses stimulate vasodilation to increase the blood flow, the release of soluble mediators, and the elevation of cellular metabolism. Inflammation is a tightly controlled process that aims to restore and maintain the homeostasis following an infection or injury. Various components of the infected/damaged tissue are altered due to the presence of pathogens or dead cells (Libby 2007). This change in the chemical composition of the tissue stimulates the release of inflammatory mediators such as histamine and prostaglandin. The release of these mediators induces vasodilatation and thus increases blood flow to the affected area, as well as increases the transport and migration of inflammatory cells into the affected region. Moreover, the permeability of the endothelium is increased to promote infiltration of immune cells (Libby 2007).

1.10.1 Role of platelets in inflammation

While the primary physiological role of platelets is to maintain haemostasis, they also play a critical role in inflammation through adaptive immune responses (Weyrich and Zimmerman 2004). Platelets promptly come into contact with pathogens entering the circulation or any damage to tissues (Shiraki, Inoue et al. 2004). Platelets contribute to inflammation via various immune functions through their ability to interact with different types of leukocytes, and their capacity to release a large number of immunomodulatory molecules (Palabrica, Lobb et al. 1992).

α-granules contain various cytokines, chemokines and growth factors, and they undergo regulated exocytosis upon activation to release of their contents into the

extracellular environment (Morrell, Aggrey et al. 2014). Cytokines are packaged in platelet granules during the process of megakaryocyte development (Veljkovic, Cramer et al. 2003). Despite the absence of a nucleus, the platelets can synthesise and secrete proteins such as IL-1β and IL-18 by using megakaryocyte-derived mRNA molecules (Lindemann, Tolley et al. 2001, Allam, Samarani et al. 2012). Levels of cytokines released from activated platelets are elevated during several pathological conditions such in cancer (Peterson, Zurakowski et al. 2012, Fu, Fu et al. 2015), showing an active uptake of these cytokines due to either platelet hyperactivity or changes in megakaryopoiesis in response to a disease. This indicates that underlying pathologies can influence platelet reactivity and their role in regulating immune responses.

It is well established that the formation of platelet-leukocyte aggregates is one of the principal processes in pathological conditions such as inflammation, atherothrombosis (May, Langer et al. 2007), and rheumatoid arthritis (Joseph, Harrison et al. 2001). The adhesive molecules that are located on platelet surface, such as P-Selectin, facilitate direct interactions between platelets and immune cells (Frenette, Johnson et al. 1995, Chen and Geng 2006).

An increase in the number of platelet-leukocyte aggregates in the circulation reflects the role of platelets in inflammation. This interaction acts as an important diagnostic marker for inflammatory processes and thrombosis, (McCabe, Harrison et al. 2004), and myocardial infarction (Michelson, Barnard et al. 2001, Htun, Fateh-Moghadam et al. 2006). Furthermore, this interaction modulates immune responses, resulting in local cytokine release (Schrottmaier, Kral et al. 2015). Various secretory

mediators are released upon platelet activation, which contribute to modulation of immune and inflammatory responses (McNicol and Israels 2008).

Pro-inflammatory cytokines released from platelets also stimulate the migration of monocytes into the affected tissue. IL1β, IL-6 and TNFα are pro-inflammatory cytokines that are released via different stimuli such as histamines and prostaglandins (Galley and Webster 1996). IL-1β and TNFα stimulate the activation of the transcription factor nuclear factor-κB (NF-κB) in immune cells. The activation of NF-κB via the phosphorylation and degradation of IκB leads to its translocation into the nucleus, where NF-κB promotes the transcription of pro-inflammatory cytokines (Rousell, Haddad el et al. 1997).

1.10.2 Platelets in inflammatory diseases (e.g. sepsis)

The main cause of a systemic inflammatory response is the presence of a microbial population within the circulatory system, which allows pathogenic molecules to access every region of the host system through the vasculature and initiate infection on a large scale. However, a response from the immune system begins to remove pathogens in order to prevent any damage that might occur to the host tissue. Sepsis, a life-threating condition, develops due to the dysregulated response of the immune and haemostatic systems in response to pathogens (Vincent and Abraham 2006). Sepsis presents as a severe systemic inflammatory response to bacterial, fungal or viral infections in the form of a high fever and an increased number of leukocytes at the initial stage. Notably, the increase in vascular permeability that is caused by disrupted endothelial barrier function also increases the possibility of sepsis (Goldenberg, Steinberg et al. 2011).

Alterations to platelet number and distribution are common in human sepsis (Aydemir, Piskin et al. 2015). Because sepsis is the main cause of thrombocytopaenia, there is a significant link between the number of platelets in circulation and sepsis (Semple and Freedman 2010). Platelets and fibrinogen together form neutrophil aggregates around endothelial cells during inflammation (Kirschenbaum, McKevitt et al. 2004). Formation of a thrombus in the vasculature (Semeraro, Ammollo et al. 2010), leads to a low level of platelets and coagulation proteins, which leads to bleeding complications in most cases of sepsis (Iba, Gando et al. 2016). Furthermore, it has been found that atherosclerosis-related systemic microvasculature dysfunction causes symptomatic of organ damage in sepsis (Goldenberg, Steinberg et al. 2011, Lee and Liles 2011). The sequestration of platelet aggregates was also found in organs such as the lungs and kidenys.

Platelets also influence the clinical outcomes of sepsis through destabilisation of the endothelial barrier. Angiopoietin 1 (Ang-1) is a well-known protein that supports vasculo-protective effects by suppressing blood leakage and causing inhibition of vascular inflammation (Brindle, Saharinen et al. 2006, Simoes, Vassilakopoulos et al. 2008). Notably, platelets are the major source of Ang-1, which is stored in α-granules (Li, Huang et al. 2001) . In sepsis patients, it has been shown that the level of Ang-1 is significantly decreased (Ricciuto, dos Santos et al. 2011, Claushuis, van Vught et al. 2016). Therefore, thrombocytopenia can lead to adverse outcomes by reducing the supply of Ang-1 to the endothelium. Another mechanism of the contribution of platelets to sepsis is via their interaction with immune cells (Vieira-de-Abreu, Campbell et al. 2012). Platelets can initiate inflammatory responses as well as the clearance of pathogens, which may result in vascular and tissue damage and thus the exacerbation of sepsis symptoms (Herter, Rossaint et al. 2014, Xu, Zhang et al. 2016).

1.11 Significance of natural products and essential oils in human health

Natural products have long had a vital role in the treatment and prevention of numerous human diseases. For many years, natural products that are derived from plants have been an important source for drug discovery. For example, more than 40 new pharmaceutical drugs that were developed between 1990 and 2000 were derived from natural products (Newman, Cragg et al. 2003). In addition, according to the World Health Organisation (WHO), ~80% of people worldwide still depend on traditional medicine derived from plant sources for their healthcare at various stages.

1.11.1 Medicinal plants

For thousands of years, human beings have used medicinal plants containing a vast array of compounds that are now known for their therapeutic effects. Indeed, medicinal plants have played a vital role for the development of new drugs. Several plant compounds also act as templates for novel drug design. With the huge discovery and development of pharmaceuticals, the use of medicinal plants as a source for new drugs has been increasing in medicine, including as whole plant preparations or isolated compounds or isolated essential oils from plants (Maffei, Gertsch et al. 2011). Additionally, more studies using medicinal plants were encouraged by the WHO to develop new drugs with less side effects. Furthermore, a number of evidences demonstrate that high consumption of fruit and vegetables can protect human health from CVD by inhibiting platelet function (Estruch, Martínez-González et al. 2006, Vilahur and Badimon 2013). In recent years, several studies from different research groups around the world have investigated the anti-platelet activities of compounds isolated from numerous medicinal plants (Ayatollahi, Javan et al. 2014, Yasin, Hussain

Janbaz et al. 2014, Zhang, Zhao et al. 2014). However, in a huge number of these studies, the specific mechanism of antiplatelet actions and the bioactive molecules of isolated plants are still unknown.

1.11.1.1 Flavonoids

Flavonoids are naturally occurring compounds that found primarily in plants and they often consumed as part of a regular diet. They have shown to exert many beneficial effects for human health against various diseases (Kumar and Pandey 2013, Panche, Diwan et al. 2016). Thus, they have been a potential source for the development of new therapeutic agents. The impact of flavonoids on platelet function has been investigated in numerous studies. For example, nobiletin and tangeretin that found in citrus fruits have been investigated for their antiplatelet effects in our laboratories (Vaiyapuri, Ali et al. 2013, Vaiyapuri, Roweth et al. 2015). These two compounds were found to inhibit platelet aggregation and thrombus formation with minimal effects on haemostasis. In addition, quercetin (the richest flavonoid in human diets such as in onions, grapes and wine), apigenin, luteolin, catechin and chrysin are structurally diverse flavonoids, and they have been shown to inhibit platelet function (Guerrero, Lozano et al. 2005, Wright, Moraes et al. 2010). In addition, flavonoids from *Justicia procumbens* and *Cephalotxus wilsoniana*, have been shown to exert anti-platelet activity by inhibiting platelet activation induced by adrenaline, and purple juice has also demonstrated antiplatelet effects by suppressing platelet aggregation induced by ADP (Freedman, Parker Iii et al. 2001). Overall, flavonoids are well known for their impact on the control of platelet activation. However, it is difficult to achieve required concentrations of many of these molecules in the circulation via the

consumption of regular diets. Hence, dietary supplements of many of these compounds became available in the market.

1.11.1.2 Alkaloids

Alkaloids are another group of organic molecules found in plants and they account for about 20% of plant compounds and are secondary metabolites of plants (Amirkia and Heinrich 2014, Khan 2016). Plants with rich source of alkaloids are known to have anticoagulant and antiplatelet properties (Ain, Khan et al. 2016). For example; an alkaloid, rutaecarpine from *Evodia rutaecarpa* and its derivatives 2,3-methylenedioxyrutaecarpine, 3-chlororutaecarpine and 3-hydroxyrutaecarpine have demonstrated to possess antiplatelet activity by inhibiting platelet aggregation and clot formation (Sheu, Hung et al. 1996, Son, Chang et al. 2015). Similarly, spiramine C1 is an alkaloid isolated from *Spiraea japonica*, has been reported to inhibit platelet aggregation induced by PAF (Li, Shen et al. 2002). Moreover, β-carboline alkaloids isolated from *Preganum harmala* have also demonstrated antiplatelet activities.

1.11.2 Essential oils

An essential oil is a concentrated hydrophobic liquid containing volatile chemical compounds formed by aromatic plants as secondary metabolites. Essential oils contribute to the secondary metabolism of plants and are highly enriched compounds of an isoprene structure, and they can be classified into three major compounds: phenolic, alkaloid and terpene (Bakkali, Averbeck et al. 2008). The main feature of terpene molecules is that they contain carbon double bonds (c=c). Terpene compounds can also be classified based on their quantity of isoprene structures, including hemiterpenes (C_5H_8) and monoterpenes ($C_{10}H_{16}$) such as citral, menthol and linalool in essential oils (Little and Croteau 1999). Terpene is a broad class of organic

chemical molecules and comprises around 90% of essential oils (Bakkali, Averbeck et al. 2008).

Monoterpenes are the main active ingredients of essential oils, and have a number of biological functions including anti-inflammatory, antimicrobial, antioxidant and antiviral effects (Bohlmann, Meyer-Gauen et al. 1998). The lipophilic characteristics of monoterpenes including their absorption and rapid action, make them a promising agent in the modulation of cytokine function (Cristani, D'Arrigo et al. 2007, Quintans, Shanmugam et al. 2019). Moreover, monoterpenes have been reported to stimulate the synthesis and release of anti-inflammatory cytokines such as IL-10 (Lima, Quintans-Júnior et al. 2013).

1.11.3 Eucalyptol (1,8-cineole)

Eucalyptol, which is also known as 1,8-cineole, a natural bicyclic monoterpene was identified by F. S. Cloez in 1870. It is the main compound in the essential oil obtained from *Eucalyptus globulus*. This plant is native to Tasmania, although there are around 600 different species of eucalyptus in Australia. It is also now grown in many warmer climates around the world (Brandfonbrener 2002). Eucalyptus trees have been used in traditional medicine in Australia, with its therapeutic effects firstly introduced in Chinese, Greco European and Indian herbal medicine (Mota Vde, Turrini et al. 2015). The essential oils of eucalyptus can be extracted by harvesting the leaves of the tree through steam distillation (Lis-Balchin, Deans et al. 1998).

Eucalyptus oil is used to relieve many medical symptoms, including cold and influenza, and drinking it as a tea has been shown to reduce mucus and thereby help open the blocked nasal cavities (Allan and Arroll 2014). It has also been widely used

as an ointment to relieve muscle problems due to its ability to increase the blood flow to muscle tissue (Tagaya, Tamaoki et al. 2000).

Eucalyptus oil has an antibacterial effect, as it has been shown to kill several strains of *Streptococcus* (Villecco, Catalan et al. 2008). Eucalyptus oil's antimicrobial activity has also been shown against several antibiotic-resistant strains of *E. coli* and *Pseudomonas* species (OULIA, SADERI et al. 2009, Knezevic, Aleksic et al. 2016). Likewise, eucalyptus oil has antiseptic properties and can be used for small cuts as a topical antiseptic (Hendry, Worthington et al. 2009).

Eucalyptol has many pharmacological effects. The nomenclature "1,8" refers to the single oxygen atom that is bonded to both the first and the eighth carbon atoms (Figure 1-7) (Mota Vde, Turrini et al. 2015). 1,8-cineole is a colourless organic liquid compound comprised of 90% in generic eucalyptol oil, which can be purified via distillation from the leaves of eucalyptus tree. Although eucalyptol can be used in flavouring food and in other medicinal items, such as mouthwash and cough suppressants, it is normally used in low doses. It is insoluble in water, has a boiling point of 170°C and a minty smell/spicy taste (Masadeh, Gharaibeh et al. 2013).

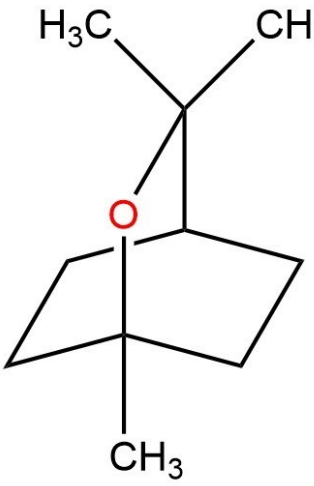

Figure 1-7: Molecular structure of 1,8-cineole ($C_{10}H_{18}O$)

1.11.3.1 Anti-inflammatory effects of 1,8-cineole

The investigation of the pharmacological effects of 1,8-cineole has become an area of interest for researchers in several fields over the last two decades. For example, Jurgens *et al*.'s (2004) *in vitro* study observed that at 0.15 µg/mL 1,8-cineole shows anti-inflammatory activity by inhibiting lipopolysaccharide-stimulated production of cytokines such as TNF-α and IL-1β in monocytes. Their study demonstrated the ability of 1,8-cineole to control the hypersecretion of airway mucus in asthma. The authors also found that 1,8-cineole can decrease the metabolism of arachidonic acid as well as decrease the cytokine production in human monocytes (Juergens, Engelen et al. 2004).

In bronchial vessels, cytokines such as TNF-α and IL-1β stimulate endothelial cells, which in turn express both the VCAM-1 and ICAM-1. The expression of these two molecules results in leukocyte migration to the site of infection (Garcia, Xia et al. 2000). Leukotrienes, a family of eicosanoid inflammatory mediators that are synthesised at high levels during asthma are also able to induce the migration of leukocytes to the subendothelial region of vasculature (Gimbrone, Brock et al. 1984, Barnes, Chung et al. 1998). Juergens and collaborators demonstrated a significant inhibition of cytokines, leukotriene and thromboxane B2 in human blood monocytes after treatment with 1,8-cineole for 3 days with a dose of 200 mg, 3 times a day (Juergens, Stöber et al. 1998). Similarly, Li and collaborators observed a significant effect of 1,8-cineole in the expression of ICAM-1 and VCAM-1, as well as suppression of proinflammatory cytokines and transcription factors on the cell surface of mice after exposure to the influenza virus. The effect on the 1,8-cineole treated group was similar to the oseltamivir-treated group (positive control), which is an antiviral drug used to treat the influenza virus (Li, Lai et al. 2016).

Chemokines such as IL-8, bind to a GPCR on inflammatory cells and regulate their adhesion to pulmonary interstitium (Yamagata and Ichinose 2006). *In vivo* experiments have demonstrated the effectiveness of 1,8-cineole in the reduction of IL-8 and the number of leukocytes that adhered to a bronchoalveolar lavage in HDM-stimulated bronchial epithelial cells and in the HDM-induced murine asthma model (Lee, Park et al. 2016).

Furthermore, the anti-inflammatory effects of 1,8-cineole were evaluated by investigating patients who were diagnosed with severe asthma. Thirty-two patients were recruited in a double-blind placebo-controlled trial, with the majority of those patients taking 200 mg of 1,8-cineole 3 times a day. The asthma patients who received 1,8-cineole remained stable compared to the placebo group. This was the first study to show the clinical anti-inflammatory effects of 1,8-cineole in the treatment of bronchial asthma (Juergens, Dethlefsen et al. 2003).

Moreover, 1,8-cineole has been investigated under *in vivo* settings in which rats were injected with trinitrobenzene sulfonic acid (which is an exogenous agent used to induce colitis in laboratory animals in order to model inflammatory bowel disease) as a marker for drug activity against human inflammatory bowel disease (Chen, Wang et al. 1999). 1,8-cineole was given to male rats 24 hours before pre-treatment with trinitrobenzene sulfonic acid. The administration of trinitrobenzene sulfonic acid resulted in severe inflammation as well as colonic ulceration. The animals that were pre-treated with 1,8-cineole demonstrated a significant decrease in the degree of inflammed tissue. This study suggests that 1,8-cineole may have an anti-inflammatory effect on gastrointestinal inflammation (Santos, Silva et al. 2004).

TLR4 plays an important role in inflammation, and it should be noted that in a signalling pathway that is associated with TLR4, p38 MAP kinase (MAPK) plays a critical role in inflammation response (Bhattacharyya, Brown et al. 2007). Conversely, it has been demonstrated that 1,8-cineole decreased the expression of TLR4 in mice after inducing lung inflammation by lipopolysacharide (LPS), compared to the effect of prednisone (positive control group), which is an anti-inflammatory drug (Zhao, Sun et al. 2014).

In an *in vivo* study conducted by Nascimento and collaborators, a reduction of tracheobronchial resistance was observed by 1,8-cineole after bronchospasm was induced by carbachol. The effect of 1,8-cineole was similar to the effect obtained with phenoterol, a drug used to treat asthmatic crises (Nascimento, Refosco et al. 2009). In addition, in the same study, the authors observed that 1,8-cineole also relaxed tracheal rings precontracted by carbachol and high potassium (K^+ 80 mM). The authors suggests that 1,8-cineole either may act as an antagonist on the transmembrane influx of Ca^{2+} or may have an intracellular effect as a second messenger.

1.11.3.2 Cardiovascular effects of 1,8-cineole

The cardiovascular effects of 1,8-cineole was investigated by Soares and collaborators as suggested that 1,8-cineole may induce a negative inotropic effect on heart tissue in rats, as it caused a reduction in the influx of Ca^{2+} via voltage-operated calcium channels in the sarcolemma of cardiac myocytes (Soares, Damiani et al. 2005). The authors suggested that this effect of 1,8-cineole could be related to its negative regulation of the influx of Ca^{2+} through cell membranes. Furthermore, it was

observed in the tracheal smooth muscle that 1,8-cineole also inhibits Ca^{2+} through the voltage-gated calcium channel (Pereira-Gonçalves, Ferreira-da-Silva et al. 2018).

1.11.3.3 Anti-cancer effect of 1,8-cineole

Recently, the effect of 1,8-cineole on cancer cells was established as it was observed that 1,8-cineole induced apoptosis in leukaemia cells (Moteki, Hibasami et al. 2002). Roettger et al. (2017) found that 1,8-cineole acts as an inhibitor of the WNT signalling pathway, which is known to lead to cancer progression, for example through GSK-3 (Ser 9/21), β-catenin and WNT11, in head and neck squamous cell carcinoma cell lines (Roettger, Bruchhage et al. 2017).

Since 1,8-cineole has a useful role in treating inflammatory diseases, it may be beneficial in the treatment of thrombotic diseases. In addition, as discussed above, under *in vivo* settings, platelets contribute to the inflammatory or immune responses. This project focuses on investigating the role of 1,8-cineole in the modulation of platelet function.

1.12 Hypothesis

1,8-cineole modulates platelet function, thrombosis and haemostasis through distinct mechanisms and therefore it will act as a powerful antiplatelet agent.

1.13 Research objectives

Previous work on the anti-inflammatory effects of 1,8-cineole has focussed largely on the ability of 1,8-cineole to modulate inflammation. However, the anti-platelet effects of 1,8-cineole have not yet been established. Therefore, the overarching aim of this project was to assess the potency of 1,8-cineole in platelets and establish the mechanisms through which, it affects platelet function.

The main research questions addressed in this study therefore include:

- Does 1,8-cineole inhibit platelet functions and *in vitro* thrombus formation under arterial flow conditions?
- Does it affect *in vivo* haemostasis in a tail bleeding model in mice?
- Does it inhibit platelet-mediated inflammatory responses?
- What are the mechanisms through which 1,8-cineole inhibits platelet function?

2- MATERIALS AND METHODS

2.1 Materials

2.1.1 Platelet agonists

In this study, various platelet agonsits were used to determine the impact of 1,8-cineole in the modulation of platelet reactivity. The cross-linked collagen-related peptide (CRP-XL) was obtained from Professor Richard Farndale (University of Cambridge, UK). Horm collagen was purchased from Nycomed, Austria. Thrombin and ADP were purchased from Sigma-Aldrich, UK. Thromboxane A2 receptor agonist, U46619 was obtained from Tocris, UK. All other chemicals used were obtained from Sigma-Aldrich, UK unless otherwise specified.

2.1.2 Antibodies

Information regarding the antibodies used for this study is listed in Table 2.1, along with their applications and concentrations used.

2.1.3 Animals

To perfom the tail bleeding assay, C57BL/6 mice were obtained from Envigo, UK.

Table 2.1 List of antibodies used for this study.

Antibody	Host	Clone number	Application	Dilution	Source and catalogue no.
FITC conjugated anti-human fibrinogen	Rabbit	FL-D6	Flow cytometry	1:50	Dako (Glustrub, UK) F0111
PE/Cy5 anti-human CD62P monoclonal antibody	Mouse	AK-4	Flow cytometry	1:50	BD Biosciences, UK. 551142
PE-conjugated anti-human CD45	Mouse	HI30	Flow cytometry	1:50	BD Biosciences, UK. 555483
FITC-conjugated anti-human CD42b	Mouse	HIP1	Flow cytometry	1:50	BD Biosciences, UK. 561855
Phospho-AKT (S473)	Rabbit	EP2109Y	Western blotting	1:1000	Abcam, UK ab81283
Phospho-LAT (Y200)	Rabbit	EP983(2)Y	Western blotting	1:1000	Abcam, UK ab68139
Phospho-Src (Y527)	Rabbit	36D10	Western blotting	1:1000	Cell signalling, UK. 21055
Phospho-SYK (Y323)	Rabbit	EP573-4	Western blotting	1:1000	Abcam, UK Ab62338
Anti- p38/MAPK	Rabbit	E229	Western blotting	1:1000	Abcam, UK Ab170099
Anti-ERK1/2	Rabbit	SP327	Western blotting	1:1000	Abcam, UK Ab17942
Phospho-VASP (Ser157)	Rabbit	5C6	Western blotting	1:1000	Abcam, UK Ab58555
Anti-human 14-3-3ζ	Mouse	D7H5	Western blotting	1:2000	Santa Cruz Biotechnology, USA Sc-293415

2.2 Methods

All the experimental procedures using human blood from healthy volunteers. A written informed consent was obtained from human volunteers according to the procedures approved by the University of Reading Research Ethics Committee (Reference no: UREC 17/17, approved on the 10th of May 2017). The blood samples were collected from aspirin-free healthy volunteers after obtaining informed consent. The blood was taken via venepuncture in VACUETTE® blood collecting tubes containing 3.2% (w/v) citrate.

2.2.1 Preparation of human platelet-rich plasma (PRP) and isolated platelets

For the preparation of PRP, the whole blood was centrifuged at 102 g for 20 minutes at 20°C to obtain PRP. The PRP was then transferred to another tube and rested in a water bath at 30°C for 30 minutes before use in various assays. Where isolated platelets were needed, the whole blood was transferred to a 50 mL falcon tube and mixed with 15% (v/v) acid citrate dextrose (ACD) [2.5% (w/v) sodium citrate, 1.5% (w/v) citric acid and 2% (w/v) D-glucose] at 1:9 ratio (ACD: blood) to prevent the activation of platelets, and transferred into 4 ml blood collection tubes prior to centrifuging at 102 g for 20 minutes at 20°C. The resultant PRP was collected in a 50 ml falcon tube and mixed with 3 mL of ACD and 125 ng/mL prostaglandin I_2 (PGI_2) (to prevent platelet activation) and centrifuged at 1413 g for 10 minutes at 20°C. The resultant supernatant was discarded in Kloresept disinfectant and the platelet pellet was resuspended in modified Tyrodes-HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$ and 5 mM D-

glucose, pH 7.3) in the presence of 10 µL PGI_2 (125 µg/mL), and centrifuged at 1413 g for 10 minutes at 20°C. Finally, the platelet pellet was resuspended in modified Tyrodes-HEPES buffer at a density of 4×10^8 cells/mL and rested for 30 minutes at 30°C before use.

2.2.2 Preparation of 1,8-cineole

1,8-cineole in liquid form with a purity of 99% was purchased from (Sigma-Aldrich, UK) and prepared as 1 mM stock concentration by mixing 168.5 µl of 1,8-cineole stock with 831.5 µl of 100% ethanol. This was further diluted with modified Tyrodes-HEPES buffer to the required concentrations for assays, and the final concentration of ethanol in platelets was kept at 0.01% (v/v). A vehicle control with ethanol at a concentration of 0.01% (v/v) was included in all the experiments and this did not affect the platelet function in any of the experiments performed in this study.

2.2.3 Platelet aggregation assay

Light transmission aggregometry (LTA) was designed by Professor Born in 1960s and is being performed using isolated platelets or PRP. LTA is a method of measuring platelet aggregation in response to platelet agonists and is still the primary, gold standard technique to determine the impact of various molecules on platelet activation. LTA provides a number of details regarding platelet function including platelet shape, and the rate/kinetics of aggregation. The aggregometer is a fixed optical wavelength spectrophotometer, which provides with incubation chamber warmed at 37°C to mimic the body temperature, with continuous stirring of the sample using a magnetic stirrer.

This test is based on the detection of the increase in light transmission through platelet samples following the addition of an agonist. The change in optical density as the light pass through the sample is recorded when platelets are stirred with an agonist. Firstly, the light beam passes through the cuvette containing platelet poor plasma (PPP) which is considered as 100% light transmission corresponding to 100% aggregation. PRP sample is set at 0% aggregation prior to the addition of an agonist. The difference between light transmission of the reference (PPP) and the sample (PRP) is the output. This output of the light transmission during platelet aggregation automatically transferred in a graph. Figure 2.1 is a representative diagram of the platelet aggregation using LTA. The trace shows typical changes that can be explained by platelet shape change and subsequent decrease in transmission representing the aggregation, after addition of an agonist.

In this study, platelet aggregation assays were performed by light transmission (optical) aggregometry (Chrono-Log, USA). Human isolated platelets or PRP (in the presence of plasma proteins) (445 µL) were incubated with different concentrations (6.25, 12.5, 25, 50 and 100 µM) of 1,8-cineole or a vehicle control [0.01% (v/v) ethanol] (5µL) in a siliconised cuvette for 5 minutes at 37°C (with an initial 5 seconds of stirring to evenly distribute 1,8-cineole in the solution). In separation experiment, incubation of platelets with 1,8-cineole and monitoring platelet aggregation over five minutes, 1,8-cineole alone does not cause any changes in platelets aggregation. The samples were then stimulated with 50 µl of CRP-XL (to a final concentration of 1 µg/ml or 0.5 µg/ml), collagen (final concentration of 1 µg/ml or 0.5 µg/ml), thrombin (final concentration of 0.01 U/mL) or ADP (final concentration of 5 µM or 2.5 µM). The platelets were allowed to aggregate with continuous stirring (1200 rpm) at 37°C for 5 minutes and the level of aggregation was monitored as a change in light transmission. The data were analysed

by calculating the percentage of maximum platelet aggregation at 5 minutes, and the level of aggregation obtained with the vehicle control was considered as 100% to quantify the impact of 1,8-cineole on platelets aggregation.

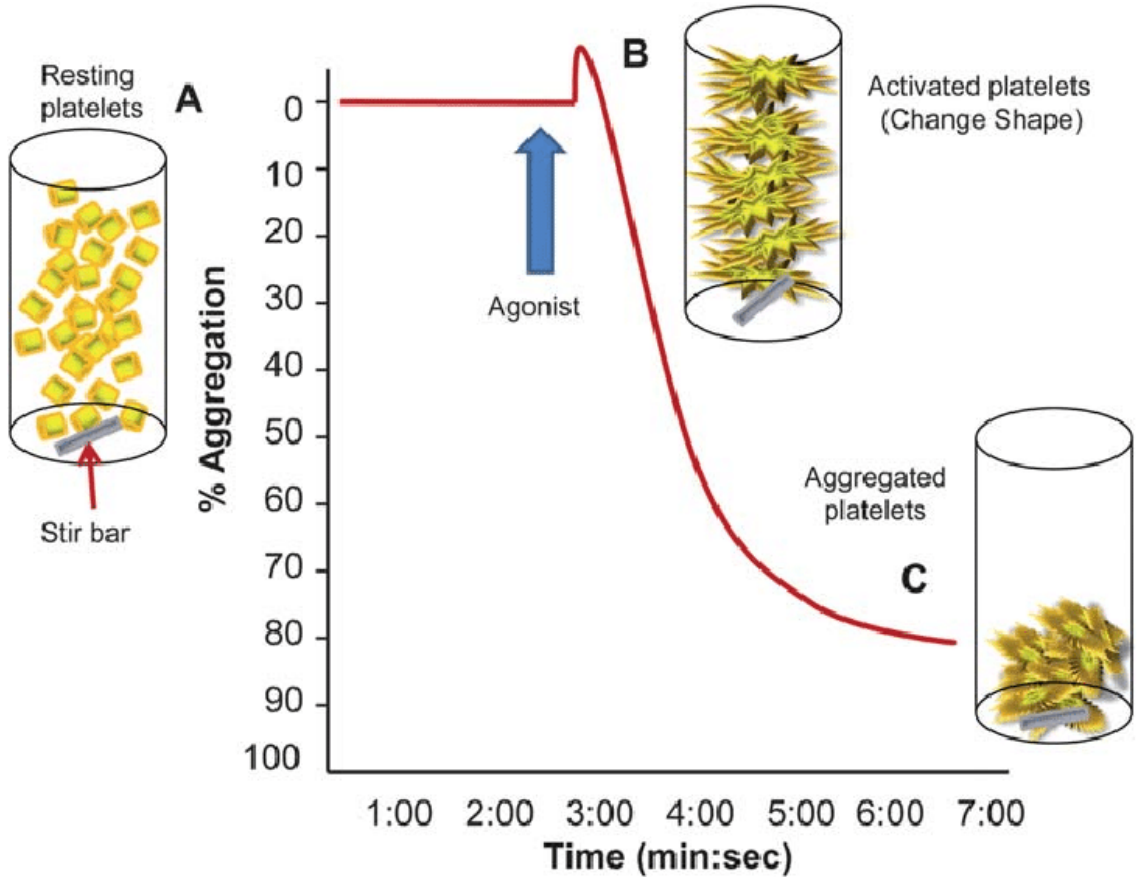

Figure 2-1: Platelet aggregation assay using LTA. The LTA measures changes in light transmission of a stirred platelet suspension (resting state) exposed to a platelet agonist. The baseline is adjusted by a cuvette containing PPP which equates to 100% light transmission. Platelets in the absence of an agonist compares to 0% light transmission (**A**). Changes in platelet shape due to the addition of a platelet agonist result in a short decrease (the small curve) in the light transmission (**B**). When platelet aggregates are forming, an increase in the light transmission is recorded and calculated as percentage of aggregation (**C**). This figure was adapted from (Santos-Martínez, Prina-Mello et al. 2011).

2.2.4 Dense granule secretion

This test is based on the assessment of adenosine triphosphate (ATP) secreted from activated platelets using a luminescence technique in aggregometry. The luciferin-luciferase reaction was used to quantify the amount of ATP released from activated platelets. By using ATP as energy, luciferin gets converted to oxyluciferin via the oxidative enzyme luciferase, which causes luminescence to be released (McElroy 1947). In this assay, a chrono-lume reagent containing luciferin and luciferase was used in lumi-aggregometry (model 700, Chronolo-Log, PA, USA). Lumi-aggregometry allows simultaneous measurement of the release of ATP from platelet granules as well as platelet aggregation (Cattaneo 2009). Upon platelet activation with an agonist, ATP secreted from dense granules is used in the reaction as a source of energy and the lumi- aggregometer is used to measure the level of luminescence released as a result of this reaction (Figure 2.2).

Human PRP (395 µL) was incubated with 50µl of chrono-Lume reagent in a glass cuvette at 37°C for 2 minutes in the dark. Various concentrations (6.25, 12.5, 25, 50 and 100 µM) of 1,8-cineole or a vehicle control were added to PRP and incubated for further 5 minutes prior to activation with 50 µl of collagen (0.5 µg/mL). The level of luminescence was monitored for 5 minutes using the AggroLink 8 software (Chrono-Log, PA, USA), with the luminescent gain adjusted until the ATP response was in the 20-60% range according to the manufacturer instructions. The final setting was saved and applied to all the subsequent experiments.

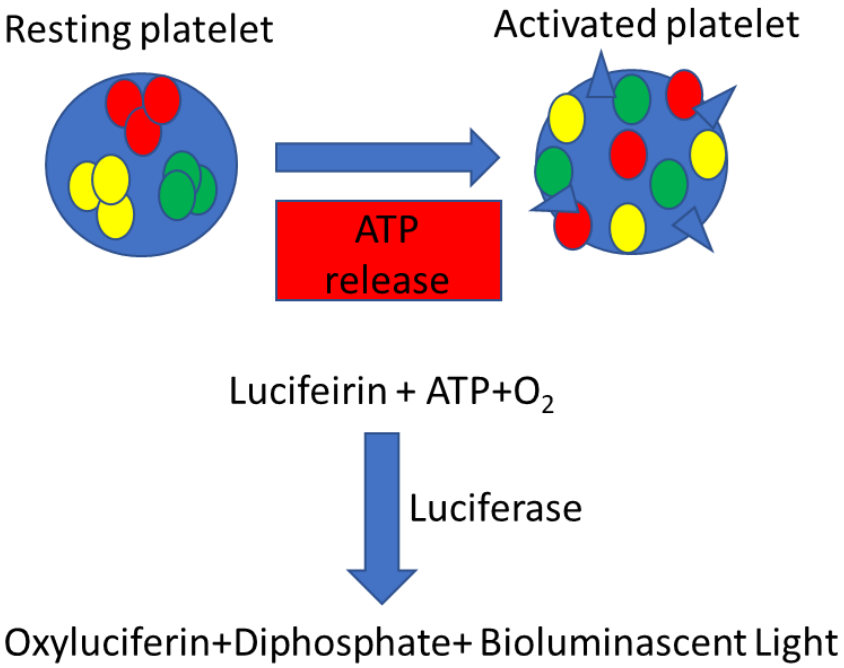

Figure 2-2: Mechanism of ATP secretion: Adenosine triphosphate (ATP) is released from dense granules during platelet activation. The reaction equation of firefly luciferase assay. The assay is based on luciferase's requirement for ATP in producing light. According to the reaction equation, the light production by luciferase is continuous and related to the amount of ATP released.

2.2.5 Flow cytometry-based assays

Flow cytometry is a laser-based, sensitive technique that is widely used to detect and measure a range of biophysical features of cells. A sample can be analysed by a flow cytometer using multiple parameters. The cells are normally suspended in a fluid and then injected into the flow cytometer, where the cells will illuminate due to the laser beam passing through the solution. The light then is captured by a detector and sent as a signal into data file on computer software for analysis. The light is divided into forward scatter (FSC) and side scatter (SSC). Forward scatter reflects the size of the cell and allows for the distinguishing of cells based on their size. Whereas the SSC data estimate the granularity of the cell (Figure 2.3).

The level of P-selectin exposure (as a marker for α-granule secretion from platelets) and the level of fibrinogen binding (as a marker for inside-out signalling to integrin αIIbβ3) on the platelet surface upon activation were measured using PE/Cy5-labelled mouse anti-human CD62P antibody and FITC-conjugated polyclonal rabbit anti-human fibrinogen antibody, respectively. Briefly, human isolated platelets or PRP (5 µl) were treated with different concentrations (6.25, 12.5, 25, 50 and 100 µM) of 1,8-cineole or a vehicle control for 5 minutes at room temperature in the presence of 2 µl anti-CD62P and 2 µl anti-fibrinogen antibodies. Platelets were then activated with CRP-XL (0.5 µg/mL) for 20 minutes at room temperature. Following this, 0.2% (v/v) formyl saline was added to fix the platelets. The levels of fibrinogen binding and P-selectin exposure were measured by Accuri C6 flow cytometry (BD Biosciences, UK) by collecting 5000 events in a gated region for platelets and the data were analysed using Accuri C6 plus software. The median fluorescence intensity was used to assess the level of fibrinogen binding and P-selectin exposure on the platelet surface. The level of fibrinogen binding and P-selectin exposure in the treated samples was

calculated by taking the level of median fluorescence intensity obtained with the vehicle control as 100% for easier comparison.

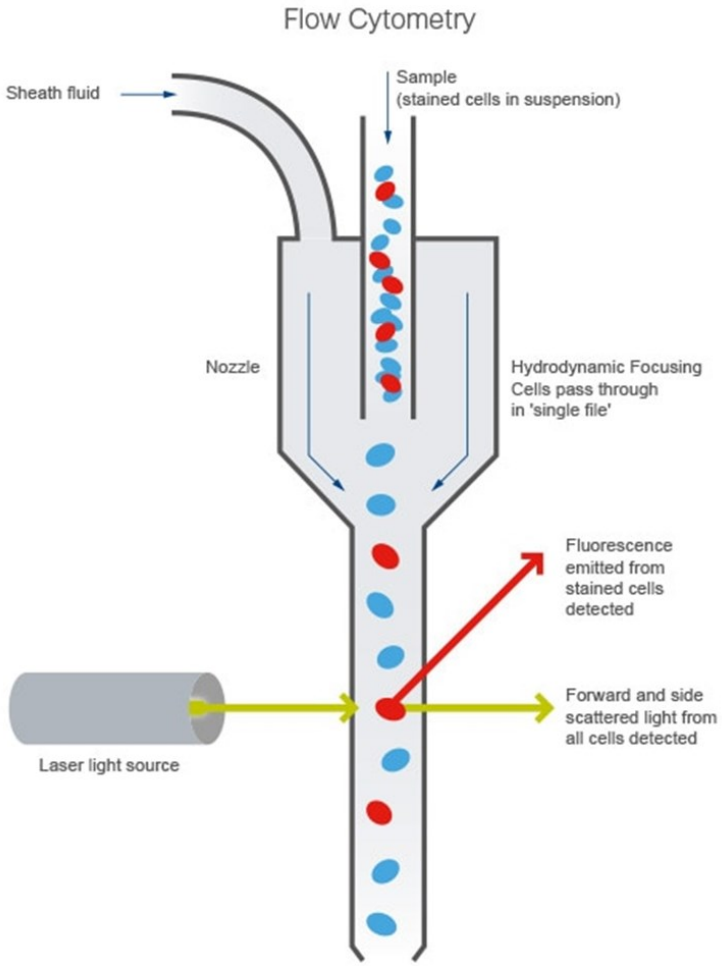

Figure 2-3: Overview of the working principle of flow cytometer. Sheath fluid focuses on cell suspension, causing the cells to pass through a laser beam as one cell at a time. Forward and side scattered light is detected, as well as fluorescence emitted from stained cells. This figure was adapted from www.abcam.com.

2.2.6 Intracellular calcium mobilisation

The intracellular calcium levels in platelets were measured using Fluo-4 AM calcium-sensitive dye (Life Technologies, UK), which binds free intracellular calcium. Two mL of human PRP were loaded with 2 µl (i.e. 2 µM final concentration) of Fluo-4 AM dye and incubated for 45 minutes at 30°C in the dark. After this, the PRP was centrifuged at 350 g for 15 minutes and, the resulting platelet pellet was resuspended in 500 µl of modified Tyrodes-HEPES buffer. The isolated platelets loaded with free acid of Fluo-4 AM were incubated with a vehicle control [0.01% (v/v) ethanol] or different concentrations (6.25, 12.5, 25, and 50 µM) of 1,8-cineole before activating with 0.5 µg/ml CRP-XL. The level of fluorescence intensity was measured by a NOVOstar plate reader at 37°C for 5 minutes using an excitation wavelength of 480 nm and emission at 520 nm. The data were analysed by measuring the percentage of peak calcium levels released at around 90 seconds, where the maximum level of calcium was released in the positive control samples.

2.2.7 Platelet spreading assay

Glass coverslips were placed in a 24-well plate and they were coated with 300 µl of fibrinogen (100 µg/ml) dissolved in modified Tyrodes-HEPES buffer for 45 minutes. After the incubation, the supernatant was removed and 1% (w/v) BSA was added onto the coverslips to prevent non-specific binding of platelets to the glass surface and incubated for 1 hour. The coverslips were then washed 3 times with PBS (10 mM phosphate buffer, 2.7 mM KaCl, and 137 mM NaCl, pH 7.4). Human isolated platelets at a density of $2X10^7$ cells/mL were treated with a vehicle control [0.01% (v/v) ethanol] or different concentrations (6.25, 12.5, 25, and 50µM) of 1,8-cineole for 5

minutes before loading them on to coverslips and incubating for 45 minutes at 37°C. The unbound platelets were removed, and coverslips were then washed 3 times with PBS. The adhered platelets were fixed with 0.2% (v/v) formyl saline for 10 minutes. Once again, the coverslips were washed 3 times after removing the fixative solution. The attached platelets were permeabilised by adding 300 µl of 0.2% (v/v) Triton X-100 in PBS for 5 minutes at room temperature. Following incubation, the solution was then removed, and the coverslips were washed 3 times with PBS. Then the adhered platelets were stained by adding Alexa-Fluor 488-labelled phalloidin onto the coverslips and incubating in the dark for 30 minutes at room temperature to label platelet filamentous actin. The coverslips were then mounted onto microscopic slides using Prolong Gold Antifade [Life Tech (USA)] to preserve fluorescence and then kept at 4°C until visualisation. The slides were imaged using a 100X oil immersion objective in a Nikon A1-R confocal microscope (Nikon, Japan). The level of platelet spreading was calculated by counting the number of platelets on 10 random images of each population (obtained from different donors) that were captured. The platelets were marked as ‘adhered but not spread’ or ‘spreading’ where the cells extended filopodia, and ‘spread fully’ where lamellipodia was formed.

2.2.8 Clot retraction

Clot retraction is a process that occurs after the clot has been formed in order to bring the wound edges together to facilitate wound healing. This activity is driven through outside-in signalling mediated by integrin αIIbβ3, resulting in the contraction of fibrin mesh, and thus reduces the size of blood clot. The complete clot retraction occurs approximately around 2 hours after the formation of blood clot formation.

To measure the effect of 1,8-cineole on clot retraction, human PRP (200 µL) was obtained and mixed with 5 µl of red blood cells (for visualisation of the clot) and a vehicle control or different concentrations (6.25, 12.5, 25 and 50µM) of 1,8-cineole. The final volume was made up to 950 µl by adding modified Tyrodes-HEPES buffer. To initiate the fibrin clot formation, 50 µl of thrombin (final concentration of 1 U/mL) was added to the PRP in each test tube. A blunt glass capillary was placed in the middle of each test tube, to enable the clot formation around this. The clot formation was monitored for 2 hours at room temperature and photographs were taken every 30 minutes. Finally, the clots (without any liquid) were removed from the glass capillaries in each test tube and weighed. The clot mass was calculated for each sample in order to determine the level of clot retraction.

2.2.9 *In vitro* thrombus formation under arterial flow conditions

The *in vitro* thrombus formation assay is able to measure various parameters of thrombi that form under arterial flow conditions on a collagen-coated surface at physiological shear rate. Human whole blood was incubated with 5 µM of a lipophilic dye, DiOC6 (3,3'-Dihexyloxacarbocyanine Iodide) (Sigma Aldrich, UK) at 30°C for 30 minutes. Vena8 BioChip (Cellix Ltd, Ireland) microfluidic channels were coated with collagen (400 µg/ml) for one hour. Following blocking, the channels were gently washed with modified Tyrodes-HEPES buffer. The fluorescent-labelled human whole blood was pre-incubated with a vehicle or various concentrations (6.25, 12.5 and 50µM) of 1,8-cineole for 5 minutes before perfusion over the collagen-coated microfluidic channels at a shear stress of 20 dynes/cm^2 for 10 minutes. The level of thrombus formation was observed using a Nikon A1-R confocal microscope using 20X objective. Fluorescence images of thrombi were captured every 30 seconds

continuously for 10 minutes. The mean fluorescence intensity, volume and number of thrombi were calculated using NIS Elements software (Nikon, Japan) and the images were analysed using ImageJ (National Institute of Health, USA).

2.2.10 Tail bleeding assay

A tail bleeding assay in mice is commonly performed to assess the haemostatic action of platelets under physiological settings. This assay involves transection of the tail tip and measuring the bleeding volume and/or time. The bleeding assay was the first test designed *in vivo* to evaluate platelets ability to perform a haemostatic plug by recording the time for stopping the bleeding (Quick 1975). University of Reading Research Ethics Committee and the British Home Office have approved the experimental procedures used in tail bleeding assay in mice. In brief, C57BL/6 (both males and females) mice (12 weeks old; Envigo, UK) weighing around 20-25 g were anaesthetised using ketamine (80 mg/kg) and xylazine (5 mg/kg) administrated via the intraperitoneal route and placed on a heated mat (37°C). After around 10-15 minutes of anesthetisation, the vehicle control [0.01% (v/v) ethanol] or 1,8-cineole (6.25 and 12.5 μM - final concentration was calculated based on the estimated volume of blood in accordance with their weight) was administered via femoral artery and incubated for 5 minutes. A distal (3 mm) segment of the tail tip was dissected using a scalpel blade and the tail tip was placed in sterile saline. The time taken to cessation of bleeding was measured up to 20 minutes, when the assay was terminated.

2.2.11 Lactate dehydrogenase (LDH) cytotoxicity assay

LDH is a cytosolic enzyme present in numerous cell types and is well-known as an indicator of cellular toxicity. Upon damage of the plasma membrane the cell will start to release LDH into the surrounding culture medium. The released LDH can be measured by an enzymatic reaction whereby LDH catalyses the conversion of lactate to pyruvate by reducing NAD+ to NADH. The reduction of NADH is then used by diaphorase to reduce a tetrazolium salt to form a red formazan. Thus, an LDH cytotoxicity assay was used to determine whether 1,8-cineole has toxic effects on platelets.

An LDH cytotoxicity assay was performed using a Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher, UK) according to the manufacturer's instructions. Human PRP was incubated with different concentrations (6.25, 12.5, 25, 50 and 100 μM) of 1,8-cineole or a vehicle control for 5 minutes prior to adding the reaction mixture (provided in the kit) and incubating for 30 minutes at 37°C under 5% CO_2. After the incubation, the reaction was terminated by adding the stop solution (provided in the kit) and the absorbance of this mixture was read at 490 nm and 650 nm using a spectrophotometer (Molecular Devices, UK). A lysis buffer provided with the kit was loaded onto a set of wells as a positive control for maximum LDH release. Additionally, modified Tyrodes-HEPES buffer was loaded onto another set of wells for the detection of spontaneous LDH release from platelets as a negative control.

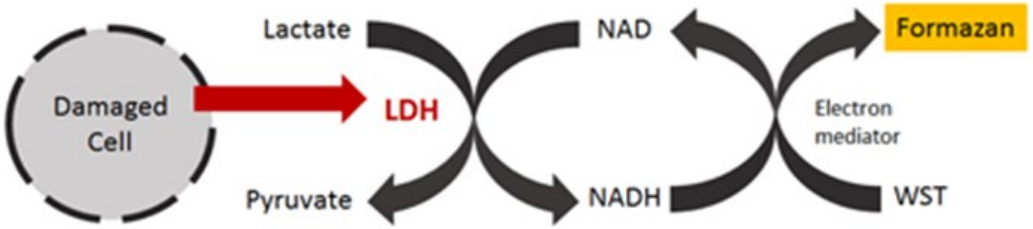

Figure 2-4: Measuring LDH Cytotoxicity. LDH catalyses dehydrogenation of lactate to pyruvate, reducing NAD to NADH. NADH reduces a water-soluble tetrazolium salt in the presence of an electron mediator to produce red formazan dye. The amount of formazan dye produced is proportional to the amount of LDH released into the medium, indicating cytotoxicity. This figure was adapted from enzo lifescinces website.

2.2.12 Measurement of ROS production

ROS production is associated with platelet aggregation upon stimulation with agonists such as collagen and thrombin (Fuentes, Gibbins et al. 2018). The intracellular ROS level was measured in CRP-XL-stimulated platelets using a reagent, 2′,7′-Dichlorofluorescin diacetate (H_2DCFDA) (Cambridge Bioscience, UK), a cell permeable, non-fluorescent probe used as an indicator for ROS in cells. Upon cleavage of the acetate group via intracellular esterases and oxidation, the H_2DCFDA is converted to a highly fluorescent signal (Pignatelli, Pulcinelli et al. 2000). H_2DCFDA was thus widely used as marker to analyse intracellular ROS generation in various cell types. We investigated whether 1,8-cineole affects ROS production in platelets in response to CRP-XL stimulation.

ROS production was measured in human isolated platelets (2×10^8/mL). Platelets were incubated for 30 minutes in the dark at 37^{o}C with 10 µM dihydrodichloro fluorescein diacetate (H2DCFDA). After the incubation, platelets were treated with different concentrations of 1,8-cineole (6.25 µM - 50 µM) for 5 minutes at 37^{o}C prior to being stimulated with 1 µg/mL CRP-XL for 10 minutes. After stimulation, samples were then diluted 10-fold in modified Tyrode's-HEPES buffer containing 0.1% (w/v) BSA and analysed immediately using an Accuri C6 Flow cytometer (BD Biosciences, UK). The level of ROS was calculated from median fluorescence intensity (MFI) value and expressed as fold change relative to resting platelet samples (unstimulated platelets). To estimate any inhibitory effects of 1,8-cineole in CRP-XL-induced platelet ROS production, the level of ROS obtained in a positive control (stimulated platelets) was set as 100%.

2.2.13. Quantification of various inflammatory molecules released from platelets

The human sP-Selectin sandwich ELISA (enzyme-linked immunosorbent assay) was designed to measure the amount of sP-selectin in a solution. An anti-human sP-selectin has been pre-coated in the wells of the supplied microplate. Samples and standards are then added into these wells to bind the immobilised (capture) antibody. The sandwich is then formed by the addition of the second (detector) antibody conjugated with an enzyme [usually a horse-radish peroxidase (HRP)]. A substrate (based on the enzyme conjugate used) solution is added that reacts with the enzyme-antibody-target complex to produce measurable signal. The intensity of this signal is directly proportional to the concentration of target present in the original specimen.

Here, a human sP-selectin ELISA kit (Theromfisher Scientific, UK) was used for the detection of the levels of sP-selectin released from human platelets. To investigate the effect of 1,8-cineole on sP-selectin release, human isolated platelets were prepared as described before, and pre-incubated for 5 minutes with different concentrations of 1,8-cineole (6.25 µM – 50 µM) before treated with a vehicle or CRP-XL (1 µg/mL) for five minutes under stirring conditions to obtain resting or activated platelets, respectively. Then platelets were centrifuged at 1000 g for 10 minutes at room temperature. The supernatant was collected, and immediately used in the assay.

Briefly, 100 µl sample diluent were added in duplicates in standards and blank wells and 100 µl of prepared human sP-selectin standards with dilutions ranging from 0 - 40 ng/ml were added to standard wells. 90 µl of sample diluent also added to sample wells. Either the resting or activated platelet samples in ratio (1:10) 10 µl of supernatant collected were added to sample wells that contain 90 µl of sample diluent

provided with the kit. Human sP-selectin present in the samples or standard binds to antibody adsorbed to the microwells and 50 µl of HRP-conjugated anti-human sP-selectin antibody was added to the wells and they bind to human sP-selectin captured by the first antibody, and the plate was incubated for 2 hours at room temperature on microplate shaker. The plate was then washed three times with wash buffer [PBS with 1% (v/v) Tween 20], and 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added and the mix was allowed to incubate in the dark for 15 minutes at room temperature until the development of a colour. The reaction was then stopped by the addition of 100 µl stop solution (1 M HCl). The level of absorbance was measured at 450 nm using an ELISA microplate reader (EMax precision plate reader, Molecular Devices, UK).

For all other assays, similar protocol was followed using commercially available ELISA kits to measure cytokines release upon platelet activation including human tumour necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), human interleukin beta (IL-β), and Human Regulated on Activation Normal T cell Expressed and Secreted (RANTES).

2.2.14 Platelet-leukocyte interactions using flow cytometry

Cells in immune system such as monocytes and lymphocytes are two major classes of white blood cells. Monocytes are larger than lymphocytes and exhibit forward scatter of higher intensity in flow cytometer. While side scatter release light at a larger angle due to the interface between the laser and intracellular structure of the cells such as granules and nucleus and reflects the cellular complex and granularity. Florescent markers are often used to stain cells of interest with fluorescent dyes or fluorophores to be visible to cytometer.

Immunolabelling of platelets and leukocytes was performed using whole blood for flow cytometry analysis. Aliquots of 100 µl of whole blood were diluted fivefold by adding 400 µl of Ca^{+2}-free HEPES buffer. Diluted samples were then incubated with the following antibodies: FITC-conjugated anti-human CD42b as a marker for platelets, and phycoerythrin (PE)-labelled anti-human CD45 antibodies as a marker for leukocytes, for 20 minutes at room temperature. After the incubation, the samples were treated with different concentrations of 1,8-cineole or a vehicle control for 5 minutes in the dark at 37^{o}C. For platelet activation, 10 µM of thrombin receptor activator peptide 6 (TRAP-6) was added and incubated for 5 minutes at room temperature. After that, the samples were fixed with 1% (w/v) (final concentration) paraformaldehyde for 10 minutes at room temperature. The red cells were then lysed with 2 ml of 1x RBC lysis buffer for 10 minutes and samples were protected from light and kept at 4^{o}C until analysed by flow cytometry.

To access leukocyte population, forward and side scatter parameters were used, and a total of 20,000 events were acquired. Appropriate isotype control antibodies labelled with the same fluorophores as the primary antibodies were used to determine the basal level of fluorescence in negative controls. Total platelet-

leukocyte aggregations were taken by calculating the percentage of CD45+ cells with fluorescence from CD42b. The samples were analysed using a BD Accuri C6 flow cytometer and the data were analysed using C6 Sample Plus software. A schematic representation of this method used by flow cytometry analysis is illustrated in (Figure 2.2).

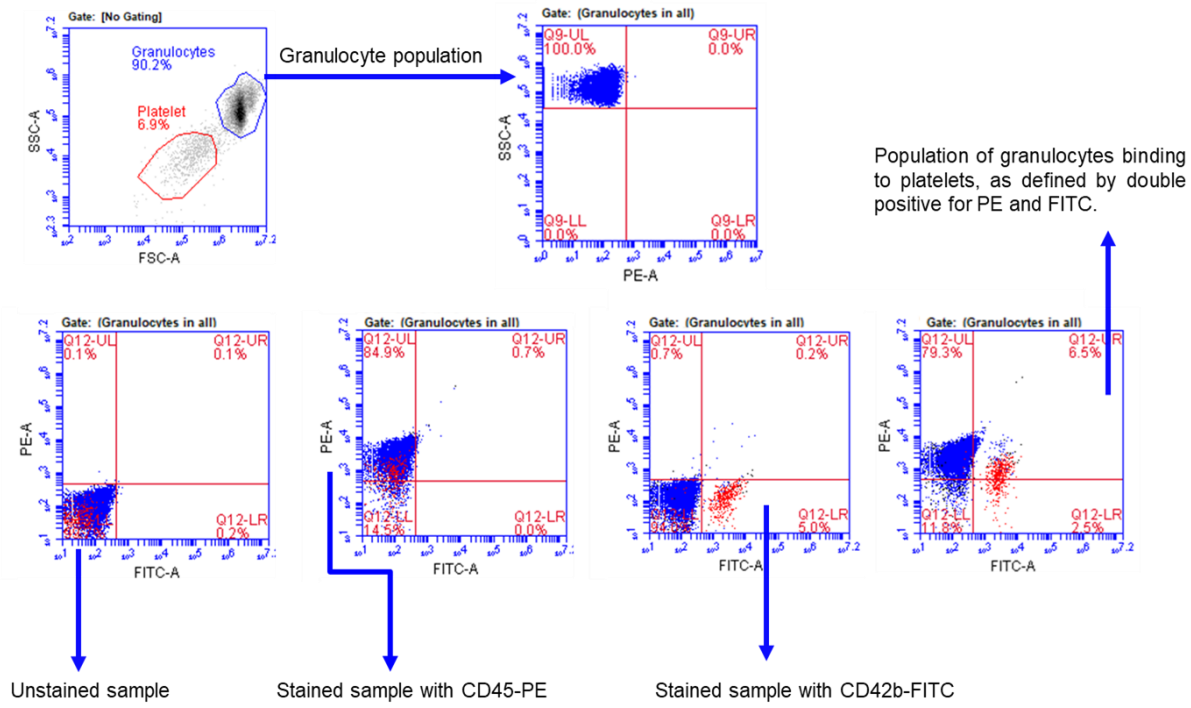

Figure 2-5: Schematic representation of flow cytometry analysis of whole blood. The granulocyte population was clearly identified in whole blood based on light scatter characteristics, size and granularity. Granulocytes stained with anti CD45 antibody while platelets stained with anti CD42b antibody. The detection limit of the positive staining was adjusted using different controls including unstained sample. Approximately 99% of the cells in granulocyte gated region were positive for the granulocyte marker, CD45 which demonstrate the specificity of marker and accuracy of cells identification according to their forward and side scatter characteristic feature. From this granulocyte gate, events that stained positively for both CD45 (PE conjugated) and platelet marker CD42b (FITC conjugated) were identified as platelet-leukocyte aggregates.

2.2.15 Immunoblotting analysis

Human isolated platelets were prepared at a density of 4 x 10^8 cells/mL, and treated with either 1,8-cineole (6.25,12.5, 25 and 50μM) or a vehicle control [0.01% (v/v) ethanol] for 5 minutes at 37°C. Following the incubation, platelets were stimulated by the addition of 0.5 μg/mL CRP-XL for 5 minutes. The samples were then immediately lysed by the addition of reducing sample treatment buffer [6X reducing sample-treatment buffer: (RSTB); 69 mM sodium dodecyl sulphate, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 25 mM Tris-HCl, pH 6.8]. The samples were boiled at 95°C for 5 minutes, before storing at -20^{o}C until use.

The samples were loaded on to 4-15% Mini-PROTEAN TGX precast protein gels (Bio-Rad, UK), which were submerged in 1X Tris/Glycine/SDS buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3). Then run at a constant voltage of 150 V for around 50 minutes (until the dye front reaches the bottom of the gel).

The proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, UK) using a Semi-Dry Transfer System (Bio-Rad, UK) at 15V for 2 hours. The membranes were then blocked by transferring them into a 50 mL falcon tube containing 10 mL of 5% (w/v) BSA for 1 hour at room temperature. Then the primary antibodies that were prepared in a dilution of 1:1000 in 2.5% (w/v) BSA were added to the membrane and they were incubated overnight at 4°C. The 14-3-3ζ rabbit polyclonal antibody (1:2000 dilution) (Santa Cruz Biotechnology, USA) was used to detect this protein as a loading control. On the next day, the primary antibodies were removed, and the PVDF membrane was washed 3 times for 15 minutes each in TBS-T buffer. Secondary antibodies, Cy5-conjugated goat anti-rabbit IgG or anti-mouse IgG (Thermo fisher Scientific, UK) were then added in a 1:4000 dilution in 2.5% BSA (w/v) solution and incubated for 1 hour at room temperature. The PVDF

membranes were washed again in TBS-T buffer 3 times for 15 minutes each and scanned using a Typhoon 9400 Variable Mode Imager system (GE Healthcare, UK) and the images were analysed using ImageJ. The level of fluorescence of specific protein bands was normalised to the loading control, 14-3-3ζ, which is a highly conserved family of phospho-serine binding proteins, that bind and regulate various of targets that involved in regulating numerous cellular pathways.

2.2.16 Quantification of cAMP levels in platelets

The cAMP levels in platelets were quantified using a cAMP ELISA quantification kit (Enzo Life sciences, UK) according to the manufacturer's instructions. Human isolated platelets were treated with a vehicle control or different concentrations of 1,8-cineole prior to activation with 0.5 µg/ml CRP-XL and measuring the level of cAMP using a cAMP ELISA kit. The amount of cAMP was quantified using the standard curve which was plotted using the control samples provided in the kit.

2.2.17 Statistical analysis

All the data obtained in this study were analysed using GraphPad Prism Version 8. Most of the data in this study are represented as mean ± SEM in bar graph formats as the data were normalised to the positive controls for each donor (to consider the individual variations in platelet reactivity). The data obtained from platelet aggregation, dense granule secretion, platelet spreading, intracellular calcium mobilisation, thrombus formation, cytotoxicity, flow cytometry-based experiments, cytokine secretion, platelet-leukocyte interactions and immunoblot analysis were analysed using one-way ANOVA with the differences between treatments investigated using a Bonferroni's post-hoc test. The data obtained from tail bleeding was analysed using the non-parametric global Kruskal-Wallis test.

Chapter-3

3-EFFECT OF 1,8-CINEOLE ON THE MODULATION OF PLATELET ACTIVATION AND THROMBUS FORMATION

3-1 Introduction

It is well-known that ischemic heart disease and strokes are the leading causes of deaths and disabilities worldwide. Their clinical symptoms are induced by the rupture of atherosclerotic lesion in the blood vessels (Braunwald 1988). Platelets play a fundamental role in the maintenance of haemostasis, however, under pathological conditions such as the rupture of atherosclerotic plaques, they induce thrombosis. Drugs that are inhibiting platelet activity have shown to be effective in the reduction of thrombotic risks. Currently available anti-platelet drugs such as aspirin and clopidogrel are widely prescribed to treat and prevent thrombotic diseases. However, these drugs are associated with severe side effects such as gastrointestinal toxicity and bleeding (Laporte, Ibanez et al. 2004). Moreover, they do not guarantee sufficient treatment and prevention in all patients due to tolerance (Bliden, DiChiara et al. 2007). Thus, there is a rising demand for the development of safer and effective anti-platelet agents that would decrease unwanted platelet activation without any undesirable effects.

Medicinal plants became a major topic of research interest over decades as a powerful resource for new medications due to the presence of various bioactive compounds and their use as potential drugs. Most of these therapeutically valuable compounds in medicinal plants include monoterpenes, flavonoids and alkaloids.

Monoterpenes have various pharmacological properties and they are known to exert significant effects on the modulation of cardiovascular system, for example by promoting vasorelaxation to reduce high blood pressure and decreasing heart rate (Aydin, Kutlay et al. 2007, Bastos, Moreira et al. 2010, Peixoto-Neves, Silva-Alves et al. 2010). 1,8-cineole is a saturated bicyclic monoterpene and a principal constituent

of eucalyptus oil which is primarily derived from its major source, eucalyptus leaves. 1,8-cineole has been reported to possess anti-inflammatory, anti-hypertensive and anti-oxidative effects as demonstrated using several *in vitro* and *in vivo* models of various pathological conditions including respiratory, cardiovascular and neurodegenerative diseases (Gupta, Prasad et al. 2016).1,8-cineole has been reported to exhibit beneficial effects in the cardiovascular system under *in vivo* and *in vitro* settings (Lahlou et al. (2002)), as intravenous administration of 1,8-cineole causes a significant reduction in blood pressure in anesthetised and conscious rats. In the same study, 1,8-cineoles displayed vasorelaxation in isolated rat thoracic aorta by inducing the reduction of the contraction caused by potassium. This effect lead the authors to suggest that the hypotensive effects of 1,8-cineole might be due to direct relaxation of vascular smooth muscle cells (Lahlou, Figueiredo et al. 2002). Despite extensive previous research on various cellular systems, it has never been established whether 1,8-cineole can be useful as an antithrombotic agent as its effects on the modulation of platelet function have not been investigated. Here, we report the impact of 1,8-cineole on the modulation of platelet activation, haemostasis and thrombus formation using a range of platelet functional assays. Due to numerous beneficial effects of 1,8-cineole, establishing their effects in modulating platelet reactivity will enable the usage of this compound to control platelet activation and thrombosis under diverse pathological settings.

3.2 Results

3-2-1 1,8-cineole inhibits agonists-induced platelet aggregation

A range of platelet functional assays are widely used to investigate the impact of therapeutically valuable compounds/molecules in the modulation of platelet activation. For example, platelet aggregation, clot retraction and calcium mobilisation in response to different agonists such as collagen, ADP or thrombin are used to determine the effects of any molecules on platelet activation. As explained in the introduction section, upon blood vessel injury, collagen will be exposed from the subendothelial region, and as the primary physiological role of platelets, they bind to collagen and release their granule contents as well as enabling the production of thrombin on their surface. All these agonists together activate a large number of platelets and they aggregate at the site of injury to form a platelet plug which helps to prevent excessive bleeding (Andrews and Berndt 2004, Barrett, Holbrook et al. 2008). Hence, platelet aggregation is known as a 'gold standard technique' for studying platelet function, by looking at the response of platelets to different agonists (e.g. thrombin, collagen, a cross-linked collagen-related-peptide (CRP-XL) and ADP) in the presence and absence of a drug or compound. Thus, measuring platelet aggregation in response to such agonists in the presence and absence of 1,8-cineole is a critical method by which the impact of 1,8-cineole on platelet activation can be established.

In the following experiments, platelet responses to various agonists in the presence and absence of different concentrations of 1,8-cineole were measured using human isolated platelets and platelet-rich plasma (PRP). A full range of 1,8-cineole concentrations (6.25-100 μM) were tested to find out the minimal and maximal effects of 1,8-cineole on the modulation of platelet activation. For all the aggregation data

figures, the panels show representative aggregation traces for each agonist in the presence or absence of 1,8-cineole as well as the cumulative data (mean ± the SEM) for aggregation as the experiments were performed using platelets obtained from different donors. A one way-ANOVA was used to assess the statistical significance with Bonferroni post-hoc multi comparison test.

3-2-1-1 1,8-cineole inhibits collagen-stimulated aggregation in human isolated platelets

To determine the impact of 1,8-cineole on platelets alone (i.e. in the absence of plasma proteins and other blood cells), its effects were first determined in human isolated platelets. First collagen was used as an agonist and it activates platelets by binding to GPVI and integrin $\alpha_2\beta_1$. Human isolated platelets (at a density of $4x10^8$ cells/mL) were prepared as described in methods section and incubated with a vehicle control [0.01% (v/v) ethanol, which did not exert any effects on platelets] or a range of different concentrations of 1,8-cineole for 5 minutes (this time point was decided based on the information published in the literature and our initial optimisation experiments) at 37ºC. Following the incubation, platelets were placed in an aggregometer that was prewarmed to 37°C with continuous stirring (1200 rpm), prior to stimulation by the addition of different concentrations of collagen (0.5 µg/mL or 1 µg/mL), and the level of aggregation was monitored for 5 minutes. The time set for monitoring aggregation response as 5 minutes demonstrated that this effect represents true inhibition of 1,8-cineole, rather than a result of a delay in the aggregometer response.

The treatment of human isolated platelets with 1,8-cineole resulted in inhibition of collagen-stimulated platelet aggregation (Figure 3-1). 1 µg/mL collagen-stimulated platelet aggregation was significantly inhibited in the presence of 1,8-cineole at high

concentrations (50 and 100 μM), whereas lower concentrations (6.25, 12.5 and 25 μM) of 1,8-cineole failed to reach a significant reduction in aggregation (Figure 3-1 A and B). The absence of significant effects of 1,8-cineole at lower concentrations could be due to the potent actions of 1 μg/mL collagen used in these experiments. Therefore, the concentration of collagen was reduced to 0.5 μg/mL to determine if lower concentrations of 1,8-cineole would now provide significant effects on platelet aggregation. Indeed, when a concentration of 0.5 μg/mL collagen used to stimulate platelets, 1,8-cineole was able to show significant inhibitory effects on platelet aggregation at all the concentrations tested (Figure 3-1 C-D). A concentration of 50 μM resulted in 85% inhibition and a concentration of 25 μM displayed around 60% inhibition. Similarly, 1,8-cineole at both concentrations of 6.25 and 12.5 μM displayed about 60% reduction in collagen-induced platelet aggregation in comparison to the vehicle control (Figure 3-1C and D). These data demonstrate that 1,8-cineole is able to clearly inhibit collagen-induced platelet activation with a concentration of as low as 6.5 μM when collagen was used at a low concentration. When the concentration of collagen was increased, the inhibitory effects of 1,8-cineole can only be observed at higher concentrations.

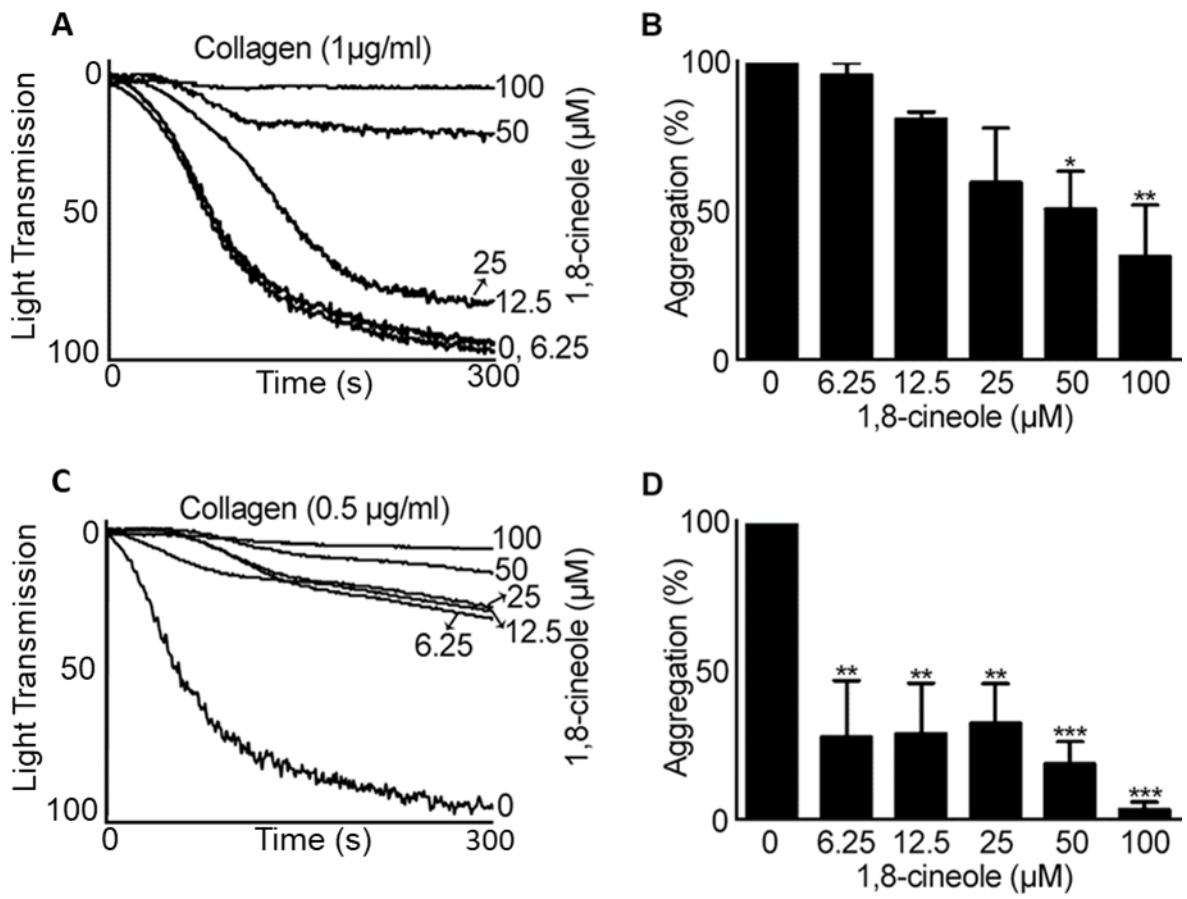

Figure 3-1: Effect of 1,8-cineole on collagen-stimulated aggregation in human isolated platelets. Human isolated platelets were incubated with different concentrations of 1,8-cineole or a vehicle control [0.01% (v/v) ethanol] for 5 minutes prior to stimulation with 1 µg/mL **(A** and **B)** or 0.5 µg/mL **(C** and **D)** collagen and the level of aggregation was monitored by optical aggregometry. The percentage of aggregation was calculated by considering the aggregation obtained with the vehicle control as 100% (**B** and **D**). The aggregation traces shown are representative of 4 individual experiments. Cumulative data represent mean ± S.E.M (n=4). The p values (*p<0.05, **p<0.01 and ***p<0.001) shown are as calculated by one-way ANOVA followed by a Bonferroni *post-hoc* test.

3-2-1-2 1,8-cineole inhibits CRP-XL-induced aggregation in human isolated platelets

As mentioned above, it is well known that collagen activates platelets via binding both integrin $\alpha_2\beta_1$ and GPVI. In order to dissect out the selective roles of GPVI, CRP-XL was developed previously as a selective agonist for GPVI and used in several assays including aggregation to investigate whether the inhibition of platelet function by a given compound was selectively mediated through GPVI signalling pathway (Asselin, Gibbins et al. 1997, Nieswandt and Watson 2003). CRP-XL has been proven to be unable to recognise and bind integrin $\alpha_2\beta_1$ for platelet activation. For example, in people diagnosed with GPVI deficiency, CRP-XL fails to induce platelet activation (Nieswandt and Watson 2003). Here, to determine the effects of 1,8-cineole selectively via GPVI signalling pathway, aggregation assays were performed using human isolated platelets upon stimulation with CRP-XL.

Human isolated platelets ($4x10^8$ cells/mL) were prepared as described earlier, and incubated with a vehicle control [0.01% (v/v) ethanol] or different concentrations of 1,8-cineole (6.25 µM-100 µM) for 5 minutes at 37°C. After the incubation, platelets were placed in an aggregometer and stimulated by the addition of different concentration of CRP-XL (0.5 µg/mL and 1 µg/mL) and the aggregation response was recorded for 5 minutes by optical aggregometry.

The results demonstrated that, 1,8-cineole was able to decrease platelet aggregation upon stimulation with 1 µg/mL CRP-XL (Figure 3-2 A and B). Similar to collagen, the concentrations of 1,8-cineole at 100 µM and 50 µM displayed around 60% inhibitory effects, but there was no significant inhibition observed with low concentrations of 1,8-cineole. The inability of low concentrations of 1,8-cineole to exert any inhibitory effects could be due to the potent actions of 1 µg/mL CRP-XL.

Therefore, the concentration of CRP-XL was reduced to 0.5 μg/mL to determine if the low concentrations of 1,8-cineole would then inhibit platelet aggregation. As expected, at concentrations of 25 and 50 μM, 1,8-cineole caused around 70% inhibition and at concentrations of 6.25 and 12.5 μM displayed about 40% to 50% reduction in CRP-XL induced platelet aggregation comparing to the vehicle control (Figure 3-2 C and D). These results demonstrate that 1,8-cineole is able to modulate platelet activation via GPVI-mediated signalling pathway.

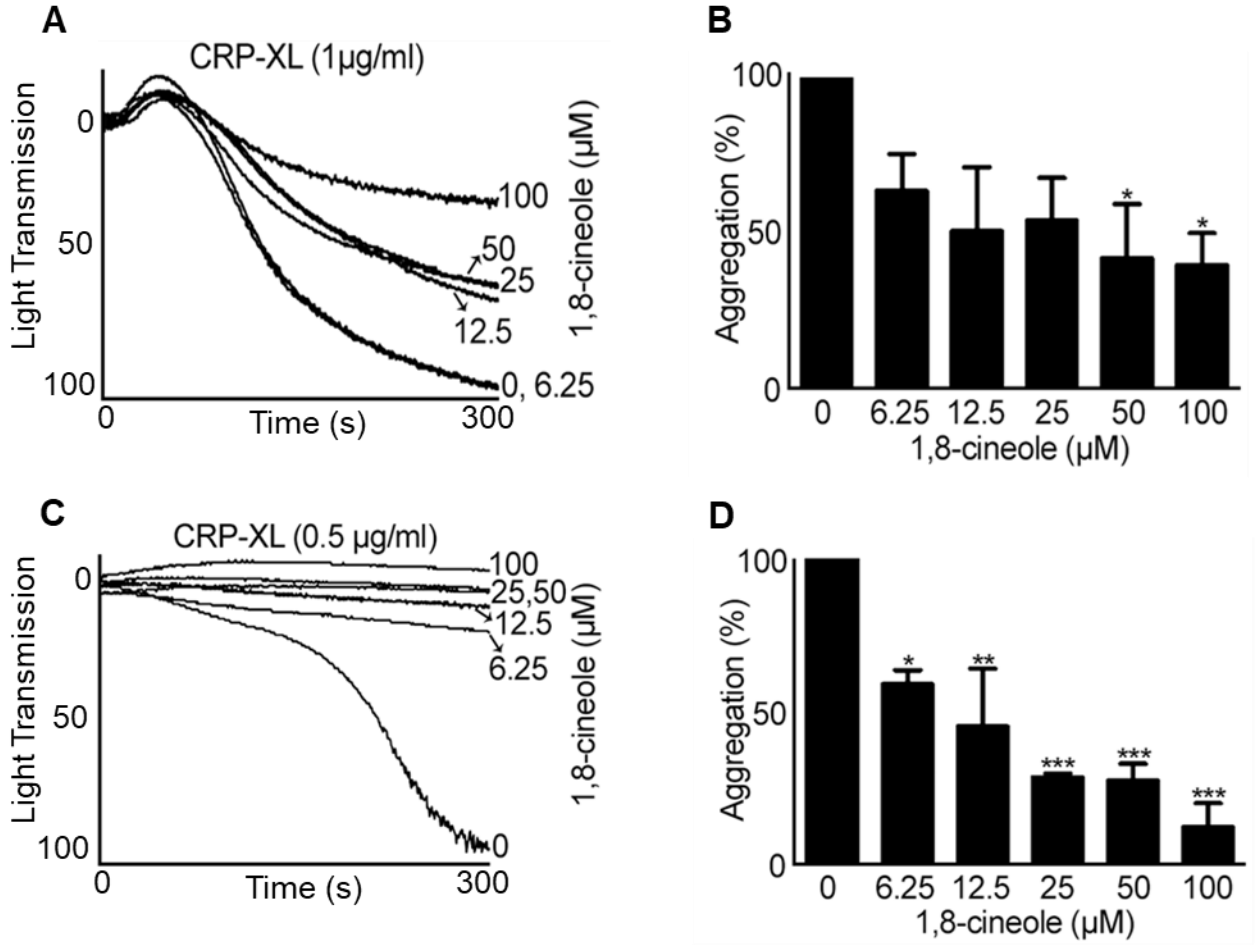

Figure 3-2: Effect of 1,8-cineole on CRP-XL-stimulated aggregation in human isolated platelets. Representative traces show the level of aggregation obtained when human isolated platelets ($4x10^8$ cells/mL) treated for 5 minutes with a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole, prior to stimulation with 1 µg/mL (**A** and **B**) or 0.5 µg/mL (**C** and **D**) CRP-XL. Bar graphs show the percentage of aggregation obtained with 1,8-cineole. The data were normalised by considering the maximum aggregation obtained for the vehicle control (CRP-XL alone) at 5 minutes as 100%, and the level of inhibition in 1,8-cineole treated platelet samples was calculated accordingly. The aggregation traces presented here are representative of 4 individual experiments. Cumulative data represent mean ± S.E.M (n=4). The p values (*$p<0.05$, **$p<0.01$ and ***$p<0.001$) shown are as calculated by one-way ANOVA followed by a Bonferroni *post-hoc* test.

3-2-1-3 1,8-cineole (at high concentrations) inhibits platelet aggregation induced by GPCR agonists

It is well known that activated platelets release various pro-thrombotic mediators such as ADP and TxA_2. Thrombin is generated on the surface of platelets at the site of injury via coagulation cascades. All these substances enhance the aggregation response by activating more circulating platelets and recruiting them to the growing thrombus. Thrombin mediates platelet activation by binding to protease-activated receptors (PARs) such as PAR-1 and PAR-4. It cleaves a part of the N-termini of these receptors which acts as tethered ligands for PAR-1 and PAR-4, and their binding subsequently activates platelets (Kahn, Nakanishi-Matsui et al. 1999, Woulfe 2005, Offermanns 2006). Similar to thrombin, ADP stimulates platelet activation by binding and signalling via two GPCRs, P2Y1 and P2Y12 (coupled to Gq and Gi, respectively) (Jin, Daniel et al. 1998, Hollopeter, Jantzen et al. 2001).

After establishing the impact of 1,8-cineole on collagen- and CRP-XL-induced platelet activation, other agonists such as thrombin and ADP that activate platelets via GPCRs were used to determine whether the actions of 1,8-cineole are only limited to GPVI stimulated signalling or they represent general effects on platelet inhibition. To determine the impact of 1,8-cineole on GPCRs-mediated platelet activation, aggregation assays were performed using thrombin and ADP as agonists.

Human isolated platelets ($4x10^8$ cells/mL) were prepared and incubated with a vehicle control [0.01% (v/v) ethanol] or different concentrations of 1,8-cineole (6.25,12.5, 25, 50, and 100 µM) for 5 minutes at 37°C as described previously. Following incubation, platelets were stimulated with thrombin (0.1 U/mL) and the level of platelet aggregation was monitored by optical aggregometry.

Figure 3-3 shows that 1,8-cineole had an inhibitory effect upon thrombin-stimulated platelet aggregation only at a highest concentration tested (100 μM). 100 μM of 1,8-cineole caused approximately 50% inhibition on thrombin-induced platelet aggregation. However, 1,8-cineole in lower concentrations (6.25, 12.5, 25 and 50 μM) has failed to show any inhibitory effect on thrombin-stimulated platelet aggregation (Figure 3-3 A-B). Again, as similar to above, the potent activity of 0.1U/ml thrombin might have prevented the observable effects of low concentrations of 1,8-cineole.

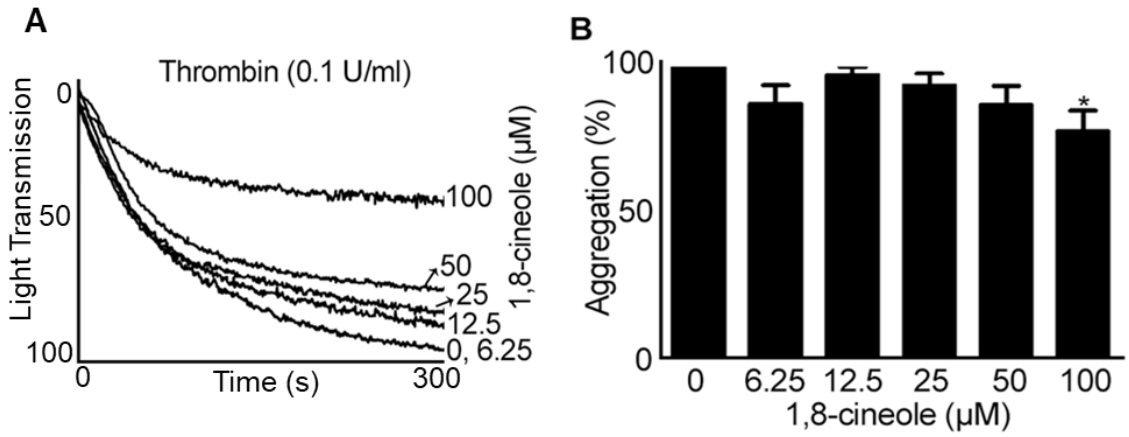

Figure 3-3: Effect of 1,8-cineole on thrombin-stimulated aggregation in human isolated platelets. Human isolated platelets ($4x10^8$ cells/mL) were incubated with different concentrations of 1,8-cineole or a vehicle control [0.01% (v/v) ethanol] for 5 minutes prior to addition of thrombin (0.1 U/mL). Aggregation was measured for 5 minutes at 37°C with constant stirring (1200 rpm) in an optical aggregometer. (**A**) Representative traces from aggregation of 3 individual experiments. (**B**) Percentage aggregation of samples in the presence and absence of different concentrations of 1,8-cineole at 5 minutes and the data were normalised to the level of aggregation obtained with the vehicle control. Cumulative data represent mean ± S.E.M (n=3). The p value (*$p<0.05$) shown is as calculated by one-way ANOVA followed by a Bonferroni *post-hoc* test.

Besides thrombin, the effect of 1,8-cineole on ADP-stimulated platelet aggregation in PRP was investigated. Since ADP does not activate isolated platelets very well, here PRP was used to determine the effects of 1,8-cineole upon activation with ADP. Human PRP was prepared and incubated with 1,8-cineole (6.25,12.5, 25, 50, and 100 μM) or a vehicle control [0.01% (v/v) ethanol] for 5 minutes at 37°C. After the incubation period, platelets were stimulated with ADP (2.5 or 5 μM), and the level of responses were recorded by optical aggregometry with continuous stirring (1200rpm) at 37°C for 5 minutes.

Figures 3-4 A and C show typical aggregation responses to ADP at concentrations of 2.5 and 5 μM. 1,8-cineole was unable to inhibit platelet aggregation stimulated by ADP (5 μM) at all the concentrations tested (Figure 3-4 A-B). However, 1,8-cineole exhibited inhibitory effects on low concentration (2.5 μM) of ADP-induced platelet aggregation, but only the highest concentration of 100 μM causing approximately 60% inhibition, while other concentrations of 1,8-cineole (6.25, 12.5, 25, and 50 μM) were unable to cause a reduction in platelet aggregation (Figure 3-4 C-D). These results are consistent with the data obtained with platelet aggregation upon stimulation with 0.1 U/mL thrombin, where 1,8-cineole caused significant inhibition on platelet aggregation only at a high concentration (100 μM). These data suggest that 1,8-cineole negatively regulate platelet aggregation induced by GPCR agonists but only at higher concentrations.

When comparing these data with the effects of 1,8-cineole on isolated platelet aggregation with collagen and CRP-XL, it can be observed that 1,8-cineole at concentrations of 6.25, 12.5, 25 and 50 μM did not show a significant inhibitory effect on platelet aggregation upon stimulation with ADP or thrombin as it caused with

collagen or CRP-XL at low concentrations. Therefore, the inhibitory effects of 1,8-cineole on platelet aggregation is largely likely to be mediated via GPVI signalling pathway although we cannot rule out the possibilities of its minimal effects through other signalling pathways.

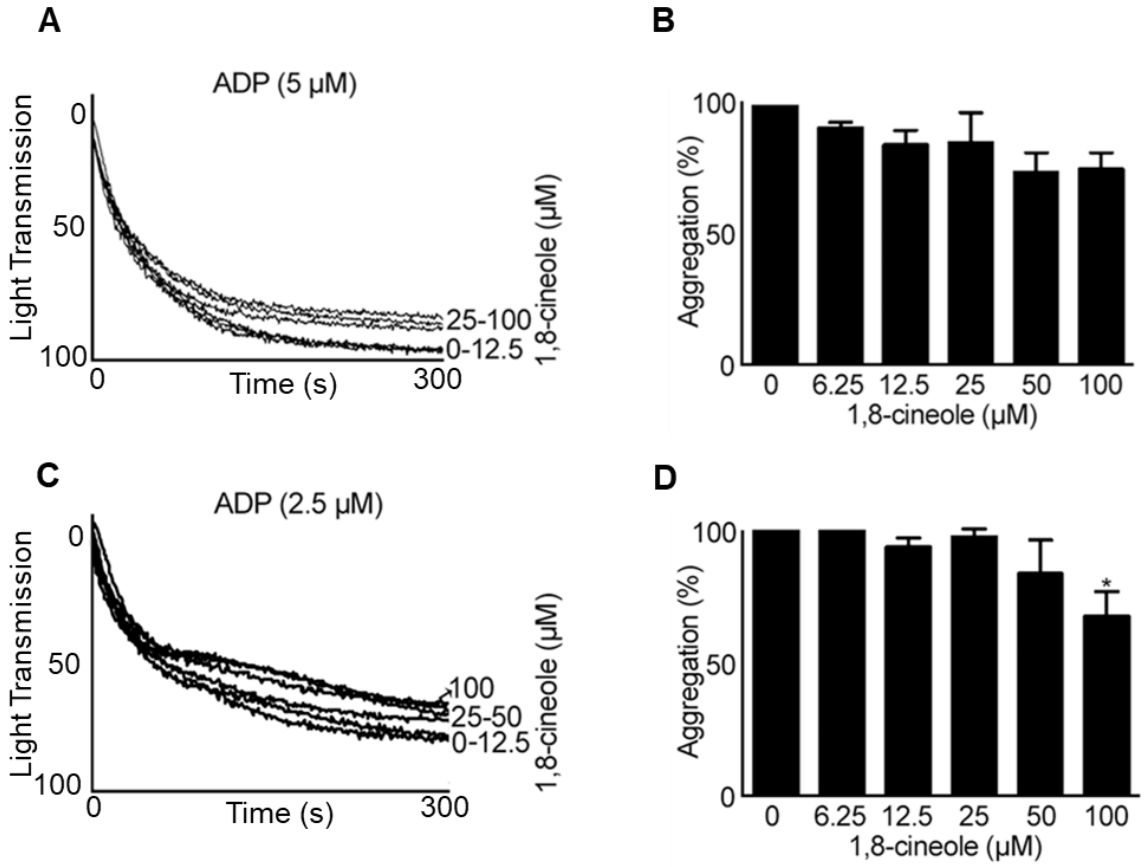

Figure 3-4: Effect of 1,8-cineole on ADP-stimulated aggregation in PRP. Human PRP was incubated with different concentrations of 1,8-cineole or a vehicle control [0.01% (v/v) ethanol] for 5 minutes prior to stimulation with 5 µM **(A** and **B)** or 2.5 µM **(C** and **D)** ADP and the level of aggregation was monitored by an optical aggregometry. The percentage of aggregation was calculated by considering the aggregation obtained with the vehicle control as 100%. The bar charts represent percentage aggregations obtained in the presence and absence of different concentrations of 1,8-cineole. Cumulative data represent mean ± S.E.M (n=3) The aggregation traces presented here are representative of 3 individual experiments. The p value (*p<0.05) shown is calculated by one-way ANOVA followed by a Bonferroni *post-hoc* test.

3-2-1-4 1,8-cineole inhibits collagen- and CRP-XL-stimulated aggregation in human PRP

The effect of 1,8-cineole on isolated platelets presented so far shows its direct impact on human isolated platelets (except for ADP-induced platelet aggregation) in the absence of plasma proteins and other blood cells. However, in order to investigate whether these effects can be translated under physiological settings, the effects of 1,8-cineole in PRP (i.e. in the presence of plasma proteins) and whole blood (i.e. in the presence of other blood cells and plasma proteins) need to be determined using different platelet functional assays. Here, the aim of this experiment is to investigate the impact of 1,8-cineole on platelet aggregation stimulated by collagen and CRP-XL (as they showed promising effects in isolated platelets) in the presence of plasma proteins using human PRP. As similar to isolated platelets, these experiments were performed using optical aggregometry.

Human PRP was prepared as described before (section 2-1) using whole blood obtained from healthy volunteers. PRP was incubated with different concentrations of 1,8-cineole (6.25, 12.5, 25, 50 and 100 μM) or a vehicle control for 5 minutes at 37°C, with stirring for 5 seconds to ensure that the 1,8-cineole had been fully mixed with the PRP. After incubation, collagen (0.5 μg/mL and 1 μg/mL) or CRP-XL (0.5 μg/mL and 1 μg/mL) was added to the mixture, and the level of aggregation was monitored for 5 minutes.

The results demonstrate that 1,8-cineole at a concentration of 100 μM displayed a significant inhibitory effect on 1 μg/mL collagen-stimulated platelet aggregation in human PRP (Figure 3-5 A-B). However, 1,8-cineole at lower concentrations (6.25, 12.5, 25 and 50 μM) had no significant inhibitory effects on platelet aggregation when 1 μg/mL collagen was used. However, at a lower

concentration of collagen (0.5 μg/mL), 1,8-cineole caused a significant inhibition on platelet aggregation, at all the concentrations tested (6.25, 12.5, 25, 50 and 100 μM) (Figure 3-5 C-D). At all the concentrations, 1,8-cineole displayed at around 60-70% inhibition although the response was appeared to be saturated as higher concentrations did not show increased inhibitory effects compared to the lower concentrations.

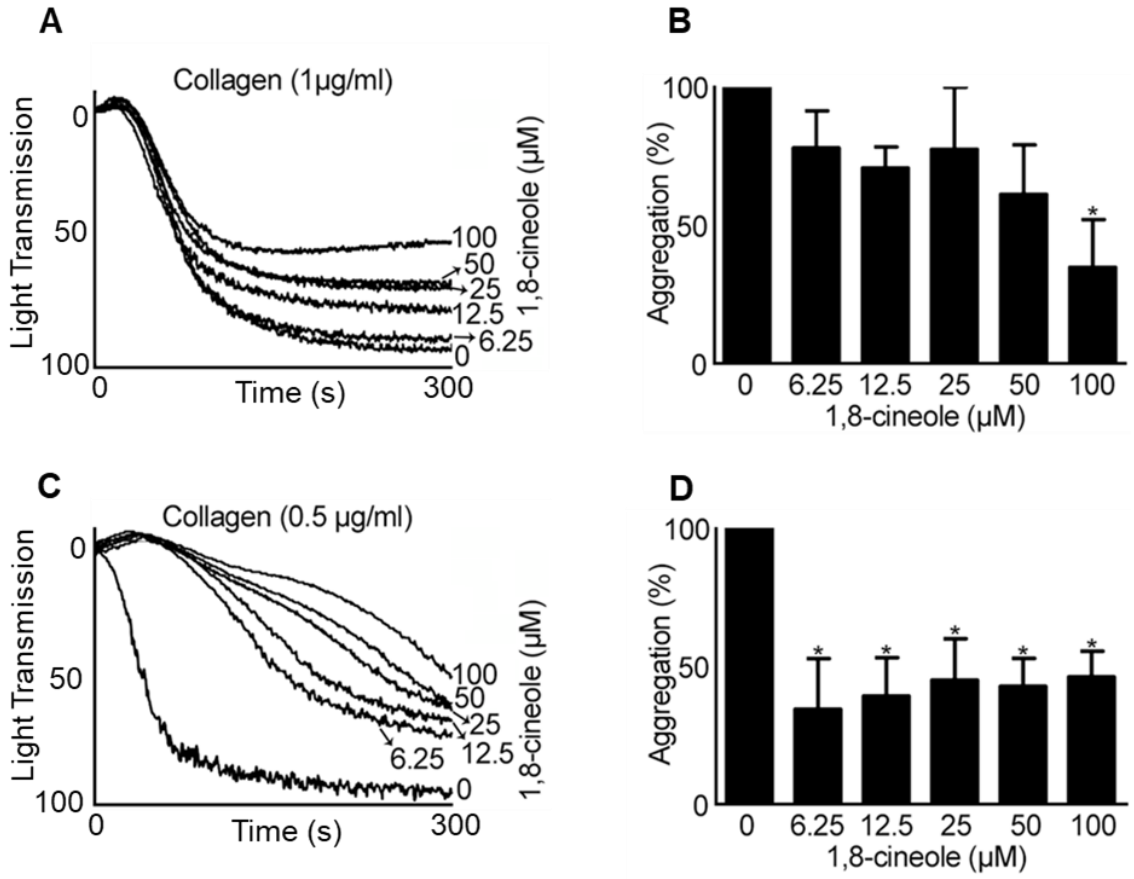

Figure 3-5: Effect of 1,8-cineole on collagen-stimulated platelet aggregation in human PRP. Optical aggregometry was used to determine the effect of 1,8-cineole on collagen-induced platelet aggregation in human PRP. PRP was incubated with different concentrations of 1,8-cineole or a vehicle control [0.01% (v/v) ethanol] for 5 minutes prior to stimulation with 1 µg/mL (**A** and **B**) or 0.5 µg/mL (**C** and **D**) collagen. The percentage of aggregation was calculated by considering the aggregation obtained with a vehicle control as 100%. The bar charts represent percentage aggregations obtained in the presence and absence of different concentrations of 1,8-cineole. The aggregation traces presented here are representative of 3 individual experiments. Cumulative data represent mean ± S.E.M (n=3). The p values (*p<0.05) shown is as calculated by one-way ANOVA followed by a Bonferroni *post-hoc* test.

Similar to these results, 1,8-cineole was unable to show any inhibitory effect on 1 µg/mL CRP-XL -induced platelet aggregation (Figure 3-6 A-B). However, 1,8-cineole showed its ability to significantly inhibit platelet aggregation on 0.5 µg/mL CRP-XL-induced platelet aggregation in PRP. A clear dose dependant inhibitory effects of 1,8-cineole were observed when 0.5 µg/mL CRP-XL was used. It displayed significant inhibitory effects at all the concentrations (12.5, 25, 50 and 100 µM) except at 6.25µM (Figure 3-6 C-D).

Overall, when comparing these data to the data obtained for isolated platelets, 1,8-cineole was still effective in inhibiting collagen or CRP-XL-stimulated platelet aggregation in PRP (i.e. in the presence of plasma proteins) at low concentration of collagen and CRP-XL. These data demonstrate that 1,8-cineole is likely to affect platelet function under physiological settings.

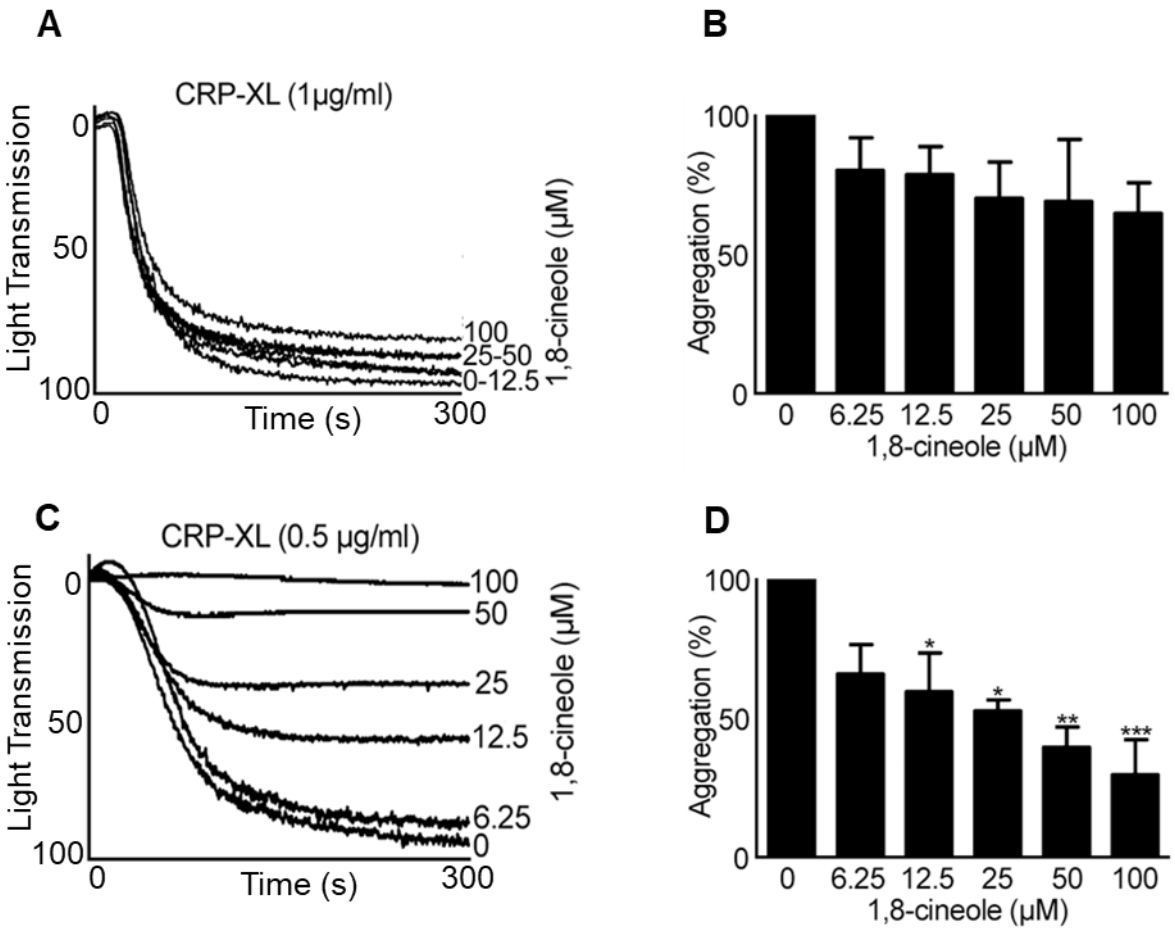

Figure 3-6: Effect of 1,8-cineole on CRP-XL-induced aggregation in human PRP. Human PRP was incubated with different concentrations of 1,8-cineole or a vehicle control [0.01% (v/v) ethanol] for 5 minutes prior to stimulation with 1 µg/mL (**A-B**) or 0.5 µg/mL (**C-D**) CRP-XL and the level of aggregation was monitored by an optical aggregometer. The percentage of aggregation was calculated by considering the aggregation obtained with the vehicle control as 100%. The bar charts represent percentage aggregations obtained in the presence and absence of different concentrations of 1,8-cineole. The aggregation traces presented here are representative of 3 individual experiments. Cumulative data represent mean ± S.E.M (n=3). The p values (*p<0.05, **p<0.01 and ***p<0.001) shown are as calculated by one-way ANOVA followed by a Bonferroni *post-hoc* test.

3-2-2 1,8-cineole inhibits inside-out signalling to integrin αIIbβ3 in platelets

Platelet membrane contains a number of different receptors for various roles. Integrin $\alpha_{II}b\beta_3$ (a highly abundant platelet surface receptor) is one of the most prominent receptors in platelet membrane (Shattil, Kashiwagi et al. 1998). Platelet aggregation is associated with a conformation change in integrin αIIbβ3 through inside-out signalling which transforms the receptor from a low-affinity state to a high binding affinity state for fibrinogen and von Willebrand factor (vWF) (Nieswandt, Varga-Szabo et al. 2009, Estevez, Shen et al. 2015, Estevez and Du 2017) . Hence, the level of fibrinogen binding on the surface of platelets can be measured as a marker for inside-out signalling to integrin αIIbβ3. Therefore, to determine the effect of 1,8-cineole on inside-out signalling to integrin αIIbβ3, the level of fibrinogen binding (as a marker for inside-out signalling) was measured in platelets stimulated with CRP-XL (0.5 µg/mL) using fluorescent-labelled anti-fibrinogen antibodies by flow cytometry.

Human isolated platelets ($2x10^8$ cells/mL) or PRP were prepared as described before and incubated with different concentrations of 1,8-cineole or a vehicle control for 5 minutes, with FITC-conjugated anti-fibrinogen antibodies at room temperature. Platelets were then stimulated with CRP-XL (0.5 µg/mL) for 20 minutes at room temperature prior to fixing with 0.2% (v/v) formyl saline and analysis by flow cytometry. 1,8-cineole showed a significant inhibition in the fibrinogen binding levels in CRP-XL activated platelets at all the concentrations tested (6.25 µM- 50 µM). Figure (3-7 A) shows a decrease in the level of fluorescence in samples incubated with 1,8-cineole compared to the positive control (CRP-XL). The fibrinogen binding on platelet surface was inhibited by 1,8-cineole in a concentration dependent manner; over 60% inhibition was observed at the lowest concentration of 6.25 µM, whereas a concentration of 12.5

μM caused around 75% inhibition, and 25-50 μM were found to be strong in inhibiting fibrinogen binding with almost 95% inhibition. This data shows a potent inhibitory effect of 1,8-cineole on inside-out signalling to integrin αIIbβ3. These data are similar to the level of inhibition observed with CRP-XL-induced platelet aggregation.

Similar to previous experiments, the effect of 1,8-cineole was also investigated in PRP in the presence of plasma proteins to determine whether 1,8-cineole will exhibit the same inhibitory effects on inside-out signalling to integrin αIIbβ3. Indeed, 1,8-cineole shows a clear inhibitory effect on fibrinogen binding to αIIbβ3 in PRP at concentrations of 12.5, 25 and 50 μM (Figure 3-7 B).

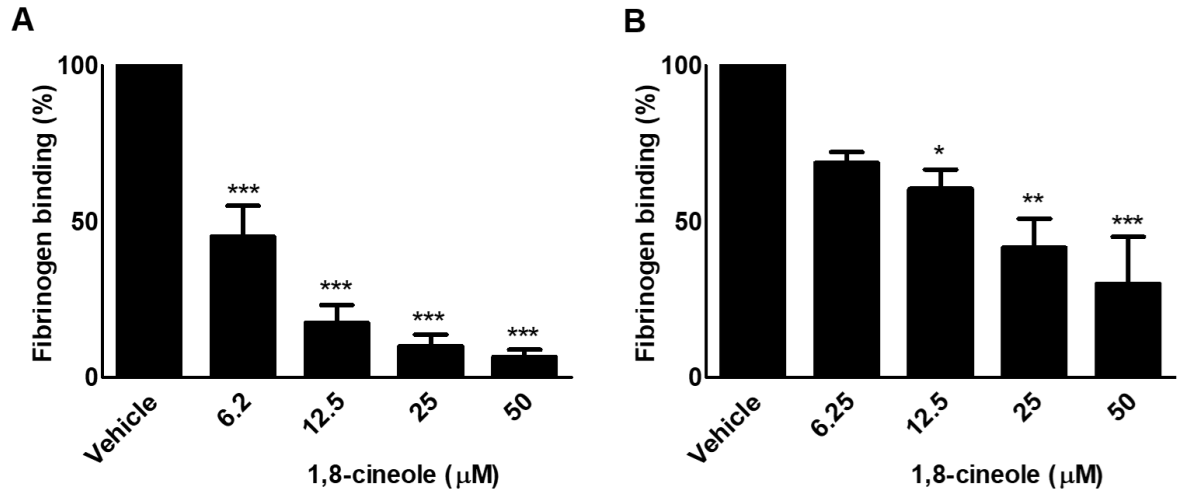

Figure 3-7: Effect of 1,8-cineole on inside-out signalling to integrin αIIbβ3. Human isolated platelets (**A**) and PRP (**B**) were incubated with a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole for 5 minutes prior to the addition of CRP-XL (0.5 µg/mL) for 20 minutes at room temperature. The level of fibrinogen binding (as a marker for inside-out signalling to integrin αIIbβ3) on the platelet surface was quantified using FITC-conjugated anti-human fibrinogen antibodies by flow cytometry. The extent of inhibition in treated samples was calculated by considering the level of fluorescence obtained with the vehicle control as 100%. Cumulative data represent mean ± S.E.M (n=3). The p values (*$p<0.05$, **$p<0.01$ and ***$p<0.001$) shown are as calculated by one-way ANOVA followed by a Bonferroni post-hoc test.

3-2-3 1,8-cineole inhibits granule secretion in platelets

Platelet cytoplasm contains both α and dense granules. Platelet α-granules contain many protein components that are critical for the positive feedback mechanisms during platelet activation and thrombosis. These proteins include fibrinogen, fibronectin, P-selectin and vWF which have critical roles in platelet adhesion, aggregation and thrombus formation (Stenberg, Shuman et al. 1984, Maynard, Heijnen et al. 2007). Notably, p-selectin (CD62P) present in α-granules is a useful marker to quantify α-granule secretion in platelets as during platelet activation P-selectin is exposed to the surface of platelets.

P-selectin is a transmembrane adhesion receptor and it plays a major role in initiating platelet-leukocyte interactions (Koedam, Cramer et al. 1992). Fluorescently labelled anti-CD62P antibodies can be used to measure the level of P-selectin exposure from α-granules during platelet activation by flow cytometry. The effect of 1,8-cineole on α-granule secretion in platelets was analysed by measuring the level of P-selectin on platelet surface when platelets were stimulated by CRP-XL in the presence and absence of various concentrations of 1,8-cineole.

Human isolated platelets at a density of $2x10^8$ cells/mL or PRP were prepared as described before and incubated with various concentrations of 1,8-cineole or a vehicle control for 5 minutes at room temperature. Anti-CD62P (Cy5/PE conjugated) antibody was added to platelet samples (isolated platelets or PRP) at a concentration of 1:100. Following incubation, platelets were stimulated with CRP-XL (0.5 µg/mL) and the samples were incubated for 20 minutes at room temperature. After that, reactions were stopped by fixing the samples with 0.2% (v/v) formyl saline. The level of P-

selectin exposure was measured by collecting 5000 events within the gated region that had been set for platelet population using a flowcytometer.

The results demonstrate that 1,8-cineole decreases the level of fluorescence in treated samples compared to the positive control (CRP-XL) in isolated platelets (Figure 3-8 A). P-selectin exposure was inhibited by 1,8-cineole in a concentration dependent manner; 50% inhibition was observed at the lowest concentration (6.25 μM) of 1,8-cineole, whereas a concentration of 12.5 μM caused approximately 60% inhibition. Higher concentrations (25 and 50 μM) of 1,8-cineole were found to be strong in inhibiting CRP-XL-induced P-selectin exposure as they showed 74% and 80% of inhibition, respectively. This data shows that 1,8-cineole is able to inhibit α-granule secretion in platelets.

In PRP, 1,8-cineole also caused a significant reduction to the level of P-selectin exposure on the cell surface at concentrations of 12.5, 25, and 50 μM compared to the control samples (Figure 3-8 B). These data demonstrate that 1,8-cineole was able to affect α-granule secretion in platelets both in the absence (isolated platelets) and presence of plasma proteins and other factors present in plasma.

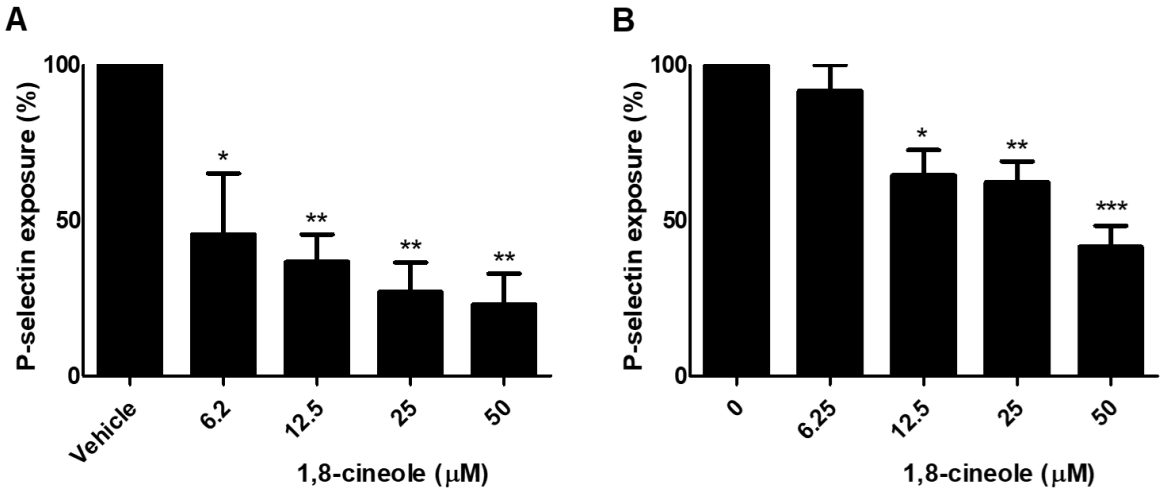

Figure 3-8: 1,8-cineole inhibits α-granule secretion in platelets. Human isolated platelets (**A**) and PRP (**B**) were incubated with a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole for 5 minutes, after which PE/Cy5 anti-human CD62P antibodies were added to samples, followed by the addition of CRP-XL (0.5 µg/mL) and incubated for 20 minutes at room temperature. The level of α-granule secretion was determined by quantifying the level of P-selectin exposed (as a marker for α-granule secretion) on the platelet surface using flow cytometry. The bar graph shows the effect of 1,8-cineole on the α-granule secretion and was calculated with respect to the positive control (CRP-XL alone) which was considered as 100%. Cumulative data represent mean ± S.E.M (n=3). The p values (*$p<0.05$, **$p<0.01$ and ***$p<0.001$) shown are as calculated by one-way ANOVA followed by a Bonferroni post-hoc test.

Unlike α-granules, a number of small, non-proteineous molecules such as ATP, ADP and serotonin are stored in dense granules, and they also play critical roles in the early process of platelet activation due to their rapid secretion when platelets are activated (Gear and Burke 1982, Jonnalagadda, Izu et al. 2012). Therefore, we investigated whether 1,8-cineole affects dense granule secretion upon platelet activation similar to their inhibitory effects in α-granule secretion. This assay was performed using lumi-aggregometry method (Feinman, Lubowsky et al. 1977).

In this assay, the luciferin-luciferase reaction was used to quantify the amount of ATP released from platelets. In brief, by using ATP as energy, luciferin gets converted to oxyluciferin via the oxidative enzyme luciferase, which causes luminescence to be released (McElroy 1947). In this assay, a chronolume reagent containing luciferin and luciferase was used. Upon platelet activation, ATP secreted from dense granules is used in the reaction as a source of energy and the lumi-aggregometer is used to measure the level of luminescence released as a result of this reaction.

Human isolated platelets ($4x10^8$ cells/mL) were treated with different concentrations of 1,8-cineole or a vehicle control for 5 minutes. The chronolume reagent was added to the platelets in the last two minutes of incubation to allow the reaction of luciferin-luciferase to occur. The secretion of ATP was monitored for 5 minutes after the addition of collagen (0.5 µg/mL) as an agonist.

The results (Figure 3-9 A-B) show that 1,8-cineole caused a significant inhibition in ATP release; approximately 60%, 70%, 60%, 85% and 90% of inhibition was achieved at 6.25, 12.5, 25, 50 and 100µM of 1,8-cineole respectively, in comparison to the vehicle control.

The data presented in the platelet granule secretion section shows the efficacy of 1,8-cineole in the inhibition of both granule secretion from platelets upon activation. This could be one of the primary mechanisms for 1,8-cineole to control platelet activation.

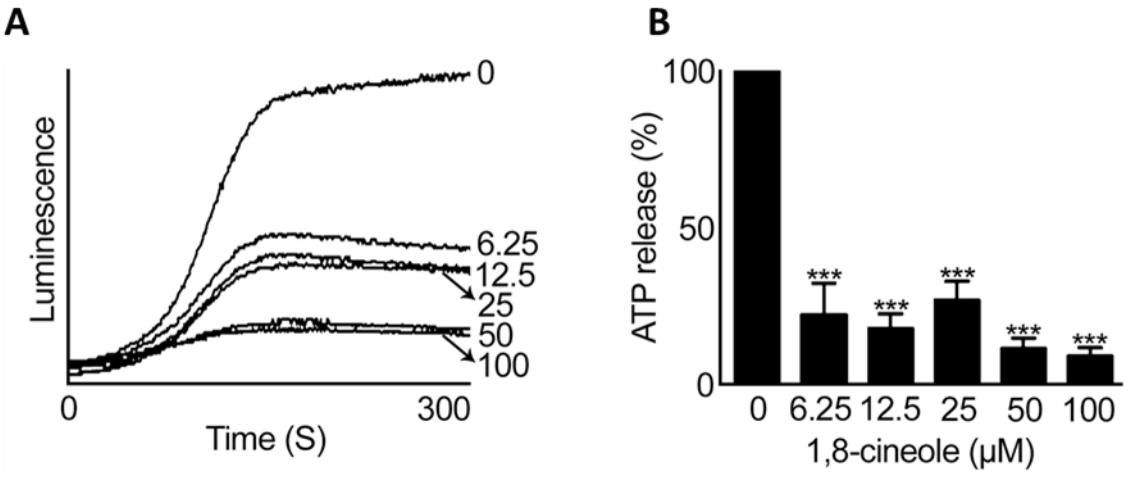

Figure 3-10: 1,8-cineole inhibits dense granule secretion in platelets. The effect of 1,8-cineole on dense granule secretion from platelets was quantified by measuring the level of ATP release upon platelet activation using lumi-aggregometry. Human isolated platelets (4 x 10^8 cells/mL) were incubated with different concentrations of 1,8-cineole or a vehicle control [0.01% (v/v) ethanol] for 5 minutes with a chronolume luciferin-luciferase reagent was added 2 minutes before stimulation with collagen (0.5 μg/mL). ATP release upon platelet activation was monitored for 5 minutes by lumi-aggregometry. (**A**) Representative traces shown are from 3 individual experiments. (**B**) ATP secretion obtained in the positive control was used as 100% to calculate the percentage of release in the presence of 1,8-cineole. Cumulative data represent mean ± SEM (n=3). The p value (***p<0.001) shown was as calculated by one-way ANOVA followed by a Bonferroni post-hoc test.

3-2-6 1,8-cineole affects intracellular calcium mobilisation in platelets

Platelet activation requires a number of intracellular signalling mechanisms. One of the key steps in platelet activation is the increase in intracellular calcium level which triggers downstream signalling pathways to support platelet adhesion, granule release and aggregation. Calcium mobilisation from intracellular stores (dense tubular system) to the cytoplasm is critical for regulating integrin activation (Stefanini, Roden et al. 2009) and other platelet functions. The mobilisation of calcium at the intracellular level is transient when platelets are stimulated by agonists (Sage and Rink 1986). Therefore, understanding the impact of any inhibitory molecule on calcium mobilisation is critical to establish their mode of mechanisms in the modulation of platelet activation, haemostasis and thrombosis. Hence, we sought to determine the impact of 1,8-cineole on intracellular calcium mobilisation during platelet activation.

Calcium mobilisation was measured using Fluo-4, a fluorescent dye that binds to free intracellular Ca^{2+} and allows the detection and visualisation of Ca^{2+} in cells, here in this case platelets. 2 mL of human PRP was incubated with Fluo-4 for 45 minutes at 37°C, with occasional mixing. After the incubation, Fluo-4 loaded isolated platelets were prepared as described in section 2-9. These isolated platelets (4×10^8 cells/mL) were then incubated with different concentrations of 1,8-cineole or a vehicle control for 5 minutes at 37°C. After the incubation, platelets were stimulated by adding CRP-XL (0.5 µg/mL) to cause an increase in Ca^{2+} level in platelet cytosol.

In vehicle control-treated platelets, peak Ca2+ was reached at around 80 seconds, whereas 1,8-cineole significantly inhibited cytosolic increase in Ca2+ levels. Figure 3-11 shows a comparison between the kinetics of cytosolic calcium release for 1,8-cineole treated samples and the vehicle control. Over 5 minutes of measurement, representative traces for 1,8-cineole-treated samples remain lower than the trace for

vehicle control and this confirms an inhibitory effect of 1,8-cineole on calcium mobilisation. Statistical calculation of the peak calcium concentration in 1,8-cineole samples was conducted and normalised to the control. 1,8-cineole at concentrations of 25 and 50 µM inhibited Ca^{2+} levels up to 40%, and around 20% inhibition was observed at 12,5 µM (Figure 3-11 B). The lowest concentration of 6.25 µM showed no significant difference in calcium levels compared to the control. The inhibitory effects of 1,8-cineole on early signalling events such as calcium mobilisation suggest a prominent role of 1,8-cineole in the modulation of platelet activation via affecting critical signalling pathways.

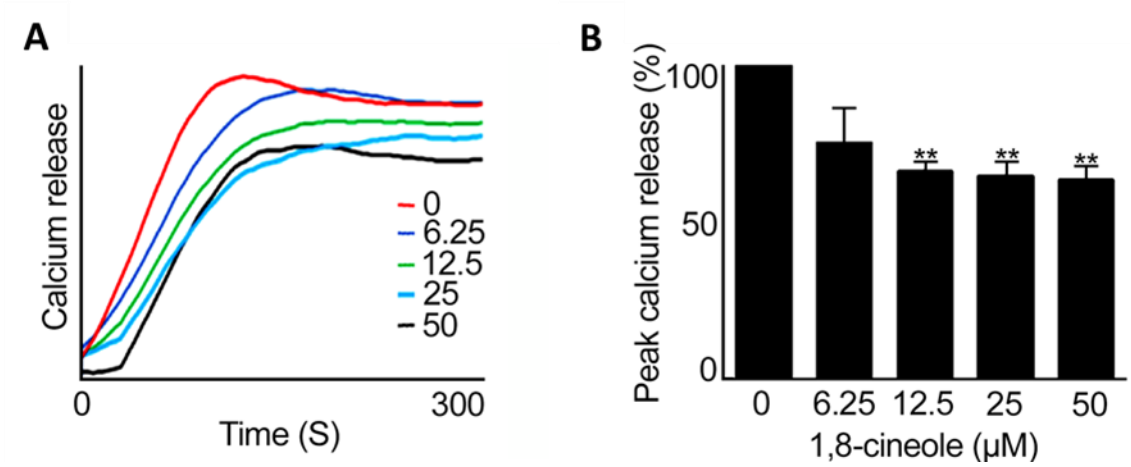

Figure 3-11: 1,8-cineole affects intracellular Ca^{2+} mobilisation in platelets. Fluo 4- loaded human isolated platelets (4 x 10^8 cells/mL) were incubated with different concentrations of 1,8-cineole or a vehicle control for 5 minutes at 37°C prior to stimulation with CRP-XL (0.5 μg/mL). The level of fluorescence (at an excitation wavelength of 340 nm and 380 nm, and emission at 510 nm) was recorded for 5 minutes using a NOVOstar plate reader, and the peak calcium level was measured as the maximum value reached in sample for 5 minutes. Traces (**A**) presented here are representative of 3 individual experiments. Cumulative data (peak calcium levels) in the presence or absence of 1,8-cineole after stimulation with CRP-XL (0.5 μg/mL) was calculated by taking peak calcium level obtained in positive control (CRP-XL) as 100% (**B**). Data represent mean ± SEM (n=3), the p value (**p<0.001) shown as calculated by one-way ANOVA followed by a Bonferroni post-hoc test.

3-3-7 1,8-cineole affects integrin αIIbβ3-mediated outside-in signalling in platelets

3-3-7-1 Platelet spreading on fibrinogen-coated surface was inhibited by 1,8-cineole

One of the main functions of fibrinogen that binds to integrin αIIbβ3, is to facilitate a cascade of intracellular (outside-in) signalling events, which cause platelet spreading and an increase in the platelet surface area by forming filopodia and lamellipodia (Furie and Furie 2008). In order to investigate whether 1,8-cineole is able to inhibit integrin αIIbβ3 mediated outside-in signalling in platelets, platelet adhesion and spreading on fibrinogen-coated surfaces were investigated.

Glass coverslips placed in a 24-well plate were coated with fibrinogen (100 µg/mL) for 45 minutes, followed by the addition of 1% (w/v) BSA for 1 hour to prevent platelet binding to non-specific glass binding. Human isolated platelets (2×10^7 cells/mL) were prepared and treated with different concentrations of 1,8-cineole or a vehicle control [ethanol 0.01 %(v/v)] for 5 minutes at 37°C and added to the coverslips pre-coated with fibrinogen (100 µg/mL). Following washing of the unbound platelets after 30 minutes, the adhered/spread platelets were permeabilised with 0.2% (v/v) Triton-X in PBS buffer. Then platelets were stained with Alexa-Fluor 488-labelled phalloidin for visualisation using a confocal microscope. Ten random images were taken of each sample, and in order to categorise the platelets at different stages of spreading, their morphology was classified into three groups: adhered, filipodia formed and lamellipodia formed. The total percentage of platelets in each group was calculated in comparison to the control group.

Figure 3-12 shows representative images of platelets either adhered or at different stages of spreading on fibrinogen in the presence or absence of different

concentrations of 1,8-cineole. Incubation of platelets with 1,8-cineole significantly inhibited their adhesion to fibrinogen at concentrations of 25 and 50 μM in comparison to the vehicle control. This inhibition indicates the ability of 1,8-cineole to negatively regulate integrin αIIbβ3 mediated outside-in signalling, adding further evidence of the impact of 1,8-cineole on reduced fibrinogen binding observed previously in samples treated with 1,8-cineole upon stimulation with CRP-XL.

Moreover, as shown in figure 3-12, platelets treated with all the concentrations of 1,8-cineole were found to exhibit reduced spreading in comparison to the vehicle control. As shown in (Figure 3-12A), vehicle-treated samples displayed a large number of fully spread platelets with apparent lamellipodia formation. However, in samples treated with 1,8-cineole (Figure 3-12B-E), a significant number of platelets were found stuck at early stages of their spreading (filopodia formation), with only a few platelets moving to lamellipodia formation. In vehicle control-treated samples, around 80% of platelets were fully spread, whereas samples treated with 1,8-cineole were reduced to 40%, 50%, 65% and 90% at concentrations of 6.25, 12.5, 25 and 50 μM, respectively. Similarly, vehicle-treated platelets were found to display around 8% filopodia after 45 minutes, whereas platelets treated with 1,8-cineole have displayed an increase in filipodia formation by around 45%, 25%, 20% and 17% at concentrations of 6.25, 12.5, 25 and 50 μM, respectively in comparison to vehicle control. These results suggest that 1,8-cineole plays a significant role in the modulation of outside-in signalling mediated by integrin $\alpha_{IIb}\beta_3$.

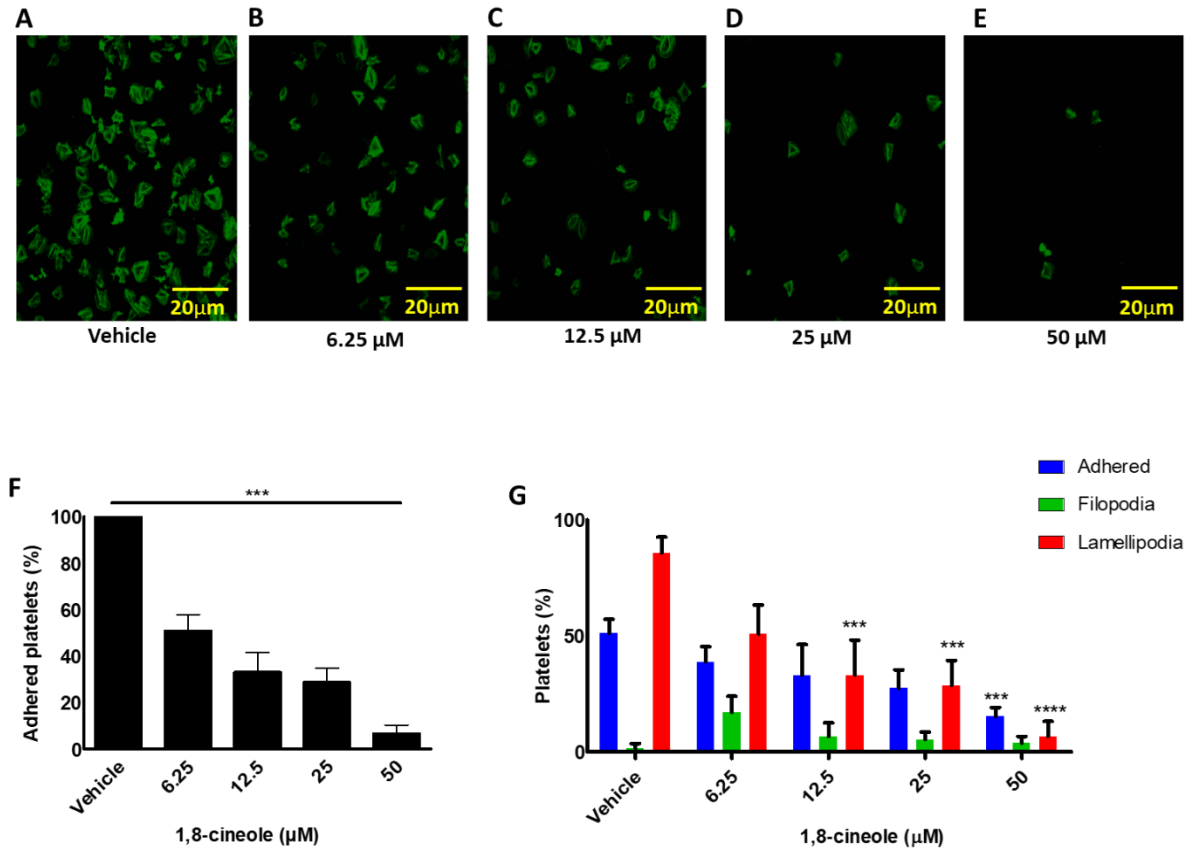

Figure 3-12: Effect of 1,8-cineole on platelet spreading. Human isolated platelets ($2x10^8$ cells/mL) were incubated with a vehicle control (**A**) or different concentrations of 1,8-cineole (**B-E**) for 5 minutes and added onto fibrinogen- (100 µg/mL) coated coverslips for 45 minutes. Then the platelets were fixed with 0.2% (v/v) formyl saline for 10 minutes and permeabilised with 0.2% (v/v) Triton X-100. Cells were stained with Alexa Fluor 488-conjugated phalloidin for visualisation. Platelet spreading on immobilised fibrinogen was analysed using a 100x oil immersion lens on a Nikon A1-R confocal microscope. Ten random images of view were recorded and for each sample, random locations on the slides were analysed. The number of adhered and spread platelets was determined by analysing the images using ImageJ. (**A-E**) representative images of platelet spreading in the absence and presence of different concentrations of 1,8-cineole. (**F**) the graph shows the percentage of platelets at various stages of spreading (adhered, filipodia and lamellipodia). Data represent mean ± SEM (n=3 individual experiments using platelets obtained from three volunteers, and for each 10 images were used for analysis). The P value shown ***p<0.001) are as calculated by one-way ANOVA followed by Bonferroni post-hoc test.

3-2-7-2 Clot retraction is inhibited by 1,8-cineole

Clot retraction is a process that occurs after the clot has been formed in order to bring the wound edges together to facilitate wound healing. This activity is driven through outside-in signalling mediated by integrin αIIbβ3, resulting in the contraction of fibrin mesh, and thus reduces the size of blood clot. The normal clot retraction occurs approximately around 2 hours after the formation of blood clot formation. The process of clot retraction depends on a number of factors that released from platelets surrounded by the fibrin clot. However, clot retraction depends mainly on the engagement of fibrinogen with integrin αIIbβ3 on platelet surface and subsequent outside-in signalling mediated by this integrin (Osdoit and Rosa 2001). Integrin $\alpha_{II}b\beta_3$ is a highly abundant heterodimeric receptor on the platelet surface and it plays a role in providing a physical link between the outside of the cell (“surrounding matrix”) and the cytoskeleton inside the platelets.

The physiological importance of clot retraction is mainly for the clearance of obstructed vessel with blood clots in order to restore normal blood flow while initiating wound healing and angiogenesis as necessary. Moreover, some clinical studies in patients who were diagnosed with Glansmann thrombasthenia reported excessive bleeding due to defects in integrin $\alpha_{II}b\beta_3$-mediated signalling, and the inability of platelets to aggregate and retract a fibrin clot. This disorder adds a clear evidence for a key role of integrin αIIbβ3 in clot retraction (Nurden 1999). Hence, the clot retraction can be analysed as another measure for integrin αIIbβ3-mediated outside-in signalling.

Given the ability of 1,8-cineole to negatively regulate platelet spreading, investigating the effect of 1,8-cineole on clot retraction would give more insight on it is effects on integrin $\alpha_{II}b\beta_3$-mediated outside-in signalling. Additionally, this effect would

be examined in an experiment that contains most of the components involved in coagulation cascade and haemostasis. Therefore, it will also help to recognise the efficacy of 1,8-cineole in a more physiological setting.

Human PRP (200 µL) was incubated with different concentrations of 1,8-cineole (6.25, 12.5, 25 and 50 µM) or a vehicle control for 5 minutes at 30°C along with modified Tyrodes-HEPES buffer (to make up the final volume of 945 µL) and 5µL of red blood cells (to allow visualisation of the clot). After that, 50 µL of thrombin (final concentration of 1 U/mL) was added to stimulate clot formation. A blunted glass pipette was added into the centre of each test tube around which the clot would form, and tubes were placed on a bench top. Clot retraction was monitored for 2 hours and images were taken at every 30 minutes. At the end of 2 hours, the remaining clots were weighed to determine the level of retraction.

The results demonstrate that 1,8-cineole significantly inhibited clot retraction at concentrations of 25 and 50 µM. The maximum retraction was noted at 50 µM (Figure 3-13A). While the vehicle control displayed a mean clot weight of 13 mg after 2 hours, 1,8-cineole-treated samples at concentrations of 12.5, 25 and 50 µM exhibited an increase in the mean clot weights to 18, 20 and 26 mg, respectively in comparison to vehicle control (Figure 3-13B). While at a lower concentration of 6.25 µM, 1,8-cineole has failed to exert a significant difference in clot weight, the other concentrations displayed a significance difference. This data suggests an inhibitory effect of 1,8-cineole on clot retraction and this is in overall agreement with the previous data obtained so far. This data indicates that the inhibition of integrin αIIbβ3 outside-in signalling as a key step through which 1,8-cineole may exert its anti-platelet effects.

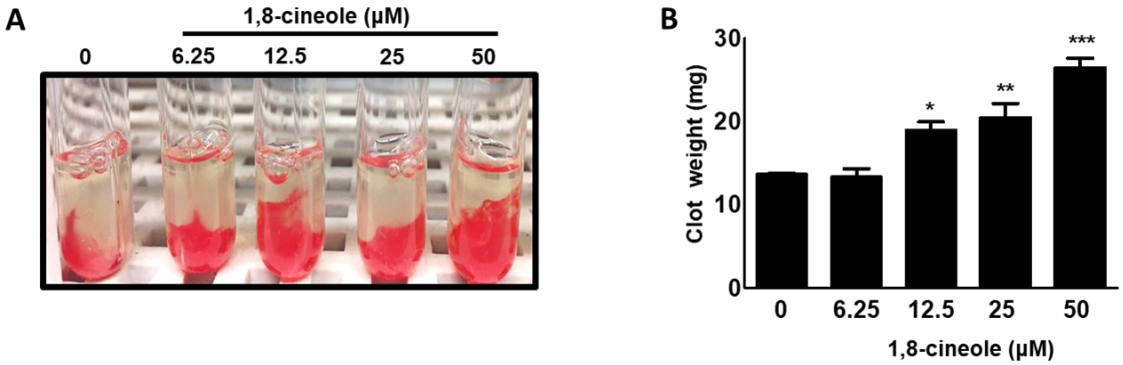

Figure 3-13: Impact of 1,8-cineole on clot retraction. Human PRP (200μl) was treated with different concentrations of 1,8-cineole or a vehicle control and the final volume was raised to 1 mL by the addition 750μl of warm modified Tyrodes-HEPES buffer to test tube. Thrombin (1 U/mL final concentration) was added to initiate the clot formation and the samples were placed in a bench top at room temperature. The clot retraction was monitored for 2 hours and images were taken at every 30 minutes, and the clot weights were measured after 2 hours. (**A**) a representative image of clot retraction after 2 hours in the presence and absence of 1,8-cineole. (**B**) Cumulative data showing the clot weight (mg) of samples treated with 1,8-cineole and compared with the vehicle control. Data represent mean ± SEM (n=4). The P values shown (*$p<0.05$, **$p<0.001$ and ***$p<0.001$) are as calculated by one-way ANOVA followed by Bonferroni post-hoc test.

3-3-8 1,8-cineole inhibits *in vitro* thrombus formation under arterial flow conditions

The data obtained so far in this chapter demonstrate the inhibitory effects of 1,8-cineole on various of platelet functions. Having observed the effects of 1,8-cineole to modulate platelet activation using isolated platelets and PRP, we then sought to determine its actions in whole blood in the presence of other blood cells (red and white blood cells). To determine the effect of 1,8-cineole in the modulation of platelet activation in whole blood, an *in vitro* thrombus formation assay on collagen-coated surface under arterial flow conditions was performed in the presence and absence of various concentrations of 1,8-cineole.

The *in vitro* thrombus formation assay is able to measure various parameters of thrombi that form under arterial flow conditions on a collagen-coated surface at physiological shear rate. Under *in vivo* settings, platelet recruitment at the site of injury requires high shear rates of above 10,000s^{-1} in order to enable interactions between GPIbα and vWF. Whereas the interaction between integrin $\alpha_{IIb}\beta_3$ and fibrinogen can occur at low physiological shear rates (600-900s^{-1}) (Savage, Almus-Jacobs et al. 1998, Nesbitt, Kulkarni et al. 2002, Nesbitt, Westein et al. 2009). Platelets can be activated at very high shear rates, as in advanced atherosclerosis, in the absence of any activator signals (Sheriff, Bluestein et al. 2010). It has been stated that physiological arterial shear stress is between 250-1750s^{-1} (10-70 dynes/cm^2) (Papaioannou and Stefanadis 2005), so for this experiment a shear rate of 20 dyns/cm^2 was chosen, this is equivalent to a shear rate of 500s^{-1}, using Colman's conversion (Colman 2006).

Human whole blood was incubated with a lipophilic dye, DiOC6 (3,3'Dihexyloxacarbocyanine iodide) at a concentration of 5 μM for 30 minutes at 30°C,

to enable platelet visualisation under a fluorescence/confocal microscope. Type I collagen (400 µg/mL) was used to coat Vena8 BioChips for one hour, and the excess collagen was washed using modified Tyrodes-HEPES buffer while non-specific binding sites were blocked with 5% (w/v) BSA. Whole blood was then incubated with different concentrations of 1,8-cineole (6.25, 12.5 and 50 µM) or a vehicle control for 5 minutes at 30°C, before perfusion through the collagen-coated microfluidic channels at a shear stress of 20 dynes/cm^2 for 10 minutes. A Nikon A1-R confocal microscope was used to observe microfluidic channels using the 20x objective, and images of thrombus formation were captured every 30 seconds over 10 minutes. NIS elements software (Nikon, UK) was used to analyse and calculate mean thrombus fluorescence intensity.

Representative images of growing thrombi over 10 minutes in 1,8-cineole-treated and a vehicle control samples are shown in Figure 3-14. Thrombi size was observed to continuously increase over 10 minutes of blood flow, and the data were normalised where the vehicle was considered as 100% thrombus formation at 10 minutes. The representative images (Figure 3-14 A) show thrombus formation over a period of 10 minutes; large and bright thrombi can be seen in vehicle control-treated sample with a significant amount of platelet accumulation occurring, resulting in a large coverage of the channel. However, this was not the case with 1,8-cineole-treated samples at concentrations of 12.5 and 50 µM (only three concentrations were selected for this assay); a reduction in platelet accumulation can be seen which results in a reduced level of florescence and the growing thrombi were not the same as in the vehicle treated sample. 1,8-cineole shows an ability to clearly inhibit the development of thrombi, as the mean fluorescence intensity was largely reduced at 50 µM due to a

significant reduction in growing thrombi from 2 minutes onwards, compared to growing thrombi in the vehicle at the same time points (Figure 3-14 A).

At a concentration of 12.5 μM, 1,8-cineole reduces the formation of thrombi significantly from as early as 2 minutes, where growing thrombi were not formed compared to the vehicle sample. 1,8-cineole was unable to significantly reduce thrombus formation at the lowest concentration of 6.25 μM. The level of inhibition achieved with 50 μM and 12.5 μM of 1,8-cineole was around 60% and 45%, respectively. These results in whole blood under arterial flow conditions along with previous aggregometry and clot retraction data using PRP clearly demonstrate the potent inhibitory effects of 1,8-cineole in modulating platelet function under diverse settings.

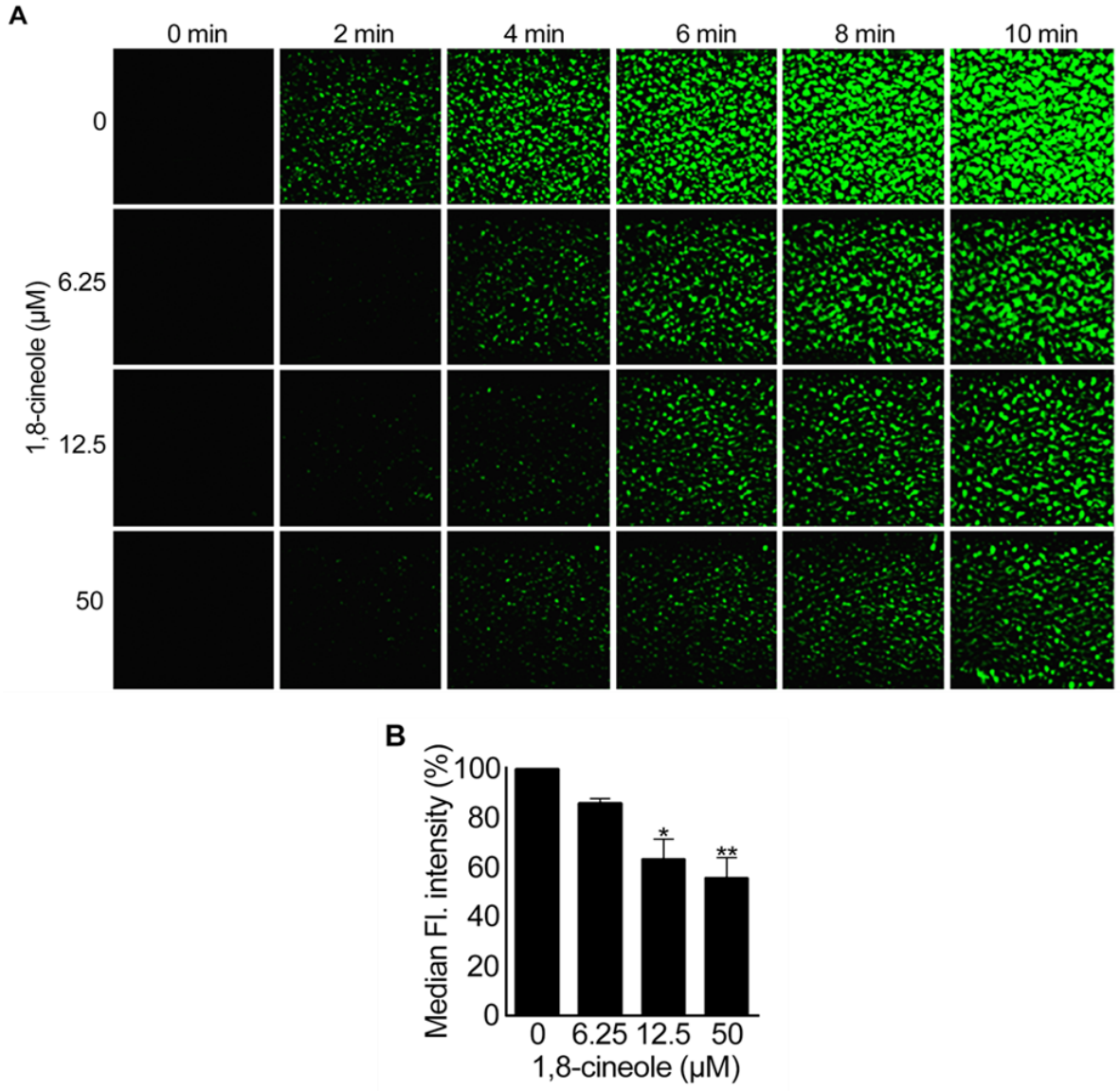

Figure 3-14: Effect of 1,8-cineole on *in vitro* thrombus formation under arterial flow conditions. DiOC6 (a lipophilic dye) (5 μM)-labelled human whole blood was incubated with a vehicle or different concentrations of 1,8-cineole for 5 minutes and perfused through the microfluidic channels (Vena8 BioChips) coated with collagen (400 μg/mL). The channel was observed using a 20x objective on a Nikon A1-R confocal microscope, with images captured every 30 seconds up to 10 minutes (**A**). Quantified data represent median fluorescence intensity of thrombi formed for 1,8-cineole-treated samples as calculated using NIS elements software (Nikon) and normalised to the level of median fluorescence intensity obtained for thrombi at 10 minutes in the vehicle treated sample (**B**). Data represents mean ± SEM (n=3). The p values (*$p<0.05$, and **$p<0.01$) shown are as calculated by one-way ANOVA with post-hoc Dunnett's test.

3-2-9 1,8-cineole affects haemostasis in mice

Haemostasis is a normal physiological response of the body to prevent excessive bleeding upon injury. To assess the haemostatic action of platelets, a tail bleeding assay in mice is commonly performed. This assay involves transection of the tail tip and measuring the bleeding volume and/or time. Anti-platelet drugs such as aspirin and clopidogrel extend the bleeding time in this model, specifying that the model is sensitive to modifications in platelet function. Thus, in order to investigate the effect of 1,8-cineole on haemostasis, a tail-bleeding assay was performed on C57BL/6 mice. The final concentrations (6.25 and 12.5 μM) of 1,8-cineole to use in this assay were calculated based on mouse weight and respective estimated volume of blood.

Twelve weeks old C57/BL/6 mice were anaesthetised prior to placing on a heated mat (37^{o}C). Then a vehicle control or 6.25 or 12.5 μM of 1,8-cineole was injected via femoral artery and incubated for 5 minutes prior to dissecting 3 mm of tail tip and monitoring the bleeding time. The mean bleeding time in the vehicle-treated control group was about 307 seconds, whereas the administration of 1,8-cineole extended the bleeding time to 498 seconds at 6.25 μM, and 837 seconds at 12.5 μM (Figure 3-15). This result indicates that 1,8-cineole is able to modulate haemostasis in mice although it was only to a minimal level at a lower concentration of 6.25 μM. Therefore, a concentration of lower than 6.25 μM may not adversely affect the haemostasis when provided for therapeutic reasons over a long period.

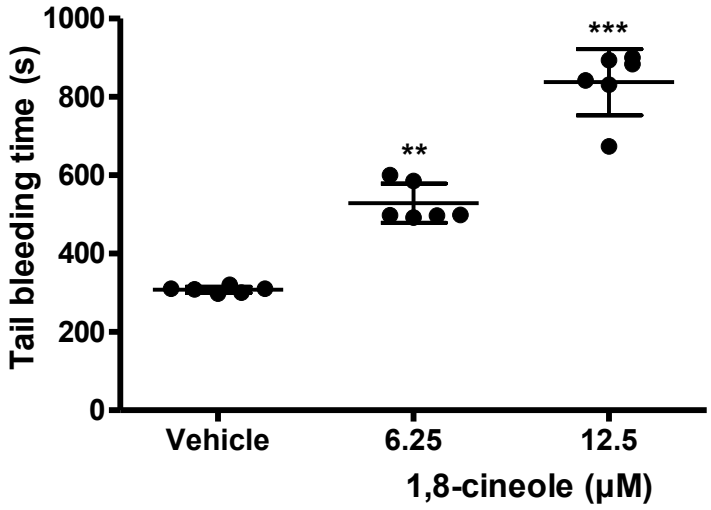

Figure 3-15: Effect of 1,8-cineole on haemostasis in mice. C57BL/6 mice (n=6 per group, 12 weeks old) were anaesthetised and a vehicle control [0.01% (v/v) ethanol] or 1,8-cineole (6.25 µM or 12.5 µM) was administered via the femoral artery. After 5 minutes of incubation, 3 mm of tail tip was dissected, and the tail was placed in sterile PBS. The time for cessation of bleeding was measured for up to 20 minutes when the assay was terminated. Data represent mean ± SEM. The p values shown (**p<0.01, ***p<0.001) are as calculated by the non-parametric Mann-Whitney test using Graphpad Prism.

3-2-10 1,8-cineole does not exert cytotoxicity in platelets

Only a limited data in the literature indicates the cytotoxic effect of 1,8-cineole in other cell types. Some studies reported that 1,8-cineole can exert cytotoxic effects, but these are mainly dependent on the cell type, and the concentration applied along with the experimental design. For example; the cytotoxic activity of 1,8-cineole in a tumour (melanoma) cell line was reported at a concentration of 20 µg/mL (Darmanin, Wismayer et al. 2009), and in human colon cancer cell lines at concentrations of 5-50 mM (Murata, Shiragami et al. 2013). Moreover, the cytotoxic activity of 1,8-cineole was also reported in leukaemia cell lines to induce apoptosis at a concentration of 7.5 µM, after 48 h of treatment with 7.5 µM of 1,8-cineole (Moteki, Hibasami et al. 2002).

Therefore, a further experiment was conducted to clarify whether the inhibitory effects observed on platelets in this study was due to the pharmacological inhibition of 1,8-cineole on platelet function or its cytotoxicity. Hence, a lactose dehydrogenase (LDH) cytotoxicity assay was performed to determine whether 1,8-cineole has toxic effects on platelets. LDH is a cytosolic enzyme present in numerous cell types and is well-known as an indicator of cellular toxicity. Upon damage of the plasma membrane the cell will start to release LDH into the surrounding culture medium. The released LDH can be measured by an enzymatic reaction whereby LDH catalyses the conversion of lactate to pyruvate by reducing NAD+ to NADH. The reduction of NADH is then used by diaphorase to reduce a tetrazolium salt to form a red formazan. The level of formazan formation is proportional to the total of LDH released into the medium and can be read using spectrofluorimetry at 490 nm and 650 nm using spectrophotometer (Molecular devices, UK).

Human PRP was incubated with different concentrations of 1,8-cineole (6.25, 12.5, 25, 50 and 100 μM), along with appropriate positive and negative controls, and the amount of LDH released was measured as a marker for toxicity using a spectrophotometer. 1,8-cineole was found to be non-toxic up to the concentrations of 50 μM, however, a mild but significant toxicity was noted at 100 μM (Figure 3-16). This result indicates that the inhibitory effects of 1,8-cineole up to 50 μM observed in previous experiments are specific pharmacological effects of this compound in platelets and not due it is cytotoxicity. Based on this data, the use of 100 μM 1,8-cineole was largely avoided in several experiments in this study.

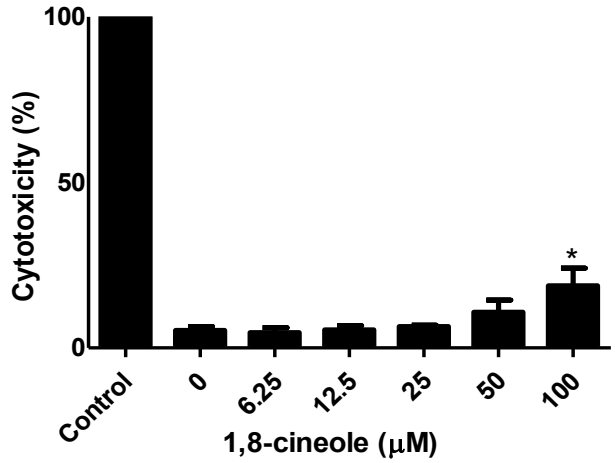

Figure 3-16: Cytotoxicity of 1,8-cineole on human platelets: Human PRP was exposed to a positive control (a detergent provided in the kit), a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole for 30 minutes and the amount of LDH released (as a marker for cytotoxicity) was measured at 490 and 650 nm using spectrophotometry. The level of LDH release for 1,8-cineole-treated samples was calculated by considering the maximum LDH release obtained with the positive control as 100%. Data represent mean ± S.E.M (n=3). The p values (*$p<0.05$) shown is calculated by one-way ANOVA with post-hoc Dunnett's test

Table 3-1: 1,8-cineole effects on agonists-induced platelet aggregation

Type of Experiment	Condition (Platelet preparation)	1,8 cineole (µM)	Agonist	Effects of 1,8-cineole in isolated platelet aggregation
Light transmittance aggregometry	Aggregation using isolated platelets	6.25	Collagen (1 µg/mL)	No inhibition effect
			Collagen (0.5 µg/mL)	60% reduction in platelet aggregation
			CRP (1 µg/mL)	No inhibition effect
			CRP (0.5 µg/mL)	40% reduction in platelet aggregation
			Thrombin 0.1U/mL	No inhibition effect
		12.5	Collagen (1 µg/mL)	No inhibition effect
			Collagen (0.5 µg/mL)	60% inhibition in platelet aggregation
			CRP (1 µg/mL)	No inhibition effect
			CRP (0.5 µg/mL)	50% reduction in platelet aggregation
			Thrombin 0.1U/mL	No inhibition effect
		25	Collagen (1 µg/mL)	No inhibition effect
			Collagen (0.5 µg/mL)	Reduced platelet aggregation by 60%
			CRP (1 µg/mL)	No inhibition effect
			CRP (0.5 µg/mL)	70% inhibition
			Thrombin 0.1U/mL	No inhibition effect
		50	Collagen (1 µg/mL)	62% reduction
			Collagen (0.5 µg/mL)	85% inhibition
			CRP (1 µg/mL)	50% inhibition
			CRP (0.5 µg/mL)	70% reduction
			Thrombin 0.1U/mL	No inhibition effect
		100	Collagen (1 µg/mL)	65% reduction
			Collagen (0.5 µg/mL)	97% inhibition
			CRP (1 µg/mL)	55% inhibition
			CRP (0.5 µg/mL)	69% inhibition
			Thrombin 0.1U/mL	50% inhibition

Table 3.1 (continued)

Type of Experiment	Condition (Platelet preparation)	1,8 cineole (µM)	Agonist	Effects of 1,8-cineole in PRP aggregation
Light transmittance aggregometry	Aggregation using Platelet-Rich-Plasma (PRP)	6.25	Collagen (1 µg/mL)	No inhibition effect
			Collagen (0.5 µg/mL)	70% inhibition
			CRP (1 µg/mL)	No inhibition effect
			CRP (0.5 µg/mL)	No inhibition effect
			ADP (5 µM)	No inhibition effect
			ADP (2.5 µM)	No inhibition effect
		12.5	Collagen (1 µg/mL)	No inhibition effect
			Collagen (0.5 µg/mL)	68% inhibition
			CRP (1 µg/mL)	No inhibition effect
			CRP (0.5 µg/mL)	45% inhibition
			ADP (5 µM)	No inhibition effect
			ADP (2.5 µM)	No inhibition effect
		25	Collagen (1 µg/mL)	No inhibition effect
			Collagen (0.5 µg/mL)	65% inhibition
			CRP (1 µg/mL)	No inhibition effect
			CRP (0.5 µg/mL)	53% inhibition
			ADP (5 µM)	No significant effect
			ADP (2.5 µM)	No significant effect
		50	Collagen (1 µg/mL)	No significant effect
			Collagen (0.5 µg/mL)	63% inhibition
			CRP (1 µg/mL)	No inhibition effect
			CRP (0.5 µg/mL)	55% inhibition
			ADP (5 µM)	No significant effect
			ADP (2.5 µM)	No significant effect
		100	Collagen (1 µg/mL)	65% inhibition
			Collagen (0.5 µg/mL)	55% inhibition
			CRP (1 µg/mL)	No inhibition effect
			CRP (0.5 µg/mL)	65% inhibition
			ADP (5 µM)	No inhibition
			ADP (2.5 µM)	60% inhibition

Table 3.2 Summary of the effects of 1,8-cineole on inside-out signalling to integrin αIIbβ3.

Type of Experiment	Assay	Agonist	1,8 cineole (μM)	Effects of 1,8-cineole on fibrinogen binding using isolated platelet	Effects of 1,8-cineole on fibrinogen binding using PRP
Flow cytometry	Measure the level of fibrinogen binding	CRP_XL (0.5 μg/mL)	6.25	60% inhibition	No inhibition
			12.5	75% inhibition	40% inhibition
			25	95% inhibition	55% inhibition
			50	95% inhibition	70% inhibition

Table 3.3 Summary of the effects of 1,8-cineole platelet Granule secretion.

Type of Experiment	Assay	Agonist	1,8 cineole (μM)	Effects of 1,8-cineole on P-selectin exposure in isolated platelet	Effects of 1,8-cineole on P-selectin exposure in PRP
flow cytometry	Measure the level of P-selectin exposure	CRP_XL (0.5 μg/mL)	6.25	50% inhibition	No inhibition
			12.5	60% inhibition	40% inhibition
			25	74% inhibition	55% inhibition
			50	80% inhibition	70% inhibition
Lumi-aggregometer	Measure the level of luminescence released	Collagen (0.5 μg/mL)	1,8 cineole (μM)	Effects of 1,8-cineole on ATP release in isolated platelet	ATP release was performed only in isolated platelets
			6.25	60% inhibition	
			12.5	70% inhibition	
			25	60% inhibition	
			50	85% inhibition	
			100	90% inhibition	

Table 3.4 Summary of the effects of 1,8-cineole on clot retraction, calcium mobilisation and thrombus formation.

Assay	Condition (Platelet preparation)	Agonist	1,8 cineole (μM)	Effects of 1,8-cineole
Clot retraction	PRP	Thrombin (1 U/mL)	6.25	No effect
			12.5	Inhibit clot retraction by 45%
			25	50% inhibition
			50	90% inhibition
Ca^{2+} mobilisation	Isolated platelet	CRP_XL (0.5 μg/mL)	6.25	No effect
			12.5	20% inhibition of Ca^{2+} mobilisation
			25	40% inhibition
			50	40% inhibition
Thrombus formation	Whole blood	Collagen (400 μg/mL)	6.25	No effect on thrombus formation
			12.5	Caused 45% reduction in thrombus formation
			50	Reduced thrombus formation by 60%

3-3 Discussion

Since platelets play critical roles in the regulation of thrombosis and haemostasis, they act as a powerful therapeutic target in order to prevent/treat thrombotic diseases. The currently used anti-platelet drugs such as aspirin and clopidogrel help saving lives, however, they exert numerous undesirable side effects. Therefore, there is an urgent need to develop safer and more efficacious anti-platelet drugs to prevent/treat thrombotic diseases. Over the last few decades, scientific research has also focussed more on medicinal plants as a source to identify/develop new drugs with reduced side effects (Bernardini, Tiezzi et al. 2018). Notably, studies on essential oils demonstrate their promising therapeutic effects for the treatment and prevention of thrombotic diseases (Edris 2007). Thus, the main aim of this chapter was to evaluate the effects of 1,8-cineole on the modulation of platelet function. This aim was achieved by using a range of different functional assays using human isolated platelets to allow the effects of 1,8-cineole to be investigated in a clear system as well as in the presence of plasma proteins “platelet-rich plasma” and in whole blood.

As the primary role of platelets is to aggregate at the site of vessel injury, initial testing was focussed on platelet aggregation assay using various agonists. 1,8-cineole has demonstrated an inhibitory effect on collagen or CRP-XL-stimulated aggregation in isolated platelets, with a significant reduction achieved only at a higher concentration of 50 or 100 μM when a higher concentration of agonists was used. However, reduced concentrations of collagen and CRP-XL (0.5 μg/mL), showed the ability of 1,8-cineole to inhibit platelet aggregation at all tested concentrations with notable levels of inhibition observed at as low as 6.25 μM. Moreover, to compare the effect of 1,8-cineole obtained in isolated platelets with PRP, same agonist was used

at same concentrations to evaluate the effect of 1,8-cineole in PRP. Nevertheless, 1,8-cineole was also found to cause inhibitory effects in PRP when concentration of agonists was decreased (e.g. 0.5 µg/mL collagen or CRP-XL), demonstrating a clear inhibition in the presence of plasma proteins. Thus, the results presented in isolated platelets and PRP demonstrate a slightly decreased potency of 1,8-cineole in the presence of plasma proteins. These findings in PRP suggest that, 1,8-cineole may bind plasma proteins, therefore, increasing the concentration of 1,8-cineole or prolonged exposure may be required to overcome this effect. The binding to plasma proteins was previously reported for various plant-derived compounds. For example, tangeretin a flavonoid rich in lemon peel and quercetin which is rich in red onions were shown to bind plasma proteins (Oh, Endale et al. 2012, Vaiyapuri, Ali et al. 2013).

Likewise, signalling through a range of different pathways such as via GPCRs is common for several plant-derived compounds in order to exert their maximal effects to control thrombus formation. Therefore, it is important to examine the effect of 1,8-cineole on these pathways. Thus, the effect of 1,8-cineole on GPCR agonists such as thrombin and ADP-stimulated aggregation was also investigated. 1,8-cineole has displayed anti-aggregatory effects upon stimulation with thrombin (0.1 U/mL) in isolated platelets and ADP (2.5 µM) in PRP only at a high concentration of 100 µM.

The lower levels of inhibitory effects of 1,8-cineole on platelet aggregation in some experiments mainly with GPCR agonist, can be attributed to the experimental time points (five minutes), as shown in representative aggregation traces described in Section 3.2.1. However, linear relationships were noticed between the concentrations of 1,8-cineole and the level of aggregation achieved. 1,8-cineole was found to inhibit the aggregation at 50% of aggregation induced by GPCRs agonists, these suggested

that 1,8-cineole may have the ability to inhibit agonist induced platelet aggregation through GPCRs if the time course of aggregation reduced to 3 minutes instead of 5 minutes, as aggregation experiments designed in this project were constant for 5 minutes. From early time points, at 3-minutes after stimulation by ADP (2.5 μM), 1,8-cineole at concentrations of (6.25, 12.5, 25 and 50 μM) was noted to diminish aggregation by 35%, 38%, and 55%, compared to the vehicle treated samples. Also, in thrombin stimulated platelet aggregation, the maximum inhibition of 1,8-cineole at concentrations of (25 and 50 μM) can be detected at 3-minutes around 40 and 50% inhibition in platelet aggregation, and lower concentrations of 1,8-cineole (6.25 and 12.5) fail to inhibit platelet aggregation induced by thrombin at 3-minutes. This might indicate the maximal inhibitory effect of 1,8-cineole achieved at early time point, might be due to the effects of 1,8-cineole in reducing the secretion of prothrombotic mediators such as ADP and TxA_2. This difference effects of 1,8-cineole on platelet aggregation at this time points; may suggest reducing the experimental time point to 3-minute instead of 5-minute.

Thus, the concentrations of 1,8-cineole required to inhibit thrombin or ADP-induced platelet aggregation are higher than those necessary for collagen and CRP-XL-induced platelet aggregation, suggesting a degree of specificity for 1,8-cineole towards GPVI signalling pathways. Notably, 100uM 1,8-cineole was later confirmed to be cytotoxic to platelets although the toxicity level was minimal. It may also be necessary to further reduce the concentrations of GPCR agonists to obtain only a minimal level of aggregation and determine the effects of 1,8-cineole to corroborate its specificity towards multiple signalling pathways. These experiments should be performed in future studies with this compound.

Similar to 1,8-cineole, other essential oils such as elemicin and eugenol isolated from Cymbopogon ambiguus are volatile monoterpenoids with anti-inflammatory effects (Janssens, Laekeman et al. 1990). Both elemincin and eugenol have been reported to possess anti-platelet effects by inhibiting ADP-induced secretion of serotonin in human platelets, while only eugenol exhibited potent inhibitory activity (IC50 - 46.6 μM) on ADP (2 μM)-induced platelet aggregation compared to aspirin (Grice, Rogers et al. 2011). Indeed, Raghavendra et al. (2009), showed inhibitory effects of eugenol on human (PRP) platelet aggregation induced by arachidonic acid (AA) (1 mM), ADP (50 μM) and collagen (0.5 μg/ml) with most inhibitory effects on AA (1 mM) induced platelet aggregation with IC_{50} values of 0.5 μM. The authors have also suggested that eugenol has an inhibitory effect on cyclooxygenase (COX-1) activity by inhibiting thromboxane A2 formation similar to aspirin (Raghavendra and Naidu 2009).

Furthermore, a previous study on rabbit isolated platelets investigated the anti-platelet aggregating activity of nutmeg oil, in which eugenol was the most active component. Eugenol was found to inhibit collagen-induced platelet aggregation (IC50 = 0.24 mM), but with less effect on ADP and thrombin-induced aggregation, similar to our findings with 1,8-cineole (Chen, Wang et al. 1996). Moreover, in another study, Srivastava et al. (1987) have demonstrated that oil of cloves where eugenol was the most active component, inhibit collagen-induced platelet aggregation, but was not able to cause a significant inhibition on ADP-induced platelet aggregation (Srivastava and Justesen 1987). In addition, the essential oil of lavender has been reported to exhibit a potent antiplatelet effect and was able to inhibit agonist-induced platelet aggregation induced by collagen, thromboxane receptor agonist (U46619), AA and ADP on guinea-

pig PRP (IC50= 191, 84, 51 and 640 µg/mL), respectively. In the same study, the antiplatelet effect of the main components of lavender essential oil [linalyl acetate (36.2%), linalool (33.4%), camphor (7.6%) and 1,8-cineole (5.8%)] were also investigated. 1,8-cineole showed anti-platelet activity by inhibiting U46619- (1 µM) stimulated platelet aggregation in guinea pig PRP with an IC50 677 µM (Ballabeni, Tognolini et al. 2004). The inhibitory effects of 1,8-cineole observed on platelet aggregation in our study are also similar to the effects observed with two naturally occurring flavonoids, tangeretin and nobiletin, where they tested for their antiplatelet effects. Tangeretin and nobiletin were examined for their effects on CRP-XL and collagen-induced platelet aggregation, where they exhibited an inhibitory effect only at higher concentration specifically, 150 µM nobiletin was able to inhibit thrombin-induced platelet aggregation (Vaiyapuri, Ali et al. 2013, Vaiyapuri, Roweth et al. 2015).

Thus, our results suggest that 1,8-cineole mainly target the GPVI signalling, granule secretion and cytosolic Ca^{2+} levels which may lead to the activation of phospholipase C (PLC), which in turn mediate subsequent thrombus formation (Puri, Colman et al. 1997). The effects of 1,8-cineole on major events such as dense and α-granule secretion, which serve an important role in controlling platelet aggregation by releasing various activated factors, such as ATP, P-selectin, ADP and Ca^{2+}, were investigated.

ATP release was measured as a marker for dense granule secretion. Indeed, 1,8-cineole demonstrated a significant inhibition on dense granule secretion at a concentration of as low as 6.25 µM (up to 60%) in human isolated platelets. The effect of P-selectin exposure and fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ were also measured as a marker for α-granule secretion and integrin $\alpha_{IIb}\beta_3$ inside-out signalling, respectively

in both isolated platelets and PRP. The effects of 1,8-cineole resulted in significant inhibition in these processes at all tested concentrations in isolated platelets. α-granules release several proteins including fibrinogen, which (in addition to plasma fibrinogen) interact with integrin $\alpha_{IIb}\beta_3$ and this interaction drives signalling inside the platelets leading to platelet-platelet interactions and subsequent formation of stable platelet aggregation. Our data showed a consistent inhibitory effect of 1,8-cineole (12.5, 25 and 50 μM) on α-granule secretion, in both isolated platelets and PRP. Similar to aggregation data obtained in PRP, it is clear that plasma proteins had an effect in diminishing the effect of 1,8-cineole. Therefore, these results on platelet activation may explain the anti-aggregating effects of 1,8-cineole, which affects multiple pathways in order to control platelet aggregation.

The effects of 1,8-cineole on the elevation of cytosolic Ca^{2+} upon stimulation with CRP-XL was also investigated. Calcium mobilisation in the cytoplasm in response to CRP-XL stimuli was significantly inhibited by 1,8-cineole in a dose dependent manner. The inhibitory effects of 1,8-cineole on Ca^{2+} were found to be similar to the inhibitory effect observed by nobiletin (Vaiyapuri, Roweth et al. 2015) and epigallocatechin gallate (EGCG) causing inhibition in Ca^{2+} in a dose dependant manner (Jin, Im et al. 2008) . Moreover, our results are in accordance with essential oils such as eugenol in inhibiting calcium mobilisation in platelets (Chen, Wang et al. 1996). Calcium mobilisation plays a critical role in various signalling cascades that are initiated by several agonists to stimulate platelet activation. Thus, its modulation by 1,8-cineole provides evidence that signalling events, especially intracellular calcium mobilisation could be affected by the treatment with 1,8-cineole in order to control platelet function.

Subsequently to the effects of 1,8-cineole on calcium mobilisation, and inside-out signalling, it was investigated whether 1,8-cineole also affects the outside-in signalling to integrin αIIbβ3 which drives platelet spreading and clot retraction. Results derived from the integrin αIIbβ3 mediated outside-in signalling in isolated platelets shown that 1,8-cineole was able to inhibit integrin αIIbβ3 mediated outside-in signalling. As demonstrated by platelet adhesion to and spreading on fibrinogen coated surface, a clear reduction was observed in the number of platelets spreading lamellipodia. This could be beneficial as platelet spreading is important in haemostasis, and as can be seen 1,8-cineole had a limited effect on platelet spreading at low concentrations, and only at high concentrations platelet spreading inhibited significantly. These effects of 1,8-cineole on outside-in signalling via integrin αIIbβ3 are similar to the effects obtained with some of flavonoids such as quercetin and chrysin as they also inhibited spreading of platelets on fibrinogen (Navarro-Núñez, Lozano et al. 2010, Liu, Xie et al. 2016). This was also further tested through a clot retraction assay. This test of a clot retraction assay combines the factors that are involved in the coagulation system, such as thrombin which is well known to contribute to the activation of factors XI, VII and V, as well as converting fibrinogen into fibrin during coagulation cascade to help the formation of a fibrin clot (Crawley, Zanardelli et al. 2007). The retraction process of the fibrin mesh is primarily driven by integrin αIIbβ3 which facilitates the interaction between fibrinogen and myosin-actin cytoskeleton (Tucker, Sage et al. 2012). This test measures the retraction over time of a thrombin stimulated clot and measures end clot weight when a vehicle sample has completely retracted. Our result shows that 1,8-cineole completely inhibited clot retraction at concentrations of 25 and 50 μM, with a trend of increasing clot weight at low concentrations (6.25-12.5 μM). The initial binding of cytoskeletal myosin depends

on the phosphorylation of β3 subunit with important downstream role of Src and PLCγ2 (Shattil, Kashiwagi et al. 1998). This inhibition effects of 1,8-cineole on clot retraction can be through the decrease of the activity of Src-family kinase as 1,8-cineole is able to attenuate this activity (discussed in chapter 5). Likewise, this effect of 1,8-cineole was similar to the inhibitory effects of Lavender oil on clot retraction induced by thrombin in rat PRP at a concentration of 270 µg/mL. Moreover, Ballabeni et al. (2004) reported that 1,8-cineole partially inhibited clot retraction in rat PRP, at a concentration of 900 µg/mL (Ballabeni, Tognolini et al. 2004). Similar to 1,8-cineole, other essential oils such as oils of Ocotea quixos (Ballabeni, Tognolini et al. 2007), Foeniculum vulgare (Tognolini, Ballabeni et al. 2007) and nut oil (P. gerardiana) (Saeed-ul-Hassan, Iqbal et al. 2017) caused an increase in clot weight and therefore the clot retraction was reduced.

The inhibition of numerous functions associated with platelet activation by 1,8-cineole suggests its ability to modulate thrombus formation. Therefore, it was hypothesised that 1,8-cineole has the ability to inhibit platelet function in whole blood. This was investigated by an in vitro thrombus formation under arterial flow conditions, where 1,8-cineole-treated whole blood was flowed over collagen-coated channels at a physiological shear rate. As described before, in in vitro thrombus formation a reduction in thrombi formation was observed in the presence of 1,8-cineole. This inhibition was noted to be due to a reduced level of growing thrombus and defect in the adhesion of platelets to collagen with a comparison to a vehicle control. It was not surprising to see decreased platelet-platelet interactions in samples treated with 1,8-cineole. As mentioned before, 1,8-cineole demonstrated its potential to inhibit the process of fibrinogen binding to $\alpha_{IIb}\beta_3$, resulting in significantly reduced thrombus growth. Indeed, the possible lack of robust signalling from platelet GPVI could be a

reason for reduced growth in thrombus formation. In addition, von Willebrand factor (vWF) has an important role in facilitating platelet GPIb attachment to collagen. It would be pertinent to investigate whether 1,8-cineole can modulate this interaction and limit the growth of thrombus formation.

The term of haemostasis is usually used to describe the cessation of bleeding by forming blood clot at site of injury (Fredenburgh and Weitz 2018). Mouse models have been used for many years to study blood coagulation (Hogan, Weiler et al. 2002). The similarity of blood plasma in mice and human specially in coagulation factors, make mice as important model in haemostasis and thrombosis research (Mackman 2004).

Bleeding time in mice is commonly used as a preclinical model, especially in genetically modified mice or for testing anti-thrombotic drugs following treatment. This assay involves transection of the tail tip, and bleeding time mainly determined by platelet interaction with damaged vessel leading to the formation of haemostatic plug. In this project, we used the tail bleeding assay as a model to investigate the haemostatic effects of 1,8-cineole. Since mice models are used as alternative methods to study the function of human platelets.

Mouse platelets are generally smaller than human platelets (mean diameter 0.5 μm vs 1–2 μm) and they are much higher platelet counts 1000–1500 × 109/L compared to 150–400 × 109/L in human (Levin and Ebbe 1994, Baker, Sullam et al. 1997). Human platelets express PAR1 and PAR4, unlike mouse platelets which express PAR3 and PAR4. The importance of PAR receptors in the overall platelet mechanism response is demonstrated by phenotype of mice lacking in PAR-4. PAR4 knockout mice shown a significantly prolonged tail bleeding time, with a failure of

aggregatory response to agonist such thrombin (Rowley, Oler et al. 2011). Nevertheless, these differences do not negatively impact the value of the mouse as a model to study platelet function.

Our result in tail bleeding assay has shown that 1,8-cineole (at 12.5 μM and 6.25 μM) extend the bleeding time in mice, which reflects the interaction between platelets and damaged blood vessel, leading to the formation of a haemostatic plug. This provides evidence for the pharmacological effect of 1,8-cineole on haemostasis, which may be contributed to its inhibitory effects on platelet aggregation, granules secretion and thrombus formation. However, the involvement of the other mechanisms must not be ignored and needs to be explored. The effect of 1,8-cineole on the bleeding time could also be due to its vasodilation effects at concentrations (0.006 - 2.6 mM) as reported in a previous study on rats (Lahlou, Figueiredo et al. 2002). Similar to 1,8-cineole, previous studies on terpenoids, such as Cyperus rotundus ethanolic extracts and (+)-nootkatone were found to exhibited significantly prolonged bleeding times in mice (Seo, Lee et al. 2011).

However, over a period of long exposure with a reduced concentration of 1,8-cineole is likely to modestly inhibit platelet reactivity within the circulation without affecting the physiological haemostasis. In our experiment in tail bleeding assay, the period of time between 1,8-cineole administered to mice was five minutes before measuring bleeding time, could be one of the reasons for the significantly increased in this haemostatic parameter. Therefore, the exposure time between 1,8-cineole administration and bleeding time measurement could be increased for further experimental repeat (e.g., 30 min). The effectiveness of anti-thrombotic agents is mostly limited by bleeding complications. Hence, the beneficial effects of 1,8-cineole

to prevent thrombotic conditions may be achieved via using a carefully designed dose of this compound.

In conclusion, essential oils extracted from medicinal plants have been extensively used for the treatment of various diseases and they are becoming as alternative therapeutics worldwide. 1,8-cineole has been used for many years for it is anti-inflammatory effects. Several studies have shown that 1,8-cineole is effective and safe in treatment of several diseases such bronchitis and inflammatory conditions. Together, the results obtained in this study demonstrated that 1,8-cineole has inhibitory effects on human platelet function in isolated platelets and in the presence of plasma proteins as well as in whole blood. Therefore 1,8-cineole could be beneficial in reducing thrombotic disease, thus, we conclude that further studies to investigate the therapeutic potential of 1,8-cineole may aid in the design and development of novel anti-thrombotic strategies.

Chapter 4

4- 1,8-CINEOLE MODULATES PLATELET-MEDIATED INFLAMMATORY RESPONSES

4.1 Introduction

Besides their primary role in haemostasis and thrombosis, platelets also contribute to inflammation via various immune functions, for example, through their ability to interact with different types of leukocytes, and their capacity to release a large number of immunomodulatory molecules (Palabrica, Lobb et al. 1992). Platelets play critical roles in the development of inflammatory diseases such as sepsis; the dysregulated responses of the immune and haemostatic systems, in reaction to pathogens, are known to be the main cause of sepsis (Vincent and Abraham 2006). The interaction between activated platelets and leukocytes results in the formation of platelet-leukocyte aggregates, which play a critical role in regulating inflammatory responses to produce and release a range of different pro-inflammatory cytokines (Schrottmaier, Kral et al. 2015). It is well established that the formation of platelet-leukocyte aggregates is one of the primary processes in pathological conditions, for example during the development of atherosclerosis (May, Langer et al. 2007). The physical interactions between platelets and leukocytes act as an important diagnostic marker for inflammatory processes and thrombosis, especially during transient ischemic attacks and myocardial infarction (Michelson, Barnard et al. 2001, Htun, Fateh-Moghadam et al. 2006).

Moreover, it has been reported that various inflammatory molecules secreted from platelets under pathological conditions are associated with inflammatory diseases such as atherosclerosis and sepsis (Davì and Patrono 2007). In addition, the level of some of the cytokines released from activated platelets are elevated in disease conditions such as cancer (Peterson, Zurakowski et al. 2012, Fu, Fu et al. 2015), showing an active role for these cytokines due to platelet activation in response to

disease conditions. This indicates that underlying pathological conditions can influence the platelet reactivity and their roles in regulating immune responses under these settings. The prevention or reduction of factors that augment platelet activation under pathological conditions is therefore critical in the prevention of cardio- and cerebrovascular, and inflammatory diseases (Brott and Bogousslavsky 2000, Goszcz, Deakin et al. 2015). Currently used anti-platelet drugs such as aspirin, ticagrelor and clopidogrel have been shown to affect the immune modulatory effects of platelets, and reduce platelet-leukocyte interactions, in addition to their role in controlling platelet aggregation and primary thrombosis (Schrör and Huber 2015). As explained in the introduction section, monoterpenes have shown an interesting functional profile to modulate inflammatory responses and they have demonstrated to modulate the production and release of various inflammatory mediators, including anti and pro-inflammatory cytokines (Andrade and De Sousa 2013).

Since 1,8-cineole is the main focus of this study, here we explored the actions of this molecule on platelet-mediated inflammatory responses. Indeed, previous *in vivo* experiments have demonstrated the efficacy of 1,8-cineole in the reduction of IL-8 and the number of leukocytes that adhered to a bronchoalveolar lavage in mice (Lee, Park et al. 2016). Furthermore, 1,8-cineole has shown to strongly inhibit the secretion of some inflammatory molecules and ROS generation in monocytes under *in vitro* conditions (Juergens, Racké et al. 2017). Moreover, in a clinical study, in patients with severe asthma, due to the anti-inflammatory and anti-oxidant effects of 1,8-cineole, the use of corticosteroid therapeutic agents was largely reduced (Worth and Dethlefsen 2012).

Thus, 1,8-cineole has a potential in treating inflammatory diseases in addition to be beneficial in the treatment and prevention of thrombotic diseases. Hence, in this

chapter, the ability of 1,8-cineole to modulate platelet-mediated inflammatory responses was investigated. Specifically, this chapter focuses on the ability of 1,8-cineole to modulate the release of various inflammatory cytokines from activated platelets, and its antioxidant effects, as well as its effects on platelet-leukocyte interactions.

4.2 Results

4.2.1 1,8-cineole affects the production of reactive oxygen species (ROS) in platelets

Platelets are well recognised to contribute to inflammation, and can release various endogenous inflammatory mediators as well as produce ROS (Krötz, Sohn et al. 2002, Bakdash and Williams 2008). Previous studies support the critical role of ROS in elevated platelet activation, by inducing changes in Ca^{2+} levels and acting as a second messenger in platelet activation induced by collagen or thrombin (Pignatelli, Pulcinelli et al. 1998, Wachowicz, Olas et al. 2002). Also ROS production is associated with platelet aggregation upon stimulation with agonists such as collagen and thrombin (Fuentes, Gibbins et al. 2018). However, excessive ROS production results in cell stress, leading to several diseases, including thrombotic conditions, as elevated ROS level can increase platelet activation (Jang, Wang et al. 2015). GPVI is one of the main receptors that mediates collagen-stimulated platelet activation. Therefore, the GPVI selective agonist CRP-XL was used to induce ROS production in platelets in this study.

We investigated whether 1,8-cineole affects ROS production in platelets in response to CRP-XL stimulation. The intracellular ROS level was measured in CRP-XL-stimulated platelets using a reagent, 2′,7′-Dichlorofluorescin diacetate (H_2DCFDA) (H_2DCFDA, Cambridge Bioscience, UK), a cell permeable, fluorescent probe used as an indicator for ROS in cells. Upon cleavage of the acetate group via intracellular esterases and oxidation, the H_2DCFDA is converted to a highly fluorescent signal (Pignatelli, Pulcinelli et al. 2000). H_2DCFDA was thus widely used as marker to analyse intracellular ROS generation in various cell types.

Briefly, human isolated platelets (4×10^8/ml) were preincubated with 10 μM H_2DCFDA at 37°C for 30 min. After incubation, 200 μl of platelets loaded with H_2DCFDA were added into a 96-well plate containing various concentrations of 1,8-cineole (final concentrations of 6.25 - 50 μM) and incubated for 5 minutes, and then activated with CRP-XL (final concentration of 1 μg/ml) for 10 minutes. Samples were then diluted 10-fold in modified tyrodes-HEPES buffer containing 0.1% (w/v) BSA and analysed immediately using an Accuri C6 Flow cytometer (BD Biosciences, UK). The level of ROS generated was calculated by measuring the median fluorescence intensity and expressed as fold change. The level of fluorescence obtained with H_2DCFDA loaded resting platelets (without exposure to CRP-XL) was used as a reference to measure the basal level ROS in platelets. To calculate the potential effects of 1,8-cineole, the level of fluorescence (ROS generation) obtained with CRP-XL induced platelets, a positive control was set as 100% for ROS production.

A representative histogram derived from flow cytometric analyses of platelet samples following stimulation with 1 μg/ml of CRP-XL, in the presence or absence of various concentrations of 1,8-cineole, is shown in Figure 4-1. Both resting (unstimulated platelets) and positive (vehicle) controls were included for each individual sample. Figure 4-1A shows the basal level fluorescence of resting platelets that have not been exposed to CRP-XL, whereas in the vehicle-treated sample (stimulated platelets), a significant increase in fluorescence after stimulation with CRP-XL (1 μg/mL) was observed. This increase in ROS production was significantly reduced when platelets were preincubated with different concentrations of 1,8-cineole prior to the addition of CRP-XL.

Figure 4-1A-B shows the inhibitory effect of various concentrations (6.25, 12.5, 25 and 50 μM) of 1,8-cineole on CRP-XL-induced ROS production in platelets. At a concentration of 25 and 50 μM, 1,8-cineole inhibited the ROS production to a similar extent (around 60%), and at concentrations of 6.25 and 12.5 μM of 1,8-cineole, approximately 40% reduction in ROS production was observed, in comparison to the control. These results indicate that 1,8-cineole is able to exert its inhibitory effects on ROS generation in platelets in addition to its antiplatelet effects that were demonstrated in the previous chapter.

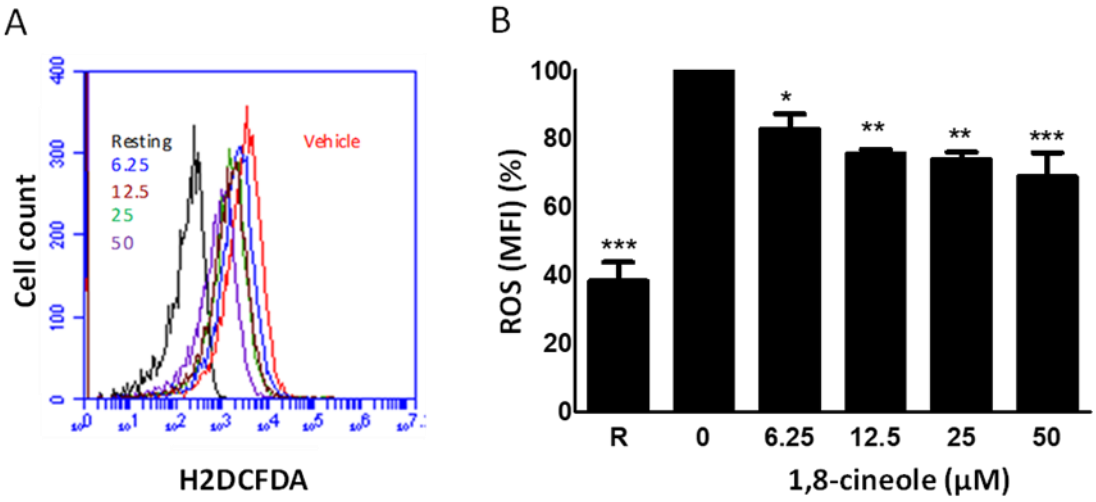

Figure 4-1: Impact of 1,8-cineole on ROS production in platelets. The intracellular ROS generation in platelets was measured using a fluorescent dye, H_2DCFDA. Human isolated platelets were labelled with H_2DCFDA for 30 minutes at 37^{o}C and treated with various concentrations of 1,8-cineole or a vehicle control for an additional 5 minutes, before being stimulated with 1 µg/mL CRP-XL. ROS production was measured in platelets by flow cytometry. (A) A representative histogram showing the ROS release. (B) The cumulative data depicts the mean ± SEM (n=6); the significance of differences was estimated using One-way ANOVA for repeated measures with Bonferroni's correction for multiple comparisons. (*p<0.05, **p< 0.01, ***p<0.001).

4.2.2 Impact of 1,8-cineole on the release of various inflammatory molecules from platelets

Upon platelet activation, various inflammatory and prothrombotic molecules are secreted from platelet granules. These molecules are either released into the circulation or translocated to the platelet membrane to facilitate binding between activated platelets and other immune cells. These molecules assist the recruitment of immune cells at the site of injury. Therefore, measuring the level of these molecules released from platelets will provide insights into the degree of platelet activation and the impact of inhibitory molecules being tested. Hence, here we have investigated the effect of 1,8-cineole on the release of a range of different inflammatory molecules released from activated platelets. These molecules were detected using enzyme-linked immunosorbent assays (ELISA) or flow cytometry.

ELISAs have been widely used to investigate the level of various platelet activation markers such as soluble P-selectin (sP-selectin) (Harrison and Keeling 2007). ELISA uses capture antibodies that are immobilised at the bottom of 96-well plates to recognise and capture a specific antigenic protein. Following this, the addition of detection antibodies conjugated with an enzyme, usually horseradish peroxide (HRP) will bind to the same antigenic protein in a different binding site. The colour development starts after the addition of a substrate solution, which reacts with the enzyme to generate a calorimetric signal which can be read by a Spectrofluorimeter to analyse the level of target protein present in the sample (Schmidt, Mazzella et al. 2012). In this study, we report the impact of 1,8-cineole on a range of different inflammatory molecules released from platelets.

4.2.2.1 1,8-cineole affects the release of sP-selectin upon platelet activation

sP-selectin is a glycoprotein, and one of the most prominent circulatory markers of platelet activation. It is present on the α-granule membrane of platelets and also on the membrane of the Weibel–Palade bodies in endothelial cells (Caine and Blann 2003, Ferroni, Martini et al. 2009). Upon platelet activation, sP-selectin is released to the circulation and it plays an important role in the upregulation of pro-inflammatory cytokines in the damaged tissue (Rondina, Weyrich et al. 2013, Yun, Sim et al. 2016).

sP-selectin released from activated platelets is found in normal human plasma at a concentration of around 15-100 ng/mL (Hartwell, Mayadas et al. 1998). Elevated levels of sP-selectin have been observed during the clinical screening of a number of diseases including thrombosis (Blann and Lip 1997), unstable angina, atherosclerosis, hypertension and inflammatory diseases (Woollard, Suhartoyo et al. 2008). In this study, the effect of 1,8-cineole on the secretion of sP-selectin upon platelet activation was investigated.

Briefly, blood samples were collected from healthy human donors and isolated platelets were prepared as described before. The platelets were pre-incubated for 5 minutes with various concentrations of 1,8-cineole (6.25 μM – 50 μM) prior to the addition of a vehicle control [ethanol (0.01%v/v)] or CRP-XL (1 μg/mL) for five minutes under stirring conditions. Then platelets were centrifuged at 1000 g for 10 minutes at room temperature. The resulting supernatant was collected and used in the assay to measure the level of sP-selectin. To determine whether 1,8-cineole affects sP-selectin secretion in CRP-XL stimulated platelets, the level of sP-selectin was measured by a commercially available human sP-selectin ELISA kit according to the manufacturer's instructions (Theromfisher, UK).

The level of sP-Selectin released from human isolated platelets in response to 1 µg/ml of CRP-XL was higher than the resting platelets with mean concentration of 32.37 ng/mL compared to 0.49 ng/mL in unstimulated cells (Figure 4-2). The incubation of platelets with 1,8-cineole significantly inhibited the secretion of sP-Selectin in response to CRP-XL. At concentrations of 25 and 50 µM of 1,8-cineole, a significant reduction on the level of sP-Selectin was observed compared to the positive control (CRP-XL alone), with an inhibitory effect of approximately 25% and 90%, with a mean concentration 24.86 ng/mL and 6.03 ng/mL, respectively compared to 32.37 ng/mL, of sP-Selectin release from stimulated platelets. Although 1,8-cineole has failed to cause a significant inhibition on sP-Selectin secretion at lower concentrations, a slight reduction can be observed at concentrations of 6.25 and 12.5 µM (mean concentration 28.55 and 27.95 ng/mL, respectively) (Figure 4-2). Suggesting that prolonged exposure to 1,8-cineole may cause significant inhibition in a smaller scale. These data demonstrate that 1,8-cineole is able to reduce the release of sP-Selectin in platelets upon stimulation with CRP-XL.

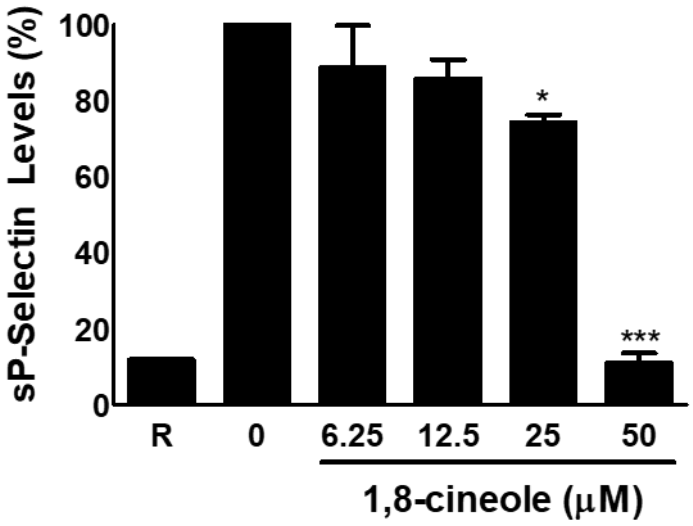

Figure 4-2: Effect of 1,8-cineole on the release of sP-selectin from platelets. Human isolated platelets were pre-treated with a vehicle control [ethanol 0.01% (v/v)] or different concentrations of 1,8-cineole for 5 minutes at 37°C and then stimulated by CRP-XL (1 µg/ml) in an aggregometer for 5 minutes at 37°C. These samples were then centrifuged at 1000 g for 10 minutes at room temperature and the resultant supernatants were immediately used. The amount of sP-selectin was determined using a commercially available human sP-selectin ELISA kit according to manufacturer's protocol. The percentage of sP-selectin released was calculated by taking the level of sP-selectin obtained with the vehicle control (CRP-XL alone) as 100%. Cumulative data represent mean ± S.E.M (n=3). p values shown (*$p<0.05$, and ***$p<0.001$) were as calculated by one-way ANOVA followed by a Bonferroni's correction for multiple comparisons.

4.2.2.2 1,8-cineole affects the release of Tumour Necrosis Factor-α (TNF-α) from platelets

Platelets activated by thrombin or collagen release α-granules and dense granules located in the platelet cytoplasm. The platelet granules contain pro-inflammatory, TNF-α, which can influence the inflammatory processes in several ways. Chronic inflammation is critical in the development of CVD, due to high level of TNF- α and IL-1β (Bruunsgaard, Andersen-Ranberg et al. 2003, Henry, Casás-Selves et al. 2015). Likewise a disease where the systemic levels of TNF- α are elevated is linked to high incidence of CVD and atherothrombotic events, suggesting that TNF-α play a significant role in development of thrombosis (Yoshida, Yilmaz et al. 2011, Manfredi, Baldini et al. 2016). The blockade of TNF-α-dependent cytokine cascades and leukocyte recruitment results in good clinical and serological outcomes during several disease conditions. Therefore, the therapies with TNF-α receptor inhibitors for rheumatoid arthritis (Jacobsson, Turesson et al. 2005) and several other inflammatory conditions were established. Here, an ELISA was used to investigate the impact of 1,8-cineole on the release of TNF-α from platelets upon activation.

Human isolated platelets were prepared as described before, and pre-incubated for 5 minutes with different concentrations of 1,8-cineole (6.25 μM – 50 μM), before treatment with a vehicle [ethanol (0.01%v/v)] or CRP-XL (1 μg/mL) for five minutes under stirring conditions to obtain resting or activated platelets, respectively. A commercially available human TNF-α ELISA kit (Theromfisher, UK) was used for the measurement of TNF-α levels in human platelets according to the manufacturer's instructions.

The concentration of TNF-α released from platelets due to the stimulation with CRP-XL was significantly increased in platelet supernatants and had a mean

concentration of 26.91 pg/mL compared to a mean concentration of non-stimulated platelets 8.69 pg/mL (Figure 4-3). The levels of TNF-α from platelets treated with 1,8-cineole were significantly lower than the positive control. In all tested concentrations of 1,8-cineole, a significant reduction of TNF-α concentration was observed. At concentrations of 6.25, 12.5, 25 and 50 μM, the levels of TNF-α were reduced by approximately 40%, 76%, 79% and 90%, with a mean of 15.08, 5.68, 4.3 and 1.88 pg/mL, respectively. This result demonstrates that 1,8-cineole is able to reduce the TNF-α secretion upon CRP-XL stimulation in human platelets.

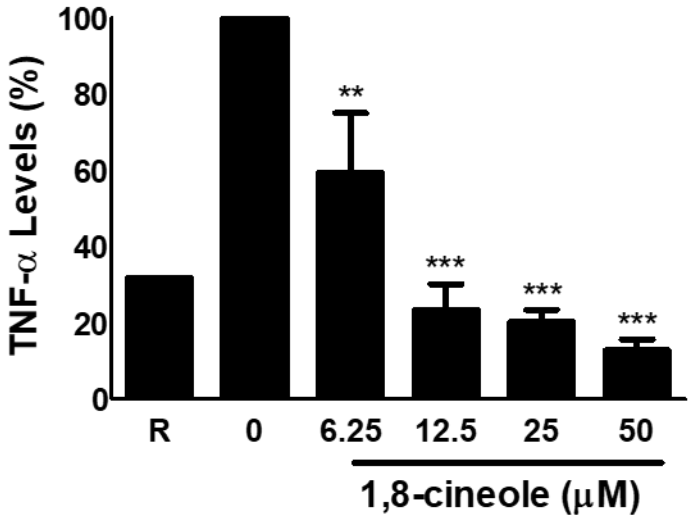

Figure 4-3: 1,8-cineole inhibits TNF-α release from agonist-activated human platelets. Human isolated platelets were incubated with various concentrations of 1,8-cineole or a vehicle [ethanol (0.01%v/v)] for 5 minutes at 37°C, prior to activation with 1 µg/ml CRP-XL under stirring conditions for 5 minutes at 37°C. Platelets were then centrifuged at 1000 g for 10 minutes at room temperature to obtain supernatants, which were immediately used in the assay. The amount of TNF-α was determined using a human TNF-α ELISA kit according to manufacturer's protocol. The percentage of TNF-α released was calculated by considering the level of TNF-α obtained with the positive control as 100%. Cumulative data represent mean ± S.E.M (n=3). p values (**p<0.01, and ***p<0.001) shown were as calculated by one-way ANOVA followed by a Bonferroni's correction for multiple comparisons.

4.2.2.3. 1,8-cineole affects the release of vascular endothelial growth factor (VEGF) from activated platelets

Platelets store a large amount of angiogenic factors within α-granules, including the most potent pro-angiogenic protein, VEGF (Pinedo, Verheul et al. 1998) (Peterson, Zurakowski et al. 2010). Several reports have shown that thrombin induced platelet activation can stimulate the release of angiogenic molecules (Li, Huang et al. 2001). Platelets are also recognised as one of the main storage site for VEGF, with approximately 80% of circulating VEGF stored in platelet α-granules (Holmes, Huang et al. 2008). Recently, it has been reported that anticoagulants such as fondaparinux (Xa inhibitor) diminish the release of VEGF from platelets, due to the ability of coagulant agents to decrease thrombin generation and therefore blocking activation through PAR1. Moreover, in the presence of PAR1 antagonists, VEGF release from platelets was also decreased (Battinelli, Markens et al. 2014).

It has been demonstrated that aspirin and other antiplatelet drugs can disrupt pro-angiogenic cytokines secreted from platelets during the crosstalk between platelets and tumour cells (Etulain, Fondevila et al. 2013, Xu, Yousef et al. 2018). Based on the above results, we hypothesised that 1,8-cineole will decrease the release of VEGF in platelets upon stimulation with CRP-XL similar to other factors shown above.

To determine whether 1,8-cineole affects VEGF secretion in platelets, we measured the level of VEGF upon stimulation with CRP-XL using a human VEGF ELISA Kit (Theromfisher, UK). Briefly, human isolated platelets were prepared as described in the methods section. Following resting, platelets were incubated with various concentrations of 1,8-cineole or with a vehicle control [ethanol (0.01%v/v)] for

five minutes. After the incubation, samples were stimulated by addition of 1 µg/ml of CRP-XL for 5 minutes, under stirring conditions in an aggregometer. Following this, samples were centrifuged immediately (1000 g for 10 min at RT), and supernatants were collected and used immediately in the assay, in accordance with the manufacturer protocol.

In Figure 4-4, a statistically significant increase (94%) was observed in the level of VEGF released from platelets that were stimulated with 1 µg/ml CRP-XL with a mean of 288 µg/mL compared to 98 µg/mL in resting cells. However, the level of VEGF released from platelets that were preincubated with different concentrations of 1,8-cineole was significantly reduced with mean concentrations of 166, 150, 156 and 120 µg/mL with inhibitory effects about 41%, 47%, 45% and 58%, at 6.25, 12.5, 25 and 50 µM respectively, upon stimulation with CRP-XL (Figure 4-4). This data demonstrates that 1,8-cineole is able to affect angiogenesis regulatory proteins such as VEGF secreted from platelets upon stimulation with an agonist.

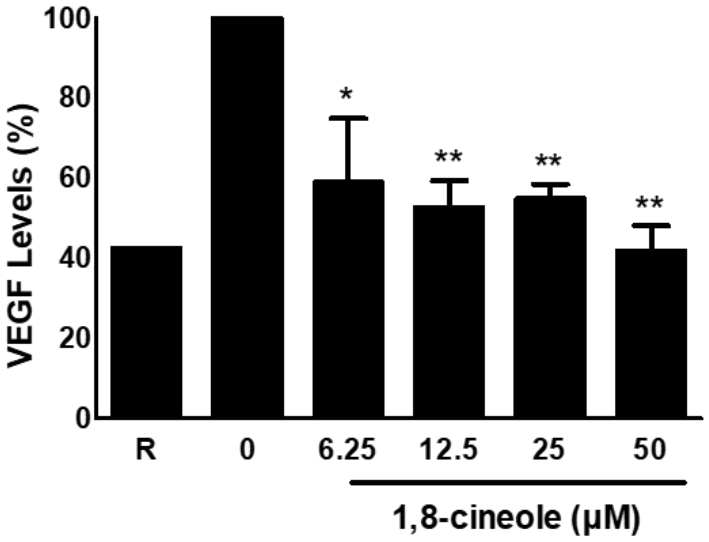

Figure 4-4: Effect of 1,8-cineole on the release of VEGF from platelets. Human isolated platelets were prepared and incubated with various concentrations of 1,8-cineole or a vehicle control [ethanol (0.01% v/v)] for 5 minutes at 37°C, before stimulation with CRP-XL (1 µg/ml) under stirring conditions in an aggregometer for 5 minutes at 37°C. Platelets were then centrifuged (1000 g), for 10 minutes at room temperature, and the resulting supernatants were used in the assay. The level of VEGF was measured using a human VEGF ELISA kit, according to the manufacturer's protocol. The percentage of the level of VEGF released was calculated by considering the level obtained with the positive control (CRP-XL alone) as 100%. The graph depicts the mean ± SEM and is representative of three independent experiments. p values shown, (*$p<0.05$ and **$p< 0.01$) were calculated using one way-ANOVA followed by Bonferroni's correction for multiple comparisons.

4.2.2.4 1,8-cineole reduces the release of interleukin-1 beta (IL-1β) from platelets

Over the last decade, it has been reported that platelet pro-inflammatory molecules contribute to the progression of atherosclerosis and ACS (Aukrust, Müller et al. 1999, Nurden 2011). Although they lack a nucleus, platelets contain a large amount of messenger RNA and therefore they have the ability to synthesise molecules such as interleukin-1 (IL-1β) (Lindemann, Tolley et al. 2001). Indeed, platelets have been described as a major source for IL-1β (Brown and McIntyre 2011, Aggrey, Srivastava et al. 2013). This pro-inflammatory cytokine was found to contribute to the progression of inflammatory and cardiovascular diseases, and has also been linked to endothelial dysfunction and coagulation diseases (Tedgui and Mallat 2006, Bozza, Cruz et al. 2008, Yang, Ko et al. 2013). IL-1β can also activate immune cells through binding to IL-1 receptor (Dinarello 2009). Here, we investigated the inhibitory effect of 1,8-cineole on IL-1β release from platelets upon stimulation with an agonist.

Human IL-1β ELISA Kit (Theromfisher, UK) was used for the quantification of the amount of IL-1β secreted. Human isolated platelets from healthy donors were prepared and incubated for 5 minutes with different concentrations of 1,8-cineole or a vehicle control, prior to activation with 0.1 U/ml thrombin under stirring condition for 5 minutes. Cells were then centrifuged immediately at 1000 g for 10 minutes at room temperature and supernatants were collected and used in the assay.

In non-stimulated platelets, the level of IL-1β release was 214.3 pg/mL. However, the release of this molecule was markedly increased after stimulation with thrombin (0.1 U/ml) with a mean of 1195 pg/mL. Pre-treatment of isolated platelets with different concentrations of 1,8-cineole prior to stimulation with thrombin resulted in significantly decreased IL-1β release (Figure 4-5). 1,8-cineole at concentrations of 6.25, 12.5, 25 and 50 μM significantly downregulated IL-1β release, and it inhibited by

40%, 43%, 75% and 80%, (823.5, 776.9, 274.9 and 142.3 pg/mL) respectively. This result shows an inhibitory effect of 1,8-cineole on the release of one of the main platelet-derived pro-inflammatory mediators upon activation. This inhibitory effect demonstrates that 1,8-cineole is able to inhibit platelet-mediated inflammatory responses.

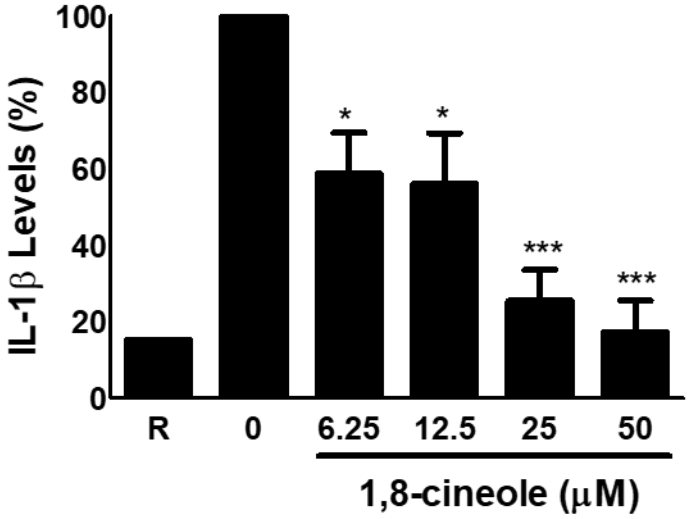

Figure 4-5: Effect of 1,8-cineole on the release of IL-1β from platelets. The concentrations of IL-1β released from platelets were measured using a human IL-1β ELISA kit. Human isolated platelets were incubated with a vehicle control [ethanol 0.01% (v/v)] or different concentrations of 1,8-cineole for 5 minutes at 37°C, and then activated with thrombin (0.1U/ml) for 5 minutes at 37°C. After the treatment, platelets were centrifuged at 1000 g for 10 minutes at room temperature, and the resulting supernatants were immediately used in the assay. The level of IL-1β was measured using human IL-1β ELISA kit according to the manufacturer's protocol. The percentage of the level of IL-1β released was calculated by considering the level of IL-1β obtained with the vehicle control as 100%. The bar charts represent the percentage of IL-1β obtained in the presence and absence of different concentrations of 1,8-cineole. The graph depicts the mean ± SEM (n=3). Statistical analysis was performed using a one-way ANOVA followed by Bonferroni's correction for multiple comparisons. p values (*$p < 0.05$ and ***$p < 0.001$) shown were calculated by comparing the treated samples with the positive control (CRP-XL alone) using one-way ANOVA.

4.2.2.5 1,8-cineole reduces the secretion of RANTES from activated platelets

Upon platelet activation, various inflammatory molecules including some chemokines are secreted into the microenvironment to support the local inflammatory responses (Page 1989, Damås, Gullestad et al. 2000). Regulated on activation normal T cell expressed and secreted (RANTES) is an important chemokine for the regulation of inflammation and pathological conditions such as the development of atherosclerosis (Gawaz, Brand et al. 2000, Von Hundelshausen, Weber et al. 2001). The main source of RANTES under *in vivo* settings is the α granules in platelets (Aukrust, Ueland et al. 1998), which further stimulates endothelial RANTES secretion (Gawaz, Langer et al. 2005). One well-established function of RANTES is to induce the adhesion of monocytes and T cells at the site of inflammation (Von Hundelshausen, Weber et al. 2001). In addition, RANTES can bind to the activated endothelial surface during atherosclerosis, and trigger the arresting and recruitment of monocytes at the inflammatory site under flow conditions (Charo and Ransohoff 2006). Given the vital role of platelet-derived chemokines in developing atherosclerosis, in this study, we further assessed the effects of 1,8-cineole on the secretion of a pro-atherosclerotic chemokine, RANTES upon platelet activation.

We examined the effect of 1,8-cineole on RANTES release in thrombin-stimulated platelets. Human isolated platelets from different donors were prepared and incubated for 5 minutes with various concentrations of 1,8-cineole or a vehicle control, prior to activation with 0.1 U/ml thrombin under stirring condition for 5 minutes. Platelets were then centrifuged immediately at 1000g for 10 minutes at room temperature and the resulting supernatants were collected and used in the assay. A human RANTES ELISA Kit (Theromfisher, UK) was used to measure the amount of RANTES released from platelets upon stimulation with 0.1 U/ml of thrombin.

As expected, the level of RANTES released from thrombin-stimulated platelets was higher than that from resting platelets with mean of concentration of 1266 pg/mL with thrombin stimulated cells compared to 118.28 pg/mL in unstimulated platelets. Our results demonstrate an inhibitory effect of 1,8-cineole on the secretion of RANTES from thrombin stimulated platelets. 1,8-cineole displayed a significant inhibitory effect at all tested concentrations with a mean concentration of 322.2, 242.1, 137.6 and 41.12 pg/mL, respectively, compared to 1266 pg/mL of thrombin stimulated cells alone, with approximately 70- 80% inhibition (Figure 4-6). These results demonstrate that 1,8-cineole is able to inhibit the secretion of platelet inflammatory molecules upon activation with different agonists. This could be attributed to the fact that 1,8-cineole inhibits α-granule secretion, as discussed in Chapter 3.

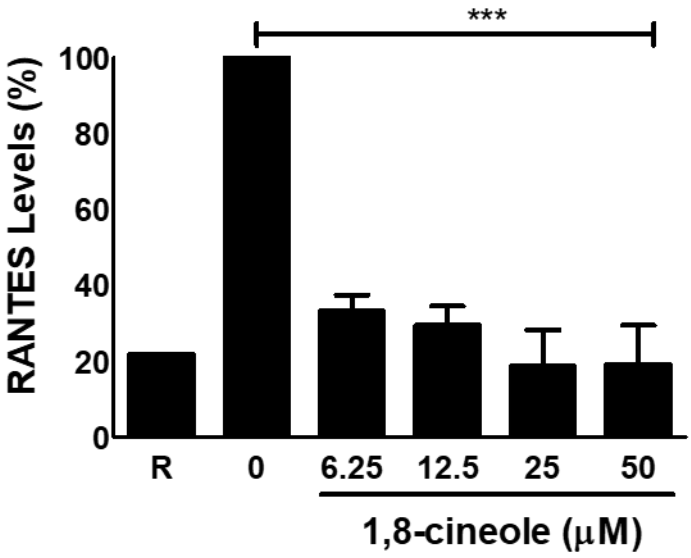

Figure 4-6: Effect of 1,8-cineole on the release of RANTES from platelets. Human isolated platelets were activated with 0.1 U/ml thrombin in an aggregometer for 5 minutes at 37°C, in the presence or absence of a vehicle [ethanol 0.01 (v/v)] or various concentrations of 1,8-cineole. Platelets were then centrifuged at 1000 g, for 10 minutes at room temperature, and resulting supernatants were collected and used in the assay. The concentrations of RANTES secreted from activated platelets were determined using a human RANTES ELISA kit according to the manufacturer's protocol. The percentage of the level of RANTES released was calculated by considering the level of RANTES obtained with the positive control (thrombin alone) as 100%. The bar chart represents percentage concentrations of RANTES obtained in the presence of different concentrations of 1,8-cineole. Cumulative data represent mean ± S.E.M (n=3). p value (***$p<0.001$) shown was calculated by one-way ANOVA followed by a Bonferroni's correction for multiple comparisons.

4.2.3 Impact of 1,8-cineole on the modulation of platelet-leukocyte interactions

Platelet-leukocyte interaction is a well-established phenomenon that occurs during inflammation. Activated platelets adhere to leukocytes (specifically monocytes and neutrophils) via P-selectin binding to PSGL1 on the leukocyte surface. P-selectin exposure on the platelet surface and RANTES secreted from activated platelets are the most important mediators to facilitate platelet-leukocyte interactions (Schober, Manka et al. 2002). Platelet-leukocyte aggregates are known as a prominent marker for pro-thrombotic conditions (Cerletti, Tamburrelli et al. 2012). Moreover, activated platelets can modify the atherogenic potential of endothelial cells by releasing various pro-inflammatory mediators, and this will lead to the formation of platelet-leukocyte aggregations in the circulation (Huo, Schober et al. 2003). In murine models, platelet-leukocyte interactions have been established to contribute to the progression of atherosclerosis. Studies in animal models show that the blockade of P-selectin significantly decreases the formation of atherosclerotic plaque in mice by reducing the level of platelet-leukocyte interactions (Dong, Brown et al. 2000). Additionally, the blockade of RANTES results in decreased formation of neointimal vascular lesions in *ApoE*$^{-/-}$ mice, further demonstrating the significance of RANTES derived from activated platelets in the development of atherosclerosis (Koenen, Von Hundelshausen et al. 2009). The above experiments measuring platelet-derived pro-inflammatory cytokines show that 1,8-cineole significantly inhibits the secretion of these molecules. Therefore, we then investigated whether 1,8-cineole is able to affect the crosstalk between platelets and leukocytes in order to further understand its impact on platelet-mediated inflammatory conditions.

Human whole blood was collected from healthy donors and incubated with different concentrations of 1,8-cineole (6.2 μM -50 μM) or a vehicle control for 10

minutes prior to stimulation with 10 μM of a thrombin receptor activation peptide (TRAP-6). To determine the level of platelet-leukocyte interactions, we used a double-labelling method on platelets and leukocytes and analysed using flow cytometry. Samples were double stained with the leukocyte pan marker, PE-labelled CD45 and a platelet marker, FITC-labelled CD42b before analysing using flow cytometry. Leukocytes were identified in the whole blood sample by their size and the expression of CD45. The positive cells within the gated region for leukocytes and platelets were identified. The results indicate that TRAP-6 significantly induces (7.7-26.2%) platelet-leukocyte interactions compared to the resting cells (Figure 4-7).

In addition, all concentrations of 1,8-cineole tested inhibited TRAP-6 induced platelet-leukocyte interactions, compared to the positive control (Figure 4-7). At all tested concentrations (6.25, 12.5, 25 and 50 μM), a significant reduction in the percentage of platelet-leukocyte interactions was observed. 1,8-cineole was able to decrease these interactions from 26.2% in TRAP-stimulated samples to 17.8%, 15.4%, 15.4% and 11.3%, treated samples with 6.25, 12.5, 25 and 50 μM, respectively (Figure 4-7 D–H). Statistical analysis shows a significant inhibitory effect in the percentage of platelet-leukocyte interactions induced by TRAP-6 (10 μM) at all concentrations of 1,8-cineole tested (Figure 4-7I).

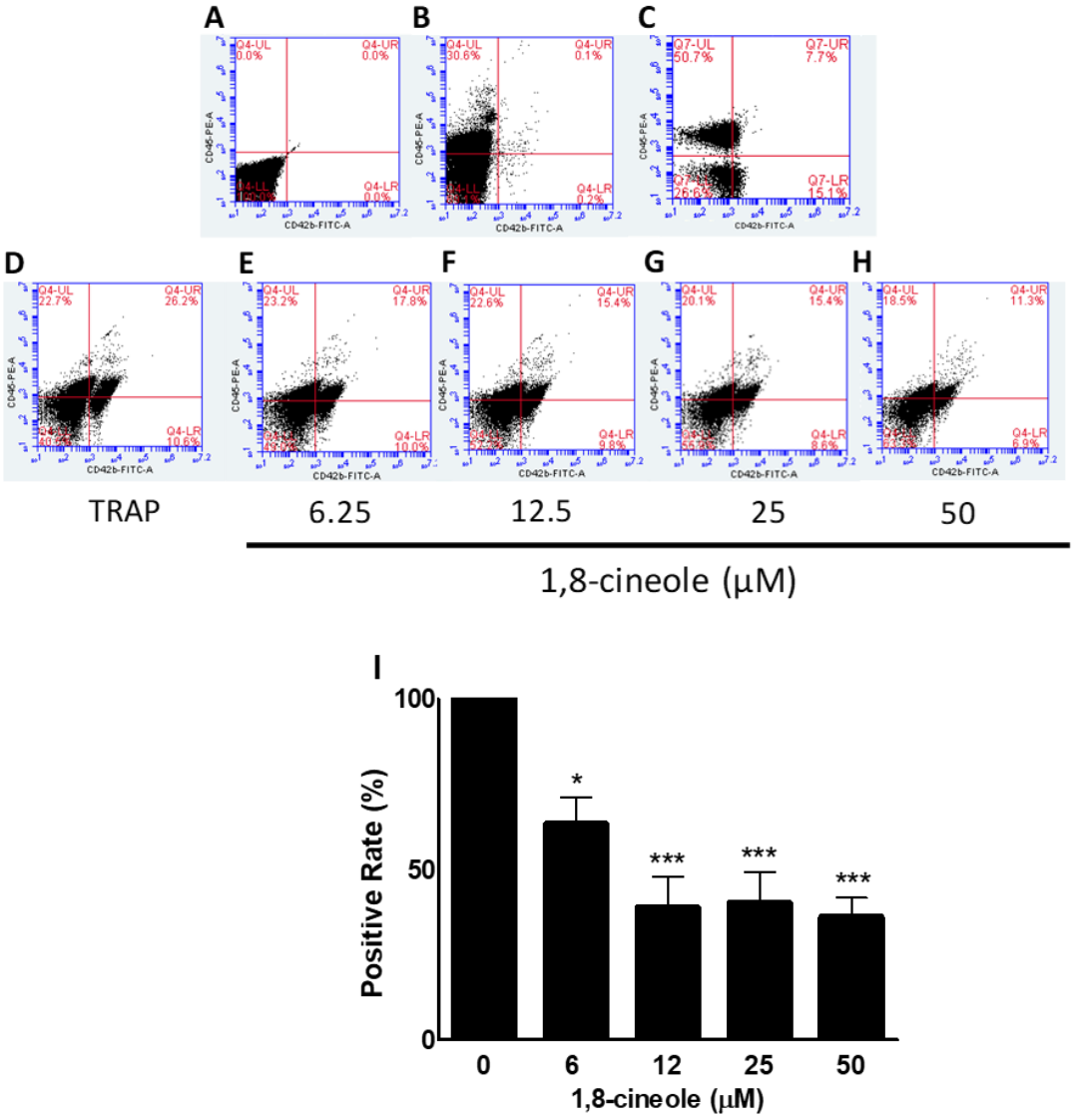

Figure 4-7: Effect of 1,8-cineole on the formation of platelet-leukocyte interactions. Human whole blood was incubated with different concentrations of 1,8-cineole or a vehicle control for 10 minutes prior to stimulation with 10 µM TRAP. Leukocytes were identified in the whole blood sample by their size and expression of CD42b in flow cytometry, using a gated region for the quantification of platelet-leukocyte aggregates by flow cytometry. Samples were unstained (**A**) or stained with the leukocyte marker, PE-labelld anti-CD45 antibodies (**B**) and the platelet marker, FITC-labelled anti-CD42b antibodies (**C**), and double stained in the presence of 10 µM TRAP (positive control) (**D**). CD42b positive events within the leukocyte gate were identified as platelet-leukocyte aggregates. Representative histograms show the level of CD42b as well as CD45 positive events in samples treated with different concentrations of 1,8-cineole (**E-H**). (**I**) Quantified data displays the percentage of inhibition by 1,8-cineole treated samples (vehicle treated sample represents 100% platelet-leukocyte aggregates). Data represent mean ± S.E.M (n=4). The statistical significance was calculated by one-way ANOVA. p values shown (*p<0.05 and ***p<0.001) are as analysed by one way-ANOVA followed by Bonferroni's correction for multiple comparisons.

Table 4.1 1,8-cineole affects the production of reactive oxygen species (ROS) in platelets.

Assay	Condition (Platelet preparation)	1,8 cineole (μM)	ROS production
ROS production in platelets	Isolated platelet	6.25	38% inhibition
		12.5	40% inhibition
		25	58% inhibition
		50	60% inhibition

Table 4.2 Summary of the impact of 1,8-cineole on the release of various inflammatory molecules from platelets

Type of Experiment	Inflammatory molecules released from activated platelets (Isolated Platelet)	1,8 cineole (μM)	Reduction in platelet secretion
Enzyme-linked immunosorbent assays (ELISA)	sP-selectin	6.25	No inhibition effect
		12.5	No inhibition effect
		25	35%
		50	89%
	TNF-α	6.25	40%
		12.5	76%
		25	80%
		50	87%
	VEGF	6.25	44%
		12.5	57%
		25	60%
		50	69%
	IL-1β	6.25	40%
		12.5	43%
		25	74%
		50	82%
	RANTES	6.25	66%
		12.5	70%
		25	81%
		50	80%

Table 4.3 Effect of 1,8-cineole on the formation of platelet-leukocyte interactions

Assay	Condition (Platelet preparation)	1,8 cineole (μM)	Platelet-leukocyte interactions
Formation of platelet-leukocyte interactions.	Human whole blood	6.25	48% inhibition
		12.5	73% inhibition
		25	78% inhibition
		50	80% inhibition

4.4 Discussion

In addition to their roles in haemostasis, platelets perform an important role by communicating with immune cells and initiating a host defence response. Indeed, in the bloodstream, platelets are in contact with pathogens when entering the circulation (Shiraki, Inoue et al. 2004). Upon platelet activation by various molecules released during pathological conditions, a large number of pro-inflammatory and pro-thrombotic molecules are released from platelets, and these molecules play an important role in facilitating the interaction between activated platelets and immune cells in order to augment inflammatory responses (Reed 2002, Lee, Park et al. 2016). The significance of thrombosis and inflammation mediated by platelets indicates their critical role in development of various inflammatory conditions such as atherosclerosis and sepsis (Wagner and Burger 2003, Khan, Kelher et al. 2006, von Hundelshausen and Weber 2007).

Previous *in vitro* and *in vivo* studies demonstrate that platelets contribute to adaptive immune and inflammatory processes, as they can directly interact with bacteria leading to platelet activation and aggregation (Page 1995, Kälvegren, Bylin et al. 2005). It has been established that platelets contribute to the pathophysiology of sepsis via numerous mechanisms including the direct interactions with bacteria. Uncontrolled infection during sepsis leads to extensive platelet activation resulting in decreased platelet count (thrombocytopenia) due to the consumption of circulating platelets through sequestration and microvasculature thrombi formation (Mavrommatis, Theodoridis et al. 2000, Vincent, Yagushi et al. 2002).

One of the main trigger for atherosclerosis is the inflammation that occurs within vascular wall, and this is correlated with platelet activation (E Tsoumani, I Kalantzi et

al. 2012). An increase in the number of platelet-leukocyte aggregates in the circulation under pathological conditions reflects the significant role of platelets during inflammation (Pitchford, Yano et al. 2003, Bozza, Shah et al. 2009, Middleton, Weyrich et al. 2016). Therefore, platelets provide a fundamental link between thrombotic and inflammatory cascades where the release of numerous inflammatory molecules contribute to collective inflammatory responses (Pitchford, Riffo-Vasquez et al. 2004, Idzko, Pitchford et al. 2015). Therefore, it is critical to understand the molecular mechanisms behind the regulation of platelet-mediated thromboinflammatory responses and develop novel therapeutic strategies to control these responses under pathological settings.

Monoterpenes in general have shown interesting properties to reduce inflammatory processes via modulating the release and functions of various inflammatory mediators. Jurgens et al. (2004) demonstrated that 1,8-cineole shows anti-inflammatory activities by inhibiting lipopolysaccharide-stimulated production of cytokines such as TNF-α and IL-1β in monocytes. The ability of 1,8-cineole to control the hypersecretion of airway mucus in asthma was also previously reported (Juergens, Engelen et al. 2004). Notably, 1,8-cineole has been investigated using *in vivo* studies in which rats were injected with trinitrobenzene sulfonic acid as a marker for drug activity against human inflammatory bowel disease (Chen, Wang et al. 1999). 1,8-cineole was given to male rats 24 hours before treatment with trinitrobenzene sulfonic acid. The animals that were pre-treated with 1,8-cineole demonstrated a significant decrease in the degree of inflammed tissue. This study suggests that 1,8-cineole has an anti-inflammatory effect on gastrointestinal inflammation (Santos, Silva et al. 2004). Similarly, 1,8-cineole has shown to decrease the expression of TLR4 in mice after inducing lung inflammation by LPS, compared to the effect of prednisolone (positive

control group), which is an anti-inflammatory drug (Zhao, Sun et al. 2014). Moreover, *in vivo* experiments have demonstrated the effectiveness of 1,8-cineole in the reduction of IL-8 and the number of leukocytes that adhered to a bronchoalveolar lavage in mice (Lee, Park et al. 2016). Together, it has been demonstrated that 1,8-cineole has significant impact on inflammatory responses in humans and animals. Given the main focus of this study on platelets, here we determined the impact of 1,8-cineole on the modulation of platelet-mediated inflammatory responses as this was not previously established.

ROS regulates a number of physiological and pathological responses in the cardiovascular system (Al Ghouleh, Khoo et al. 2011). Therefore, ROS production is an important mediator of various cell signalling pathways that play major roles in the development of inflammatory diseases including vascular inflammation and endothelial dysfunction leading to atherosclerosis and thrombosis (Krötz, Sohn et al. 2004, Madamanchi, Hakim et al. 2005, Griffith, Pendyala et al. 2009). The production of ROS from activated platelets was first observed in 1977 by Marcus et al. (Marcus, Silk et al. 1977). Under inflammatory conditions, platelets produce high quantities of ROS which in turn modulates inflammatory responses (Pratico, Iuliano et al. 1993, Cerwinka, Cooper et al. 2003). ROS is considered as a platelet activation marker (Shahbaz Ghasabeh, Ghasemzadeh et al. 2016), which is generated via NADPH oxidase (NOX_2) during platelet activation (Violi and Pignatelli 2012). A number of recent reports suggest that agonists stimulating NOX2 activation in platelets play a critical role in ROS generation and platelet activation (Arthur, Qiao et al. 2012, Walsh, Berndt et al. 2014). Accordingly, studies *in vitro* and *in vivo* have shown NOX-dependant platelet $O_{2-\cdot}$ generation also supports platelet aggregation and thrombotic functions (Krötz, Sohn et al. 2004). Our results demonstrate that 1,8-cineole is able to

inhibit CRP-XL-induced production of ROS in platelets. This inhibition of ROS production in platelets by 1,8-cineole suggests an important role for this molecule as an antioxidant and anti-inflammatory agent. It has been previously demonstrated by Juregens et, al. (2018), that 1,8-cineole has antioxidative properties, as it affected the production of ROS in human monocytes upon stimulation by LPS (Juergens, Tuleta et al. 2018). Based on our results and other studies including in humans (Pignatelli, Carnevale et al. 2011, Carnevale, Loffredo et al. 2014) and mice (Delaney, Kim et al. 2016), 1,8-cineole can be used as a potential agent to control anti-oxidant effects mediated by platelets, and this will also modulate platelet-mediated inflammatory responses. However, the mechanisms through which 1,8-cineole inhibits ROS production have to be elucidated in further studies.

The secretion of cytokines is initiated as an inflammatory response and these cytokines are a critical component during the progression of inflammatory diseases. Circulating sP-selectin, RANTES, VEGF, TNF-α, and IL-1β are some of the pro-inflammatory molecules released from platelets upon activation and they are also known as platelet activation markers (Assoian, Komoriya et al. 1983, Ferroni, Martini et al. 2009). Furthermore, IL-1β has been recognised as a critical mediator of platelet activation and endothelial cell dysfunction. (Hawrylowicz, Howells et al. 1991). The elevated levels of pro-inflammatory cytokines in the blood are believed to contribute to vascular inflammation and atherosclerosis along with other pathological conditions (Huo and Ley 2004, Projahn and Koenen 2012). Several *in vivo* studies in animal models have confirmed that platelet-derived inflammatory molecules play a critical pathogenic role in the development of atherothrombosis (Aslam, Speck et al. 2006, Clark, Ma et al. 2007, Kuckleburg, Tiwari et al. 2008). In patients with inflammatory diseases such as asthma, the level of platelet activation markers including PF-4, PAF

and RANTES were reported to be elevated, which further indicates that platelet activation contributes to inflammatory disease (O'Sullivan, Linden et al. 2005, Idzko, Pitchford et al. 2015). Therefore, establishing the impact of 1,8-cineole on the release of various inflammatory molecules from platelets would provide further insights into the understanding of its effect on platelet-mediated inflammatory responses.

Thus, we investigated the impact of 1,8-cineole on the secretion of various inflammatory cytokines such as sP-Selectin, RANTES, VEGF, TNF-α, and IL-1β from agonist-stimulated platelets. Indeed, 1,8-cineole has affected the secretion of these cytokines from platelets upon activation. These results further support the actions of 1,8-cineole on platelet-mediated inflammatory response. Although the precise mechanisms through which 1,8-cineole affects the release of cytokines from platelets are unknown, one possibility that can explain this inhibition is that 1,8-cineole is well known to modulate NF-kB activity which correlated to inflammatory responses including the synthesis and release of cytokines (Haasbach, Reiling et al. 2013, Linghu, Lin et al. 2016). Therefore, 1,8-cineole could exhibit its effects on platelets via affecting NF-kB signalling pathway. However, further studies are required to determine the mechanisms through which 1,8-cineole affects the release of cytokines from platelets.

The effects of 1,8-cineole observed in platelets in this study are similar to several studies that reported the impact of this molecule on the release of various inflammatory molecules in other cell types. Lima at.et, (2013) investigated the potential of 1,8-cineole to exert antioxidant and anti-inflammatory effects against cerulenin-induced severe pancreatitis, and found that the production of TNF-α, IL-1β, and IL-6 was increased by cerulenin, but decreased upon pre-treatment with 1,8-cineole (Lima, de Melo et al. 2013). Moreover, in bronchial vessels, cytokines such as TNF-α and

IL-1β stimulate endothelial cells, which in turn express both the vascular cell adhesion molecule (VCAM)-1 and the intercellular adhesion molecule (ICAM)-1; the expression of these two molecules results in migration and recruitment of leukocytes at the site of infection (Garcia, Xia et al. 2000). Li et al. observed a significant effect of 1,8-cineole on the expression of ICAM-1 and VCAM-1 and suppression of the production of proinflammatory cytokines on the cell surface of mice after exposure to the influenza virus. The effect in the1,8-cineole treated group was similar to the oseltamivir-treated group, which is an antiviral drug used to treat the influenza virus (Li, Lai et al. 2016). Previous studies have also demonstrated the impact of 1,8-cineole on production of TNF-α, IL-1ß, IL-6, and IL-8 in monocytes upon stimulation with LPS, and TNF-α, IL1ß, IL-4 and IL-5 in lymphocytes upon stimulation with ionomycin at a concentration of 1 mM (Juergens, Stöber et al. 1998, Juergens, Engelen et al. 2004).

In addition to ROS production and release of inflammatory molecules, platelet activation during inflammation results in formation of platelet-leukocyte aggregates. P-selectin (CD62P) exposed on the platelet surface following activation is an important adhesive molecule, which facilitates platelet-leukocyte interactions via binding to its ligand, PSGL-1 on leukocytes (Frenette, Johnson et al. 1995, Chen and Geng 2006). This binding results in direct interactions between platelets and leukocytes, which helps in efficient migration of inflammatory cells to affected tissue. In recent years, it has been suggested that the crosstalk between platelets and leukocytes contributes to the progression of CVD, and it plays a crucial role in the progression of atherosclerosis and thrombosis (Blann, Nadar et al. 2003, Gkaliagkousi, Corrigall et al. 2009). Moreover, an increase in platelet-leukocyte aggregates in the circulation has been observed in patients with ACS (Patel, Pfau et al. 2004).

While we have previously demonstrated the inhibitory effect of 1,8-cineole on P-selectin exposure and the release of RANTES upon platelet activation which are critical for the formation of platelet-leukocyte interactions, here we specifically analysed the impact of 1,8-cineole on these interactions. Flow-cytometric analysis confirmed the ability of TRAP-6 to increase the formation of platelet-leukocyte aggregates. However, 1,8-cineole strongly reduces the formation of platelet-leukocyte aggregates upon stimulation with TRAP-6. The inhibition of the release of RANTES by 1,8-cineole might also attribute to the inhibition on platelet leukocyte interactions. Our results are in agreement with other studies that observed a decrease in platelet-leukocyte interactions under *in vitro* and *in vivo* settings following a treatment with clopidogrel and prasugrel (Klinkhardt, Bauersachs et al. 2003, Harding, Sarma et al. 2006). Similar to the effect of 1,8-cineole on the formation of platelet-leukocyte aggregates, flavonoids such as flavonolignans demonstrate anti-inflammatory and anti-platelet effects, as well as inhibit IL-1β-induced interactions between platelets and leukocytes at a concertation of (10-100 μM) (Bijak, Dziedzic et al. 2017) .

In summary, the data provided in this chapter clearly demonstrate the anti-inflammatory effects of 1,8-cineole specifically on platelet-mediated inflammation. Further research is required to determine the effect of 1,8-cineole in the modulation of platelet-mediated inflammation under *in vivo* and disease settings and to establish the mechanisms behind this inhibition. However, based on the impact of 1,8-cineole on the control of platelet activation, thrombus formation and inflammation, this might act as a powerful antiplatelet agent to control thrombotic and inflammatory diseases. Hence, further preclinical and clinical studies with a clinical grade 1,8-cineole will be warranted.

Chapter 5

5- MECHANISMS THROUGH WHICH 1,8-CINEOLE MODULATES PLATELET FUNCTION

5-1 Introduction

The results obtained so far demonstrate the potential of 1,8-cineole to modulate a range of platelet functions, thrombus formation (*in vitro*) and haemostasis under physiological settings. In addition, 1,8-cineole shows an inhibitory effect on calcium mobilisation, which is directly regulated by the activation of various intracellular signalling pathways in platelets. Our data in Chapter 3 demonstrate an inhibitory effect of 1,8-cineole selectively on collagen-stimulated platelet function. Similarly, 1,8-cineole inhibits CRP-XL-stimulated dense and α-granule secretion in platelets. Moreover, outside-in signalling triggered by integrin $\alpha_{IIb}\beta_3$ was also affected by 1,8-cineole, as observed via the inhibition of clot retraction and platelet spreading. Together, these results suggested that GPVI-mediated signalling might be largely affected by 1,8-cineole in order to modulate platelet function.

We therefore extended our study to examine whether the effects of 1,8-cineole on platelets result from its ability to modulate different signalling pathways in these cells. 1,8-cineole has been reported to regulate various signalling proteins that are involved in the regulation of leukocyte-mediated inflammatory responses (as explained in Chapter 1). Here, we investigated the effect of 1,8-cineole on the level of phosphorylation of various signalling proteins involved in GPVI signalling pathway. Phosphorylation of signalling proteins, such as SYK and linker for the activation of T cells (LAT) are important for the propagation of signalling activation in platelets (Pasquet, Gross et al. 1999, Buitrago, Bhavanasi et al. 2013). In addition, we also evaluated the impact of 1,8-cineole on the modulation of cAMP signalling pathway as it is an important negative feedback mechanism in platelets in order to prevent their unwarranted activation in the circulation. The data provided in this chapter highlight some of the plausible mechanisms for 1,8-cineole to modulate platelet function.

5-2 Results

5-2-1 1,8-cineole negatively regulates GPIV signalling in platelets

GPVI has been shown to be associated with the FcRγ, a transmembrane protein which is important for the transduction of downstream signalling via this receptor in platelets (Gibbins, Okuma et al. 1997). FcRγ contains the immunoreceptor tyrosine activation motif (ITAM), which, upon collagen (or CRP-XL) binding to GPVI, causes the clustering of receptor resulting in autophosphorylation of the Src family kinases (Fyn and Lyn) in the cytoplasmic tail via their SH3 domains (Ezumi, Shindoh et al. 1998). In addition, the phosphorylation of ITAM causes recruitment and binding of SYK to ITAM via Src-homology2 domains (SH2), which leads to the autophosphorylation and activation of SYK (Li, Delaney et al. 2010). The activation of SYK is followed by further tyrosine phosphorylation of its downstream target, LAT and results in the formation of a protein complex called the 'LAT signallosome'. The LAT signallosome composed of effector and adaptor proteins such as Gads (Grb2) and SLP-76, which carry the effectors to their substrates (Hughes, Auger et al. 2008). This structure of LAT signallosome facilitates the recruitment of phospholipase Cy2 (PLCy2) and PI3K which evokes the conversion of PIP2 to PIP3 (Ragab, Séverin et al. 2007, Moroi and Watson 2015). The conversion of PIP2 to PIP3 will enables PLCy2 and Brutons tyrosin kinase (Btk) to localise at plasma membrane leading to phosphorylation and activation of PLCy2 (Pasquet, Gross et al. 1999).

Activation and phosphorylation of PLCy2, generates secondary messengers IP3 and DAG, and the binding of IP3 to its receptor on the dense tubular system facilitates calcium mobilisation from intracellular stores, while DAG leads to protein kinase C (PKC) activation (Jung and Moroi 2008, Moraes, Barrett et al. 2010). This is

followed by the upregulation of the affinity of integrin $\alpha_{IIb}\beta_3$ to allow fibrinogen binding and degranulation of platelet granules, and all of these leads to platelet activation and aggregation (Konopatskaya, Matthews et al. 2011).

The effect of 1,8-cineole in modulating GPVI signalling was investigated by examining its effects on early GPVI signalling events, which are mainly controlled by tyrosine phosphorylation of the key signalling proteins, SYK and LAT. To investigate the effect of 1,8-cineole on GPVI signalling, human isolated platelets ($4x10^8$ cells/mL), were incubated with different concentrations of 1,8-cineole or a vehicle control [0.01% ethanol (v/v)] for 5 minutes before their activation with CRP-XL (0.5 µg/ml) for 5 minutes. The platelet lysates were analysed by immunoblot analysis using appropriate antibodies. The cumulative data were obtained by quantifying the relevant protein bands using densitometry and normalising them to the loading protein, 14-3-3 ζ which is a housekeeping protein expressed ubiquitously in platelets.

Upon stimulation with CRP-XL, SYK is phosphorylated at several sites including Y323 which is a regulatory site and this phosphorylation is transient, showing high levels at around 90 seconds following CRP-XL stimulation (Asselin, Gibbins et al. 1997). While CRP-XL stimulation clearly increased the phosphorylation, upon pre-treatment with 1,8-cineole, the phosphorylation of SYK pY323 (Figure 5-1 A and B) was slightly reduced, although it was not significant after being quantified and normalised to the loading control 14-3-3 ζ, compared to the positive control (0.5 µg/mL CRP-XL).

The phosphorylation of SYK causes the activation and phosphorylation of LAT. The tyrosine residue at pY200 in LAT is phosphorylated by SFKs and SYK (Paz, WANG et al. 2001). Therefore, we measured the phosphorylation of LAT at Y200, in the presence and absence of 1,8-cineole following CRP-XL activation in platelets. The

images obtained from immunoblot analysis demonstrates that the phosphorylation of LAT (pY200) was down-regulated by 1,8-cineole. A significant inhibition with 1,8-cineole was achieved at 6.25, 12.5, 25 and 50 μM, which cause approximately 38%, 60%, 43% and 54% reduction in the phosphorylation level compared to the vehicle control, respectively (Figure 5-1 A and B).

These results suggest that 1,8-cineole, at all concentrations tested, is not able to significantly affect the activation of SYK in GPVI signalling pathway. However, a significant inhibitory effect on LAT was observed in this study. Further studies may be required to corroborate the inability of 1,8-cineole to affect the phosphorylation of SYK while its effect is prominent in LAT.

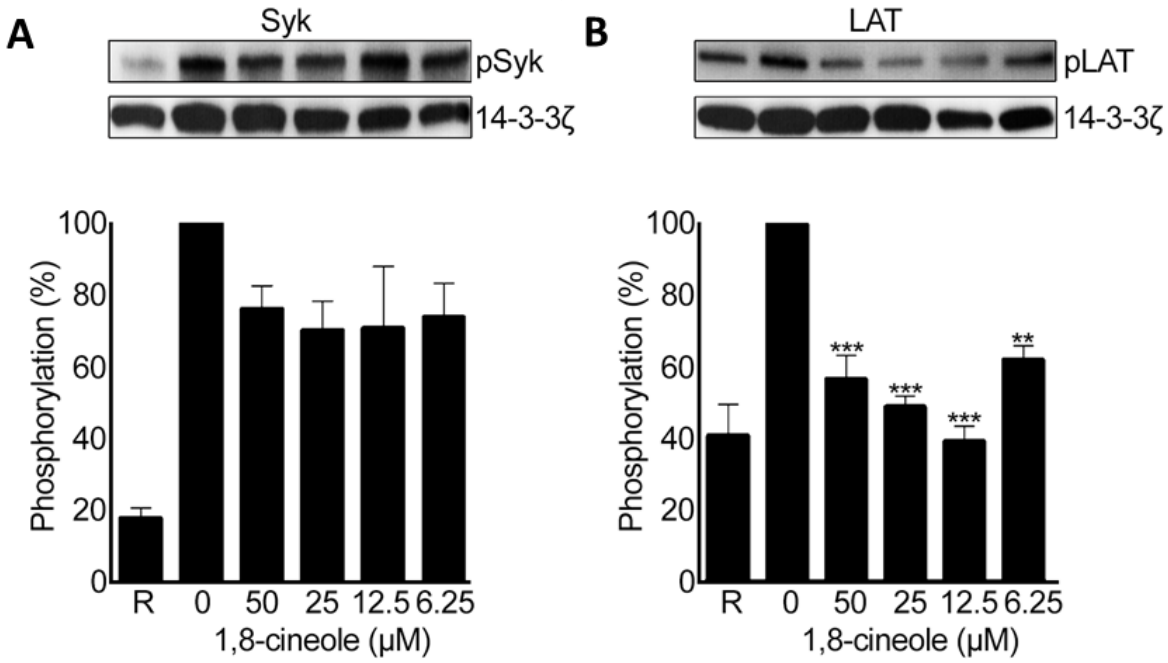

Figure 5-1: Effect of 1,8-cineole on the tyrosine phosphorylation of SYK and LAT. Human isolated platelets were treated with either a vehicle control (0) or various concentrations of 1,8-cineole for 5 minutes before being stimulated with CRP-XL (0.5 μg/mL) in an aggregometer at 37°C. The levels of phosphorylation of SYK (**A**) and LAT (**B**) were detected using primary antibodies against phosphorylated SYK at residue pY323 and LAT at residue pY200 in platelet lysates by immunoblot analysis. The level of protein, 14-3-3ζ was detected as a loading control in all the samples. The blots shown are representative of three separate experiments using platelets obtained from different donors. The relative phosphorylation levels of SYK at pY323 and LAT at pY200 are represented as the mean ± SEM (n = 3). p values shown (**p < 0.01 and ***p< 0.001), are as compared with the CRP-XL activated sample (positive control) and were analysed by one way-ANOVA followed by Bonferroni's correction for multiple comparisons.

5-2-2 1,8-cineole inhibits Src family kinases that are stimulated by CRP-XL

Signal transduction downstream of GPVI results from the coordination of phosphorylation of several intracellular proteins, especially when considering that modulation in the phosphorylation of one component is likely to influence the downstream regulation of its associated effector molecules. The Src family kinases (SFKs) are critical for the functioning and downstream signalling of GPVI (Senis, Mazharian et al. 2014). The collagen-mediated clustering of GPVI receptors causes the auto-phosphorylation of SFKs (Src, Fyn and Lyn). This caused further SFK-dependent phosphorylation of the ITAM, which contains the FcRγ -chain complex, followed by the phosphorylation of SYK (Watson and Gibbins 1998, Ellison, Mori et al. 2010). Given the inhibitory effect of 1,8-cineole on the phosphorylation of LAT, which is a downstream target of SYK phosphorylation, the effect of 1,8-cineole on CRP-XL-stimulated SFKs phosphorylation was investigated. The identification of these targeted elements of 1,8-cineole would enable the identification of underlying mechanisms through which 1,8-cineole modulates platelet function.

The phosphorylation sites of Src include an activating phosphotyrosine site (pY 416) which results from autophosphorylation, and an inhibitory phosphotyrosine site (pY527) resulting from phosphorylation by the C-terminal Src kinase and SH2 domains. The dephosphorylation of inhibitory site (pY527) is associated with an increase in Src kinase activity and platelet activation (Roskoski 2005, ELLISON, MORI et al. 2010). In platelets, Src activity is important as it allows GPVI and integrin $\alpha_{IIb}\beta_3$ to initiate platelet activation.

To investigate the effect of 1,8-cineole on the modulation of Src phosphorylation upon stimulation with a GPVI-specific agonist, CRP-XL, human isolated platelets ($4x10^8$ cells/mL) were prepared and treated with either 1,8-cineole (6.25,12.5, 25 and

50μM) or a vehicle control [ethanol 0.01% (v/v)] for 5 minutes at 37°C. Following incubation, the samples were stimulated by the addition of 0.5 μg/mL CRP-XL at 37°C for 5 minutes.

As expected, the level of Src phosphorylation at the inhibitory site upon CRP-XL stimulation was reduced in immunoblots which were developed using a phospho-specific antibody against Src pY527 (Figure 5-2). Notably, 1,8-cineole showed a significant effect by preventing the dephosphorylation of Src, while the dephosphorylation in the positive control sample remained at a low phosphorylation level. In the presence of 1,8-cineole, the phosphorylation level of Src at the inhibitory site was increased. This increase was significant at all concentrations tested, with more than an 80% increase compared to the vehicle control sample after being quantified and normalised to the loading control of 14-3-3 ζ and compared to the vehicle control (0.5 μg/mL CRP-XL). This result suggests that 1,8-cinoele is able to inhibit SFK specifically Src upon stimulation with CRP-XL in platelets. Therefore, Src might be a primary target for 1,8-cineole to modulate platelet function.

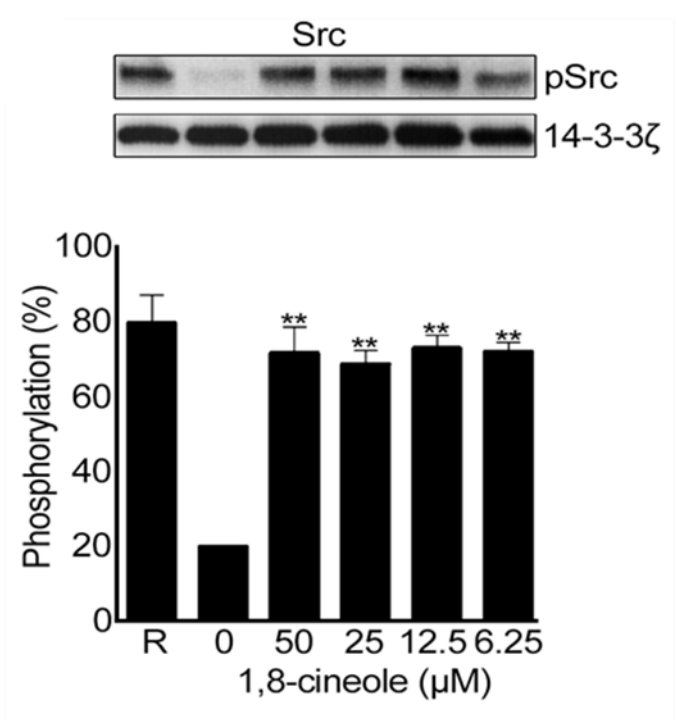

Figure 5-2: Effect of 1,8-cineole on Src phosphorylation. Human isolated platelets were treated with either a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole for 5 minutes before being stimulated with CRP-XL (0.5 μg/mL) for 5 minutes in an aggregometer at 37°C. The level of Src phosphorylation at residue Y527 was detected using platelet lysates and phospho-specific antibody against this site by immunoblot analysis. The level of 14-3-3ζ was detected as a loading control. The blots shown are representative of three separate experiments using platelets obtained from various individuals. The relative phosphorylation level of Src are represented as the mean ± SEM (n = 3) after being normalised to the loading control. p value (**p<0.01) shown is calculated when compared to positive control as analysed by one way-ANOVA followed by Bonferroni's correction for multiple comparisons.

5-2-3 1,8-cineole affects PI3K/AKT signalling

AKT has an important role in platelet activation as it is induced downstream of GPVI receptor, GPCRs and vWF receptor complex, GPIb-IX-V (Barry and Gibbins 2002, Yin, Stojanovic et al. 2008, Kim, Mangin et al. 2009). The activation of PI3K results in PIP3 production, which in turn leads to downstream activation of AKT, causing further activation of Rap1B leading to the activation of integrin αIIbβ3 (Kroner, Eybrechts et al. 2000, Lova, Paganini et al. 2003). Studies have shown that PI3K plays an important role in several cell surface receptors including cell movement, differentiation, growth and survival as a potent second messenger, and PI3K also has a critical role in platelet activation (Woulfe 2010, Guidetti, Canobbio et al. 2015). The main downstream signalling effectors for PI3K is AKT which is also known as PKB. AKT is activated and phosphorylated by PDK1 when its recruited to the plasma membrane through PIP3 binding (Chen, Zhang et al. 2013). AKT has a critical role in platelet activation by inducing downstream signalling of the vWF receptor (Yin, Stojanovic et al. 2008), GPCRs (Kim, Mangin et al. 2009) and GPVI receptor (Barry and Gibbins 2002).

1,8-cineole has been previously reported as a potential candidate to treat non-alcoholic steatohepatitis due to its inhibitory effects on AKT/PI3K pathways (Murata, Ogawa et al. 2015). Thus, we aimed to determine whether 1,8-cineole will have similar inhibitory effects on CRP-XL-induced phosphorylation of AKT in platelets.

To investigate the impact of 1,8-cineole on PI3K-mediated signalling, the phosphorylation of AKT (as a marker for PI3K signalling) was investigated in CRP-XL-stimulated platelets. The phosphorylation of AKT at serine 473 (pS473) upon stimulation with CRP-XL was investigated using platelet lysates by immunoblot analysis. Human isolated platelets were stimulated with CRP-XL (0.5 μg/mL) in the

presence of different concentrations of 1,8-cineole (6.25, 12.5, 25 and 50 μM) or a vehicle control (ethanol, 0.01 v/v%), and lysates were prepared to detect the phosphorylation of AKT (pS473) by immunoblot analysis. CRP-XL significantly increased AKT phosphorylation at 90 seconds (Figure 5-3). However, 1,8-cineole significantly inhibited the phosphorylation of AKT (pS473). At high concentrations of 50 μM and 25μM, 1,8-cineole caused around 70% inhibition in the phosphorylation level of AKT. This data demonstrates that 1,8-cineole could downregulate PI3K-mediated signalling pathways in platelets via AKT, which may affect platelet functions.

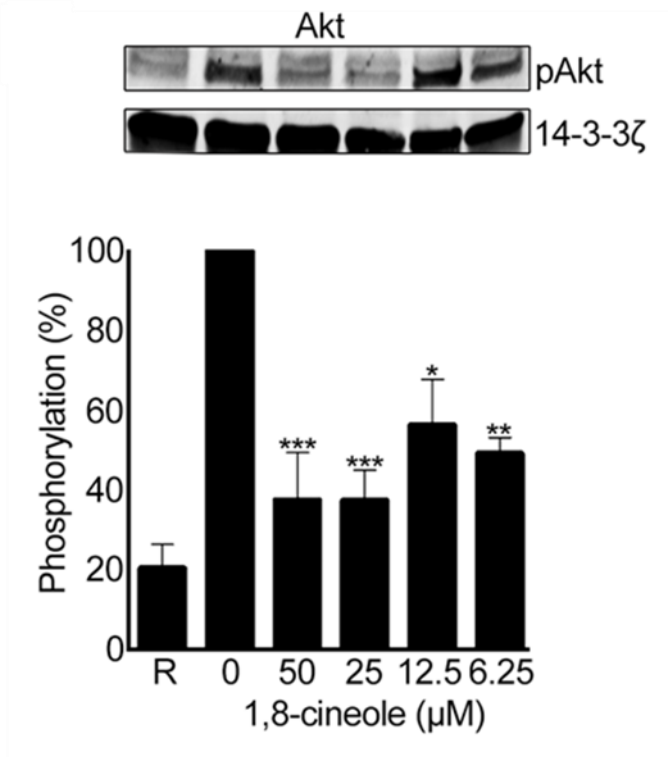

Figure 5-3: Effect of 1,8-cineole on AKT phosphorylation. Human isolated platelets were treated with a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole for 5 minutes before being stimulated with CRP-XL (0.5 μg/mL) for 5 minutes in an aggregometer at 37°C. The level of AKT phosphorylation at S473 residue was detected using platelet lysates and phospho-specific antibodies against this site by immunoblot analysis. The level of 14-3-3ζ was detected as a loading control. The blot shown is representative of three separate experiments using platelets obtained from different individuals. The relative phosphorylation levels of AKT are represented as the mean ± SEM (n = 3). p values shown (*p<0.05, **p<0.01, ***p<0.001) are as analysed by one way-ANOVA followed by Bonferroni's correction.

5-2-4 1,8-cineole affects the phosphorylation of mitogen-activated protein kinases

In platelets, GPVI-mediated signal transduction leads to the recruitment of various proteins including mitogen-activated protein kinases (MAPKs), which activate integrin αIIbβ3 and induce the synthesis and secretion of TXA2 as well as ADP from dense granules (Poole, Gibbins et al. 1997, Gibbins, Briddon et al. 1998). MAPKs are serine/threonine kinases that convert extracellular stimuli into a wide range of cellular responses (Widmann, Gibson et al. 1999), and they have a critical role in both proliferation and differentiation in eukaryotic cells. MAPKs, such as extracellular signal-regulated kinases (ERK) 1/2 and p38 (Lenormand, Pagès et al. 1993, McNicol and Shibou 1998), can be activated by various extracellular stimuli (e.g. hormones and growth factors) (Cobb, Boulton et al. 1991). Various members of MAPK family are expressed in platelets, including ERK1/2, p38, ERK5 and JNK1 (Börsch-Haubold, Ghomashchi et al. 1999). It has been demonstrated that JNK, ERK and p38 MAPKs are activated following platelet stimulation by thrombin, collagen, and other agonists, including TXA2 and ADP (Kramer, Roberts et al. 1995). Several studies have suggested that MAPKs are important in several platelet functions including aggregation (Li, Xi et al. 2001, Roger, Pawlowski et al. 2004), thrombus formation (Sakurai, Matsuo et al. 2004), Ca^{2+} mobilisation (Rosado and Sage 2001) and granule secretion (Jin, Han et al. 2007). In addition, the inhibition of the phosphorylation of ERK1/2 and p38 results in the reduction in TXA2 and arachidonic acid production, which induce platelet aggregation (Lee, Kim et al. 2010).

It is well known that bacterial LPS plays a critical role in initiating inflammatory processes via activating NF-κB and MAPK signalling pathways in immune cells (He, Wei et al. 2015). In human monocyte THP-1 cells, 1,8-cineole demonstrated an inhibitory effect on the expression of Early growth response factor 1 (Egr-1) through

inhibiting ERK signalling, which was stimulated by LPS (Zhou, Wang et al. 2007). In Chapter 4, we demonstrated the inhibitory effects of 1,8-cineole on the release of platelet-derived inflammatory cytokines upon stimulation with CRP-XL. Therefore, here we further investigated whether 1,8-cineole affected the phosphorylation of MAPKs, specifically ERK1/2 and p38 MAPK in platelets upon stimulation with CRP-XL.

Human isolated platelets were prepared and incubated with different concentrations of 1,8-cineole (6.25, 12.5, 25 and 50 μM) or a vehicle control (ethanol 0.01 v/v%) for 5 minutes, before activated with CRP-XL (0.5 μg/mL). The resulting platelet lysates were prepared to detect the phosphorylation of p38 MAPK and ERK1/2 by immunoblot analysis using appropriate phospho-specific antibodies. The immunoblot analysis as shown in Figure 5-4 A-B, revealed that 1,8-cineole could inhibit CRP-XL-induced phosphorylation of p38 MAPK (44% at 50 μM, 23% at 25 μM, 53% at 12.5 μM and 57 % at 6.25 μM). Similar effects were also observed at ERK1/2 phosphorylation with inhibitory effects on the level of phosphorylation by 47% at 50 μM, 43% at 25 μM, 51% at 12.5 μM and 46% at 6.25 μM. These results indicate that the antiplatelet activity of 1,8-cineole could be occurring via blockade of multiple platelet activatory signalling pathways including the AKT/MAPK pathways. Therefore, 1,8-cineole is likely to possess multiple targets in platelets in order to control platelet reactivity, thrombosis and haemostasis.

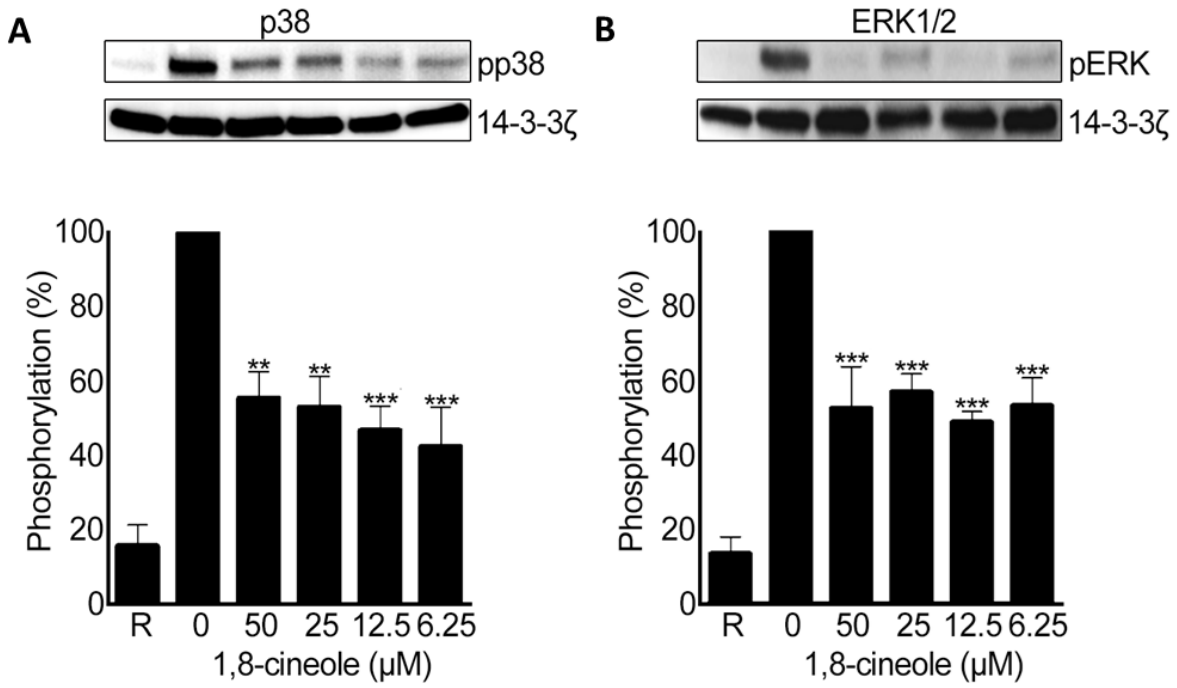

Figure 5-4: Impact of 1,8-cineole on p38 and ERK1/2 phosphorylation. Human isolated platelets were treated with either a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole for 5 minutes before being stimulated with CRP-XL (0.5 µg/mL) with constant stirring at 37°C. The levels of phosphorylation of p38 MAPK (**A**) and ERK1/2 (**B**) were detected using platelet lysates by immunoblot analysis. The level of 14-3-3ζ was detected as a loading control**.** The relative phosphorylation levels of p38MAPK and ERK1/2 are represented as the mean ± SEM (n=3). p values shown (**p<0.01 and ***p<0.001) are as analysed by one way-ANOVA followed by Bonferroni's correction for multiple comparisons.

5-2-5 1,8-cineole affects cAMP levels and phosphorylation of vasodilator-stimulated phosphoprotein (VASP) in platelets

Under physiological conditions, healthy endothelial cells constantly release nitric oxide (NO) and prostaglandin (PGI_2), which inhibit platelet function within the circulation via elevating cGMP and cAMP, respectively. Therefore, the inhibition of platelet aggregation is likely to be associated with the levels of cAMP and cGMP (Waldmann and Walter 1989, Dean, Chen et al. 1997). As several small molecule inhibitors are known to affect the levels of cAMP in platelets, we next sought to determine the effects of 1,8-cineole on cAMP levels in CRP-XL-stimulated platelets.

The levels of cAMP in platelets were measured using a cAMP ELISA kit (Cambridge Bioscience, UK). Human isolated platelets were prepared as described before and incubated with a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole (6.25-50 μM) prior to activation with CRP-XL (0.5 μg/ml). Unstimulated platelets were treated with EGTA (1 mM) as a control for maximum levels of cAMP. After that, platelets were lysed with 0.1M Hydrochloric acid (HCl), and cAMP levels were detected according to the manufacturer's protocol.

As shown in Figure 5-5, the cAMP level in CRP-XL-activated platelets was significantly lower than in resting platelets. However, pre-treatment with 1,8-cineole at all tested concentrations (6.25, 12.5, 25 and 50 μM) significantly increased cAMP levels in CRP-XL-activated platelets compared to the vehicle control.

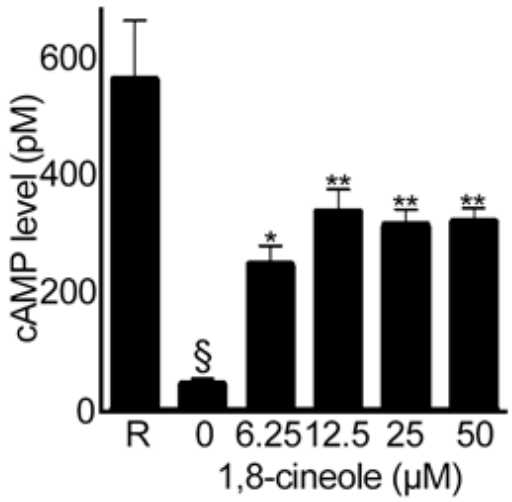

Figure 5-5: Effect of 1,8-cineole on cAMP level in platelets. The total cAMP levels in platelets that were pre-treated with a vehicle control [0.01% (v/v) ethanol] or different concentrations of 1,8-cineole (6.25–50 μM) prior to stimulation with CRP-XL (0.5 μg/mL) were determined using a cAMP ELISA kit. Resting (R) corresponds to platelets treated with EGTA (1 mM), a platelet inhibitor (to produce maximum cAMP levels). Cumulative data represent mean ± S.E.M (n=3). § indicates significant difference with respect to resting control and ∗ indicates significant difference with respect to vehicle control (0) in the absence of 1,8-cineole. p values shown (*P < 0.05 and **P < 0.01) are as calculated by one way-ANOVA followed by Bonferroni's correction for multiple comparisons.

Furthermore, to assess the effects of 1,8-cineole on cAMP-mediated signalling, the phosphorylation levels of VASP at position S157, which is a substrate for cAMP-dependent protein kinase was investigated (Wentworth, Pula et al. 2006). To investigate the effect of 1,8-cineole in this pathway, platelets were incubated with either 1,8-cineole or a vehicle control [ethanol 0.01% (v/v)] for 5 minutes prior to being stimulated with CRP-XL (0.5 µg/ml). The resting platelets showed an increase in the phosphorylation of VASP S157 compared to the CRP-XL activated sample (Figure 5-6). Additionally, the samples that were preincubated with 1,8-cineole showed an increase in the phosphorylation level of VASP (S157); but the vehicle control-treated sample (stimulated platelets) showed no increase in the phosphorylation level of VASP S157 (Figure 5-6). This was apparent that 1,8-cineole caused a significant increase in VASP phosphorylation. 1,8-cineole at concentrations of 25 and 50 µM caused approximately 70% increase, and at concentrations of 6.25 and 12.5 µM, an approximately 55% increase of VASP phosphorylation levels in comparison to positive control sample (CRP-XL alone). This observation suggests that 1,8-cineole may play an important role in affecting the phosphorylation of VASP and cAMP levels. VASP is also involved in the remodelling of the actin cytoskeleton that regulates platelet shape changes (Pula, Schuh et al. 2006).

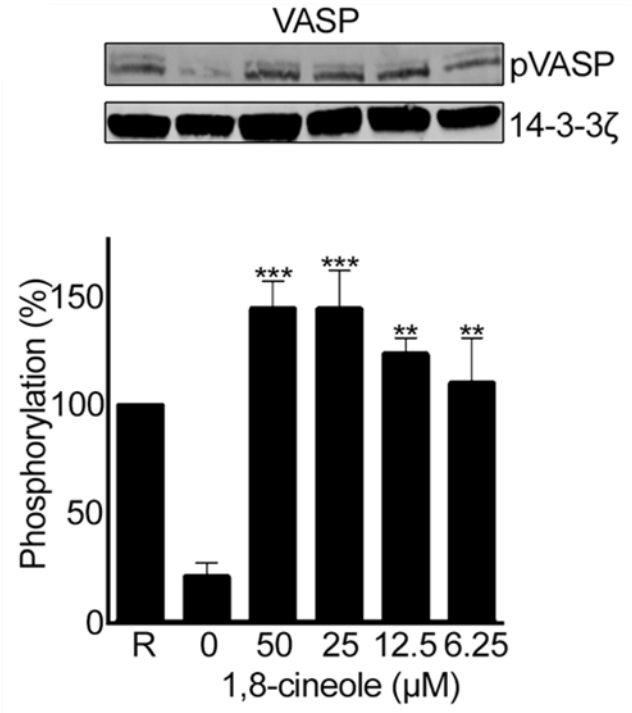

Figure 5-6: Effect of 1,8-cineole on VASP phosphorylation. Human isolated platelets were treated with either a vehicle control [0.01% (v/v) ethanol] or various concentrations of 1,8-cineole for 5 minutes before being stimulated with CRP-XL (0.5μg/mL) in an aggregometer at 37°C. The level of VASP phosphorylation in residue S157 was detected using platelet lysates by immunoblot analysis using phospho-specific antibodies for VASP S157. The level of 14-3-3ζ was detected as the loading control. The blots shown are representative of three separate experiments. The relative phosphorylation levels of VASP pS157 are represented as the mean ± SEM (n=3). p values shown (**p<0.01, and ***p<0.001) are as analysed by one way-ANOVA followed by Bonferroni's correction for multiple comparisons.

5-3 Discussion

Unwarranted activation of platelets within the vasculature results in thrombosis which is the leading cause of deaths in the world. Therefore, improved therapeutic methods to control inappropriate platelet activation are particularly important in the prevention and treatment of CVD especially thrombotic diseases. The results presented in previous chapters have shown that 1,8-cineole has a significant inhibitory effect on platelet activation, thrombus formation and platelet-mediated inflammatory responses. Due to its origin from plants, reduced side effects and increased efficacy for various diseases, understanding the impact of this molecule to control platelet activation would be highly beneficial to enable its therapeutic use for CVD. Based on the impact of 1,8-cineole on platelet function, in this chapter, we investigated the mechanisms through which 1,8-cineole modulate platelet reactivity. Here, we analysed the effect of 1,8-cineole on key signalling mechanisms in platelets.

While platelet activation induced by GPCR agonists such as thrombin and ADP were only affected at a high concentration of 1,8-cineole (100 μM), it showed a strong inhibition on collagen/CRP-XL-induced platelet activation, emphasising its effect on GPVI signalling pathway in platelets. This selective effect of 1,8-cineole towards GPVI is similar to other plant-derived flavonoids such as tangeretin, present in citrus fruits, which showed antiplatelet activity mainly through GPVI pathway (Vaiyapuri, Ali et al. 2013). Therefore, we investigated the impact of 1,8-cineole on various signalling proteins that are associated with GPVI signalling pathway.

GPVI signalling involves a sequential activation of various signalling proteins such as Src, SYK, LAT and PI3K. To determine the impact of 1,8-cineole on GPVI signalling, the phosphorylation of selected signalling proteins was analysed in the presence and absence of this molecule. Our results demonstrate that 1,8-cineole

inhibits the phosphorylation of LAT, which plays a critical role in GPVI signalling pathway, although it did not significantly affect SYK phosphorylation. This suggests that 1,8-cineole might affect different components of GPVI signalling pathway. The effects of 1,8-cineole on tyrosine phosphorylation of SYK were similar to quercetin-3-O-sulphate and quercetin-3-glucuronide, which also failed to cause a significant reduction in phosphorylation of SYK upon collagen-stimulated activation in platelets while they affected other molecules (Wright, Moraes et al. 2010).

Given the significance of SFKs in the regulation of GPVI signalling, we then analysed the impact of 1,8-cineole on the phosphorylation of SFKs such as Src. SFKs are well known to initiate primary activation of platelets together with other signalling molecules. SFK phosphorylates several downstream enzymes and cytoskeletal proteins that together initiate platelet activation. Upon platelet activation, a proportion of Src is linked with the cytoskeleton, which allows it to phosphorylate various key substrates that regulate cytoskeleton remodelling (Kralisz and Cierniewski 2000). Src is also associated with integrin $\alpha_{IIb}\beta_3$-mediated signalling, mainly via the β3 subunit (Arias-Salgado, Lizano et al. 2003).

We demonstrate that 1,8-cineole is able to affect CRP-XL-induced Src dephosphorylation at the inhibitory site of pY529 in platelets. This inhibitory effect of 1,8-cineole on Src activity is similar to other plant compounds that exhibit antiplatelet activities and inhibit SFKs in stimulated platelets, such as quercetin, genistein and apigenin (Navarro-Núñez, Lozano et al. 2010, Mosawy, Jackson et al. 2013). Moreover, it has been reported that 1,8-cineole also inhibited COX-2 and PGE_2 generation due to the inhibitory effects on the phosphorylation of ERK1/2 as well as the phosphorylation of its upstream kinases Src in UV-induced skin carcinogenesis in

HaCaT cells (Lee, Ha et al. 2017). Hence, SFKs specifically Src might be one of the key targets for 1,8-cineole to modulate platelet function.

The GPVI signalling pathway culminates on the phosphorylation and activation of PI3K signalling which results in the hydrolysis of PIP2 and subsequent liberation of IP3 and DAG. In our study, we analysed the phosphorylation of AKT at Ser 473 as a marker for PI3K signalling to determine the impact of 1,8-cineole on this signalling in platelets. Indeed, 1,8-cineole was found to decrease the serine 473 phosphorylation of AKT upon stimulation with CRP-XL. A previous study found that 1,8-cineole decreased the phosphorylation of AKT in mice that exhibited hepatic lesions that are analogous to non-alcoholic steatohepatitis (NASH) (Murata, Ogawa et al. 2015). Moreover, other studies have indicated that 1,8-cineole inactivates AKT in human colon cancer cell lines (HCT11630) (Murata, Shiragami et al. 2013).

Furthermore, studies that investigated the anti-inflammatory activities of 1,8-cineole have also indicated that it interferes with signalling pathways such as NFκB pathway. For example, in human umbilical vein endothelial cells (HUVECs), it has been reported that 1,8-cineole mainly mediates its inhibitory effects via NFκB pathway, resulting in the suppression of LPS-induced pro-inflammatory cytokine release (Linghu, Lin et al. 2016). Similarly, 1,8-cineole has been reported to inhibit ERK1/2, JNK, p-38 MAPK and AKT. For example , in human monocyte THP-1 cells, 1,8-cineole inhibits LPS-stimulated expression of Egr1 via ERK1/2 pathway (Zhou, Wang et al. 2007). Lee and collaborators reported that 1,8-cineole inhibited the phosphorylation of p38 MAPK and AKT as well as cytokine production in a model of stimulated bronchial epithelial cells in mice with induced asthma (Lee, Park et al. 2016).

Studies have demonstrated that ERK1/2 and JNK1 are linked with increased bleeding time and clot retraction, as well as the suppression of integrin $\alpha_{IIb}\beta_3$ activation

(Adam, Kauskot et al. 2010, Lee, Kim et al. 2010). Interestingly, in our study, we observed that 1,8-cineole markedly inhibited CRP-XL-induced phosphorylation of both ERK1/2 and p38 MAPK. This modulation of ERK and p38 signalling is consistent with our functional results in chapter 3, where we observed that 1,8-cineole is able to inhibit platelet granule secretion and suppress integrin $\alpha_{IIb}\beta_3$ function, which might offer a mechanistic explanation of the antiplatelet activity of 1,8-cineole. PI3K/AKT are known as the most important mediators in platelets for integrin $\alpha_{IIb}\beta_3$ activation (Guidetti, Canobbio et al. 2015). The inhibition of AKT and ERK signalling adds further evidence of antiplatelet activity of 1,8-cineole through these mechanisms.

We hypothesised that the effect of 1,8-cineole may not be only restricted to signalling molecules within the GPVI pathway. Therefore, we investigated whether well-recognised endogenous platelet inhibition mechanisms (e.g. those activated by NO and PGI_2) could also be modulated by 1,8-cineole. Platelet shape change is attenuated mainly by cAMP-dependent PKA activation rather than cGMP-dependant PKG activation (Jensen, Selheim et al. 2004). Therefore, cAMP-induced inhibition of P-selectin exposure on the platelet surface is mostly mediated by PKA activity (Libersan, Rousseau et al. 2003). An increase in VASP phosphorylation is known as a marker for cAMP-mediated signalling (Wentworth, Pula et al. 2005), which is also linked with a decrease in integrin $\alpha_{IIb}\beta_3$ activation, leading to the inhibition of platelet aggregation (Horstrup, Jablonka et al. 1994). Furthermore, the phosphorylation of VASP downregulates actin nucleation, resulting in reduced cytoskeletal restructuring, a process which is important for platelet shape change (Aszódi, Pfeifer et al. 1999).

Our results demonstrate an increase in VASP phosphorylation in the presence of 1,8-cineole compared to the positive control. In line with this result, 1,8-cineole increased the level of intracellular cAMP in platelets, which was confirmed by

measuring the cAMP level using an ELISA. These results suggest that 1,8-cineole interacts with cAMP signalling pathway and results in inhibition of platelet activation.

Overall, these data indicate that 1,8-cineole inhibits various signalling pathways via targeting multiple molecules in platelets. In addition, this study demonstrates the potential of 1,8-cineole in the inhibition of platelet reactivity and highlights that monoterpenoids such as 1,8-cineole could act as promising molecular templates for the design and development of novel antithrombotic drugs.

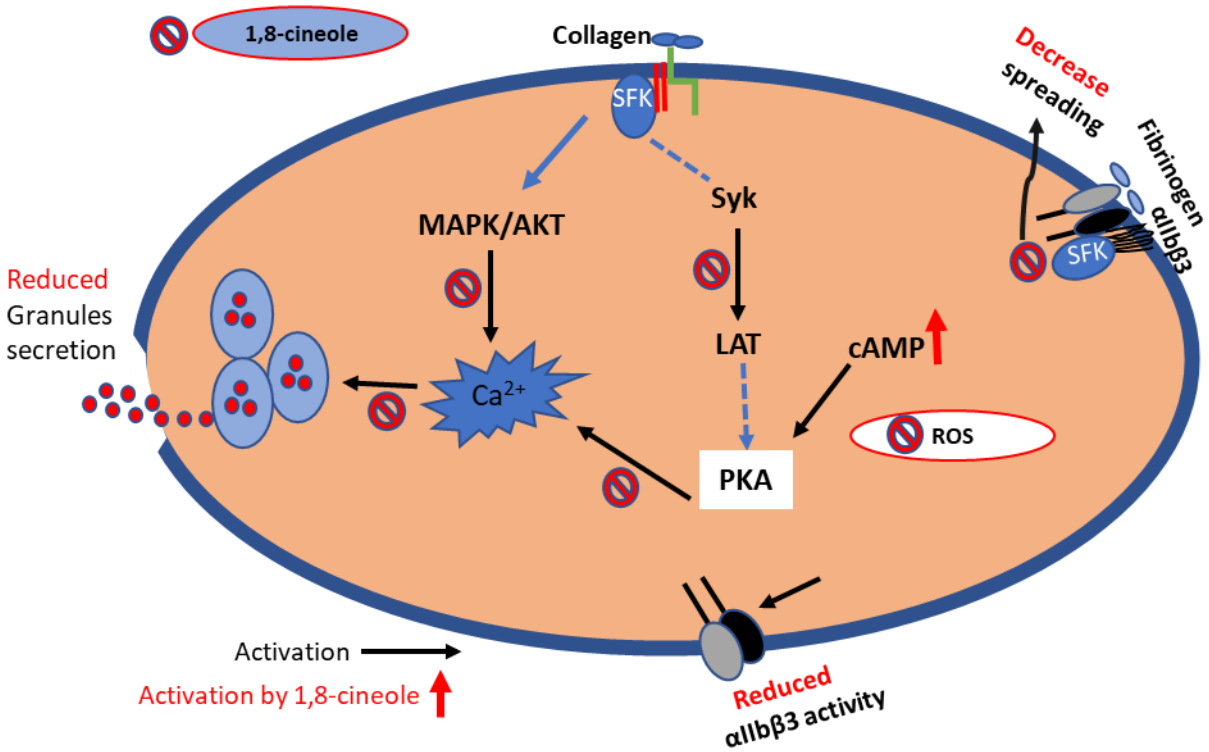

Figure 5-7: Schematic representation of the effect of 1,8-cineole on platelet signalling. Treatment of platelet with 1,8-cineole inhibit both early and late events of GPVI signalling pathway. 1,8-cineole reduced phosphorylation of Syk (Y323) and inhibit phosphorylation of LAT (Y200) and increase cAMP level, leading to a decreased in PKA phosphorylation. 1,8-cineole also decreased intracellular calcium concentration mediated by CRP-XL. ROS also inhibited by 1,8-cineole. Treatment of platelet with 1,8-cineole also inhibit the phosphorylation of SFKs downstream of GPVI (Src), MAPK, AKT and integrin αIIbβ3 receptors. The inhibition on these signalling proteins results in modulating platelet functions include aggregation, secretion and platelet spreading. The mechanism via which 1,8-cineole mediate inhibition of these signalling events still not yet clear.

GPVI: GP Glycoprotein VI, SFK: Src family kinases, Syk: spleen tyrosin kinase, LAT: Linker for activated T cells, PKA: protein kinase A, MAPK: Mitogen-activated protein kinases, AKT a serine–threonine kinase, Ca^{2+} : calcium.

Chapter-6

6- GENERAL DISCUSSION

In the circulatory system, platelets normally circulate as individual discoid bodies, although upon vascular injury, they show a marked ability to adhere to each other forming a thrombus via support of integrin αIIbβ3 and adhesion proteins, fibrinogen and von Willebrand factor. CVD, specifically thrombotic diseases such as stroke and heart attack, occur as a result of unwarranted activation of platelets. Therefore, platelets represent a powerful target for the treatment and prevention of thrombotic diseases. The current treatments for thrombotic diseases such as aspirin and clopidogrel are linked with serious side effects including prolonged bleeding. Therefore, there is a pressing priority to develop safer and more efficacious anti-platelet drugs in order to treat and prevent thrombotic diseases.

Over the last two decades, there has been a growing interest in the field of medicine plants to evaluate their therapeutic applications. A few studies have been assessing the biological values of monoterpenes. For example, the essential oils from the leaves of numerous plants have a wide range of activities including anti-inflammatory, anti-bacterial and anti-oxidant effects. Based on these effects, the essential oils are being used to treat common cold, influenza and respiratory diseases (Salari, Amine et al. 2006). The therapeutic benefits of eucalyptus oil were largely due to 1,8-cineole as it is the primary component present in this oil (Boukhatem, Amine et al. 2014). Recent studies have aimed to investigate the anti-inflammatory activities of 1,8-cineole in different cell lines and animal models. For example, under in vitro settings, the release of pro-inflammatory cytokines from monocytes and lymphocytes upon stimulation by lipopolysaccharide was significantly affected by 1,8-cineole (Lu, Lin et al. 2012). Similar to nucleated cells, platelets also release pro-inflammatory cytokines, when stimulated with agonists such as thrombin or collagen (Fuentes, Badimon et al. 2014). Despite a large number of studies reporting the impact of 1,8-

cineole on the modulation of leukocyte funcyion, the effects of 1,8-cineole on platelet reactivity, thrombus formation and haemostasis have not been established previously.

Due to effective anti-inflammatory properties, 1,8-cineole has been suggested to use as an alternative for currently used corticosteroid drugs that often have complex side effects especially in respiratory diseases. A number of clinical research reports demonstrate significant promise that 1,8-cineole can become a primary therapy or an adjunct treatment for currently used therapeutics (e.g. corticosteroids) in various disease conditions (Juergens, Racké et al. 2017). In this sense, and based on pervious observations, further evaluation of the anti-inflammatory and anti-platelet effects of 1,8-cineole may present this compound as an attractive approach for the development novel treatment strategies for inflammatory and infectious diseases, particularly those related to thrombo-inflammatory responses.

The hypothesis of this study was that 1,8-cineole inhibits platelet reactivity and thereby modulates thrombus formation, haemostasis and platelet-mediated inflammatory responses. To investigate this hypothesis, the effect of 1,8-cineole was investigated in human isolated platelets, PRP and whole blood using various platelet functional assays. Moreover, the plausible mechanisms that regulate the effects of 1,8-cineole in platelets were also determined.

6-1 1,8-cineole exerts modest anti-platelet effects

The results of this study demonstrate the inhibitory effects of 1,8-cineole on platelet activation induced by agonists that are selective for GPVI as evidenced by its effects on platelet aggregation in human isolated platelets and PRP. For example, the response of platelet aggregation to CRP-XL was significantly decreased in the presence of 1,8-cineole, indicating its impact on the GPVI signalling pathway. Furthermore, when platelets were stimulated by collagen, the inhibitory effects of 1,8-cineole were clearly shown by a reduction in aggregation, although it maintained similar levels of inhibition in PRP in the presence of plasma proteins. Furthermore, the findings presented in this study using flow cytometry-based assays show that 1,8-cineole exhibits dose-dependent effects on CRP-XL-induced fibrinogen binding at various concentrations (6.2μM-100μM). These results were consistent with the results of aggregation assays. This suggests that 1,8-cineole may have the ability to affect inside-out signalling via integrin $\alpha_{II}b\beta_3$.

Integrin $\alpha_{II}b\beta_3$-mediated outside-in signalling as measured by a clot retraction assay using PRP, and platelet adhesion and spreading on collagen, was also affected by 1,8-cineole. The effect of 1,8-cineole in an assay such as clot retraction that involves coagulation system may demonstrate the anti-coagulant actions of 1,8-cineole either directly or indirectly through platelets. 1,8-cineole may also inhibit signalling induced through integrin $\alpha_2\beta_1$ in addition to GPVI because collagen is able to activate platelets through both GPVI and integrin $\alpha_2\beta_1$.

1,8-cineole showed inhibitory effects on an agonist-induced platelet granule secretion. Granule secretions represent an important early event of positive feedback signalling that support platelet activation and subsequent thrombus formation.

Inhibition of secretion from dense and α-granules results in a decreased positive feedback leading to inhibit platelet activation. The combined effects of 1,8-cineole on inside-out signalling to integrin αIIbβ3 and outside-in signalling mediated by the same integrin may suggest the significance of 1,8-cineole on this molecule and its signalling. Consistent with in vitro data showing the inhibitory effects of 1,8-cineole on platelet aggregation, granule secretion and integrin αIIbβ3 activation, we also observed that a concentration of 6.25 μM of 1,8-cineole only causes a modest (but significant) tail bleeding *in vivo* in mice suggesting that a caution must be taken when this taken as a therapeutic agent although the lower concentrations may not have any impact.

During platelet activation, intracellular calcium mobilisation rapidly changes the cytosolic calcium levels which is required for integrin activation and other platelet functions (Sage and Rink 1986). 1,8-cineole inhibits CRP-XL-induced calcium mobilisation, which might have reflected on the inhibition of granule secretion, integrin activation and platelet aggregation. During platelet preparation, platelets were kept in suspension medium free of calcium, to avoid the influence of extracellular calcium in platelet activation during storage.

The data presented in chapter 5 confirmed that the inhibition of LAT and Syk phosphorylation by 1,8-cineole suggests that this may affect the early signalling events in GPVI signalling pathway. In addition, this may refer to an effect of 1,8-cineole on GPVI dimerization or clustering, which is a critical event in platelet activation through this receptor (Poulter, Pollitt et al. 2017), and this could be investigated in future work.

The effect of 1,8-cineole on in vitro thrombus formation under arterial flow conditions was investigated. 1,8-cineole causes inhibition of thrombus formation, consistent with other results obtained using various platelet functional assays. We found the inhibitory effects of 1,8-cineole on isolated platelets, PRP and whole blood

as well as in haemostasis in mice. However, in this study we were unable to determine the effect of 1,8-cineole under *in vivo* settings using a laser/FeCl3 injury model of thrombosis. This can be considered in future work. Together, these results presented in this study suggest that 1,8-cineole has a clear impact on the modulation of platelet functions.

6-2 1,8-cineole inhibits platelet-mediated inflammatory responses

It has been demonstrated that platelets contribute to inflammatory processes due to their origin from myeloid lineage and high number in the circulation. A potential role of ROS in platelet activation was established and many studies reported that agonists stimulating NOX activation in platelets play a critical role in ROS generation and subsequent platelet activation (Arthur, Qiao et al. 2012, Walsh, Berndt et al. 2014). Our findings indicate that CRP-XL-induced ROS generation in human platelets was significantly inhibited by 1,8-cineole. 1,8-cineole has not only diminished ROS generation but also inhibited granule secretion and aggregation, suggesting that pharmacological inhibitors targeting NOX2 might be an effective method to inhibit platelet activation. The antioxidative properties of 1,8-cineole were also previously demonstrated. Therefore, the role of 1,8-cineole in inhibiting ROS generation may suggest its impact on inflammation and oxidative stress, which ultimately affect thrombo-inflammatory conditions.

Several studies have demonstrated that the secretion of platelet pro-inflammatory mediators (e.g. RANTES, sP-selectin, TNF-α and IL-1β) plays a critical role in atherothrombosis (Aslam, Speck et al. 2006, Clark, Ma et al. 2007, Kuckleburg, Tiwari et al. 2008). Recent studies have aimed to investigate the anti-inflammatory

activities of 1,8-cineole in different cells including monocytes and lymphocytes. In this study, we assessed the ability of 1,8-cineole to modulate pro- and anti-inflammatory cytokines released from platelets.

The activity of RANTES promotes leukocyte migration and adhesion to the endothelial surface by binding to CD40 and CD40L. The release of RANTES was inhibited by 1,8-cineole suggesting its significance on this process. Moreover, 1,8-cineole has demonstrated a significant reduction in the release of other cytokines including sP-selectin, IL-1β, TNF-α and VEGF from platelets, supporting its impact on modulating platelet-mediated inflammation. The potential of 1,8-cineole to exert antioxidant and anti-inflammatory effects was also reported against cerulenin-induced severe pancreatitis, and found that pro-inflammatory cytokine production such as TNF-α, IL-1β, and IL-6 were increased by cerulenin, but decreased in the presence of 1,8-cineole (Lima, de Melo et al. 2013). Moreover, a number of studies that investigated the anti-inflammatory activity of 1,8-cineole have also indicated that it interferes with various signalling pathways. For example, in human umbilical vein endothelial cells (HUVECs), it has been reported that 1,8-cineole mainly mediates its inhibitory effects via the NFκB pathway, resulting in the suppression of pro-inflammatory cytokine release, which is induced by LPS (Linghu, Lin et al. 2016).

Several studies have shown an increase in platelet-leukocyte interactions during CVD (Totani and Evangelista 2010). Thrombosis and inflammation involve the platelet-leukocyte interactions, which reflects in atherothrombosis and other inflammatory diseases. In recent years, it has been suggested that platelet-leukocyte crosstalk contributes to the progression of CVD. Moreover, it has been observed in patients with ACS an increase of platelet-leukocyte aggregates circulating in the blood (Patel, Pfau et al. 2004).

Our findings demonstrate that 1,8-cineole significantly decreased the formation of platelet-leukocyte aggregation induced by TRAP. Thus, these data provide evidence of antithrombotic activity of 1,8-cineole and the potential for inhibition of platelet and leukocyte adhesion by reducing the formation of platelet-leukocyte interactions and cytokines release from platelets. Overall, these results demonstrate that 1,8-cineole is capable of decreasing platelet-mediated inflammatory responses.

6-3 The molecular mechanism of anti-platelet effect of 1,8-cineole

The data gathered in this study show the inhibitory effects of 1,8-cineole in the modulation of platelet function both *in vivo* and *in vitro*. Therefore, the determination of molecular mechanisms that regulate the effects of 1,8-cineole in platelets was necessary. A previous study reported that 1,8-cineole mainly mediates its effects through the NFκB signalling pathway in nucleated cells. This suggested that 1,8-cineole increases the level of IκB, which in turn decreases the activation of NFκB leading to an impact on NFκB signalling. Moreover, 1,8-cineole has been reported to control the nuclear translocation of NFκB p65 complex by stimulation of IκB leading to a limitation of the activity of NFκB (Xiao 2004). In addition, Kegang Linghu et al. (2016) demonstrated that 1,8-cineole has inhibitory effects on the NFκB pathway, therefore resulting in the suppression of pro-inflammatory cytokine release induced by LPS in human umbilical vein endothelial cells (HUVECs).

Our result demonstrated an inhibitory effect of 1,8-cineole on the signalling related to the GPVI signalling pathway. 1,8-cineole inhibits LAT phosphorylation which is involved in GPVI-mediated platelet activation upon stimulation with CRP-XL. The effect of 1,8-cineole may not only be restricted to kinases within the GPVI signalling pathway; therefore, we investigated whether a well-recognised endogenous platelet inhibition mechanism (e.g. those activated by PGI2) could also be modulated by 1,8-

cineoel. Platelet shape changes can be attenuated mainly by cAMP-dependent (PKA) activation rather than cGMP-dependant (PKG) activation (Smolenski 2012). Therefore, activation of cAMP-induced inhibition of P-selectin expression on the platelet surface is mostly mediated by PKA activity (Libersan, Rousseau et al. 2003).

Our results indicate that 1,8-cineole causing an increase in VASP phosphorylation at Ser157 (a marker for cAMP-mediated signalling) (Butt, Abel et al. 1994). Furthermore, the level of intracellular cAMP was also increased in the presence of 1,8-cineole. This effect of 1,8-cineole on the level of cAMP is consistent with our results that demonstrate an inhibitory activity of 1,8-cineole in platelet function. Upon platelet activation, a proportion of Src family kinases that are linked with the cytoskeleton allow the phosphorylation of various key substrates that regulate cytoskeleton remodelling (Senis, Mazharian et al. 2014). Src family kinases are also associated with integrin αIIbβ3, mainly with a β3 subunit (Su, Mi et al. 2008). Src family kinases are well established as the most abundant kinases in human platelets, and they are critical for initiating signals from various receptors including integrin αIIbβ3 (Senis, Mazharian et al. 2014).

Here, we demonstrated that 1,8-cineole was found to affect CRP-XL-induced Src dephosphorylation at the inhibitory site of Src (Y529). Moreover, it has been reported that 1,8-cineole also inhibited phosphorylation of ERK1/2 and the phosphorylation of c-Src (Y416), which is an upstream kinase for ERK1/2 in UVB-induced skin carcinogenesis in HaCaT cells (Lee, Ha et al. 2017). The phosphorylation of ERK1/2 was also inhibited by 1,8-cineole.

The GPVI signalling pathway is dependent on the upstream phosphorylation of PI3K. In our study, we used AKT (Ser 473) as a marker for PI3K signalling. 1,8-cineole was found to decrease the serine phosphorylation of CRP-XL-induced AKT activation.

Previous studies have indicated that 1,8-cineole inactivates AKT in human colon cancer cell lines (HCT11630) (Murata, Shiragami et al. 2013).

Moreover, in human monocytes, 1,8-cineole inhibits LPS-stimulated expression of Egr1 via the ERK1/2 pathway in human monocytes THP-1 cell lines (ZHOU, WANG et al. 2007). Lee and collaborators investigated the anti-inflammatory effects of 1,8-cineole in a model of stimulated bronchial epithelial cells in mice with induced asthma (Lee, Park et al. 2016). They found that 1,8-cineole inhibited the phosphorylation of p38 MAPK and AKT as well as cytokine production.

Studies have demonstrated that ERK1/2 and JNK1 are linked with increased bleeding time and clot retraction as well as the suppression of integrin αIIbβ3 activation (Adam, Kauskot et al. 2010, Lee, Kim et al. 2010). Interestingly, in our study, we observed that 1,8-cineole markedly inhibited CRP-XL-induced phosphorylation of both ERK1/2 and p38 MAPK. This modulation of ERK1/2 and p38 signalling is consistence with our results in Chapter 3, where we observed that 1,8-cineole is able to inhibit platelet granule secretion and the suppression of integrin αIIbβ3, which might offer a mechanism of antiplatelet activity of 1,8-cineole.

Overall, these data indicate that 1,8-cineole inhibits various signalling pathways. In addition, this study demonstrates the potential of 1,8-cineole in the inhibition of platelet function and highlights that monoterpenoids, such as 1,8-cineole, could be promising molecular templates for the design and development of novel antithrombotic drugs. Due to various reasons, we could not perform some of the planned *in vivo* experiments in this study. For example, the impact of 1,8-cineole on laser-induced thrombosis in mice was not evaluated. Moreover, the effect of 1,8-cineole on platelet function upon oral administration was also determined. Given the availability of clinical grade 1,8-cineole as an approved drug for respiratory diseases,

it will be really beneficial if the impact of 1,8-cineole on the modulation of thromboinflammation is further evaluated in future studies. As a plant-derived compound, it has limited side effects but increased bioavailability, and therefore this could be a powerful but safer anti-platelet drug in order to prevent and treat thromboinflammatory conditions under diverse settings.

6-4 Recommendation from this study

The increase in CVD events requires development of new, safer drugs that would be effective in the treatment of thrombosis and free from adverse side effects. It is well known that oxidative stress and excessive ROS production leading to several diseases, including thrombotic conditions (Jang, Wang et al. 2015). It has been suggested that, regulating ROS production and oxidative stress play an important role in reducing the risk of an appropriate platelet activation, which can leads to thrombosis (Violi and Pignatelli 2012). Many plant compounds such as flavonoids and monoterpenes have great therapeutic values for various diseases. Therefore, numerous natural compounds that showed activity to reduce the level of oxidative stress may be ideal sources for new drugs in the treatment of thrombotic conditions (Phang, Lazarus et al. 2011). 1,8 cineole has anti-inflammatory and antioxidant effects in clinical practice. 1,8 cineole has been shown to exhibit numerous beneficial effects in different pathological conditions due to it is anti-inflammatory and antioxidant effects and has been described as having positive effects on human health (Worth and Dethlefsen 2012).

The findings of this study provide evidence for 1,8-cineole's effects on platelet function under *in vitro* and *in vivo* conditions and have described the possible mechanisms through which 1,8-cineole inhibits platelet function. In addition, ROS production in platelets was also inhibited by 1,8-cineole suggests an important role for this molecule as an antioxidant to modulate platelet functions. This pharmacological effects of 1,8-cineole on platelet ROS production may represent this molecule as a future target of antiplatelet drugs.

From clinical perspective and based on our result of the effect of 1,8-cineole on extending bleeding time in mice, more extensive measurements of 1,8-cineole are required to evaluate the appropriate concentration to achieve anti-thrombotic effect with no excessive bleeding. Also, there are several questions remaining regarding to the pharmacological effects of 1,8-cineole in case of co-administration with antiplatelet drugs such aspirin, as the bleeding one of the major side effects linked with aspirin treatment. Therefore, monitoring of 1,8-cineole intake more attention should be paid, to avoid excessive bleeding. The routine doses of aspirin (usually 75-150mg daily dose) need to be examining in the presence of oral administration of 1,8-cineole, and the lowering doses of aspirin need to be investigated further to guarantee no risk of bleeding can occur. This also give an insight into decrease aspirin dose with co-administration of 1,8-cineole to reduce bleeding complications that linked with long use of aspirin. However, the interaction of 1,8-cineole and anti-platelet drugs such as aspirin has not been investigated.

Future work should also investigate the active plasma concentration upon oral 1,8-cineole administration, to identify the *in vivo* concentration that required to inhibit thrombus formation. In this study a concentration of 12.5 μM of 1,8-cineole was able to inhibit thrombus formation. Having established an inhibitory effect, thus, future work could also focus on required minimal oral dose of 1,8-cineole that could inhibit thrombus formation. In human clinical study, 1,8-cineole was given to patients at dose of 200 mg, as an adjunct treatment with prednisolone to treat bronchial asthma. 1,8-cineole showed positive clinical effect in airway inflammation, even when prednisolone dosage was decreased by 36% (Worth, Schacher et al. 2009). In a further study investigating the effect of 1,8-cineole in patients with COPD, administration of 200 mg 1,8-cineole have been shown to decrease the severity of breathing, coughing, and

dyspnea when compared to the placebo group (Kehrl, Sonnemann et al. 2004). Many Studies *in vivo* and *in vitro* have focused mainly on inflammatory condition for respiratory treatment, which various clinical trials show significant potential that 1,8-cineole may become a primary choose, or an adjunct treatment for current therapeutic such as corticosteroid. In addition, 1,8-cineole was found to reduce the systolic blood pressure in rats, when oxidative stress was induced by chronic exposure to nicotine, suggesting that this antihypertensive effect of 1,8-cineole may be related to the regulation of NO and oxidative stress in rats (Moon, Kang et al. 2014).Moreover, it has been suggested that using 1,8-cineole is a potentially effective neuroprotective candidate to treat patient with ischemic stroke, by exerts neuroprotection through its anti-oxidative properties (Ryu, Park et al. 2014).

Taken together, our results in this study and previous studies that shows the main mechanisms of 1,8-cineole are due to antioxidant and anti-inflammation effects, which support our hypothesis that 1,8-cineole could be potential compound in CVD therapeutic, especially thrombo-inflammatory disease. As the main mechanism for the treatment CVD are mainly antioxidant and anti-inflammation. Based on these, 1,8-cineole needs greater study mainly in a chronic inflammatory condition, including atherosclerotic cardiovascular disease.

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