FIBRINOLYTIC AND HAEMOSTATIC ACTIVITIES OF CAFFEIC ACID PHENETHYL ESTER AND PROPOLIS FROM MALAYSIAN STINGLESS BEE AND ROMANIAN POPLAR *IN VITRO*

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by

ABUZAR MAHMOUD ABDELRAHMAN ELNAGER

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DEDICATION

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

{ يُؤْتِي الْحِكْمَةَ مَنْ يَشَاءُ وَمَنْ يُؤْتَ الْحِكْمَةَ فَقَدْ أُوتِيَ خَيْرًا كَثِيرًا وَمَا يَذَّكَّرُ إِلَّا أُولُو الْأَلْبَابِ }

{He gives wisdom to whom He wills, and whoever has been given wisdom has certainly been given much good. And none will remember except those of understanding}

صدق الله العظيم

سورة البقرة/ الأية (269)

To the memory of my late father, who was the main inspiration to the success I obtained in my life.

Who gave me his endless love,

Who gave me his patience and support,

Who enlightened my way to achieve this work,

and

To millions of people globally

Suffering from thrombotic diseases

إن شاء الله there is a treatment and cure

I introduce my work with respect.

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Abuzar Mahmoud Abdelrahman Elnager

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AA	arachidonic acid
ACD	acid citrate dextrose
ADP	adenosine diphosphate
Amu	atomic mass unit
A.mellifera	*Apis mellifera*
APTT	activated partial thromboplastin
AST	aspartate aminotransferase
BC	before Christ
CAPE	caffeic acid pheneythyl ester
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
CD	cluster of differentiation
cGMP	cyclic guanosine monophosphate
CGK	Cheonggukjang kinase 3–5
CLT	clot lysis time
CLSI	Clinical and Laboratory Standards Institute
dl	decilitre
DD	D-Dimer
DMSO	dimethyl sulphoxide
Di-TMS	derivatized with trimethylsilyl
DTT	dithiothreitol
EDTA	Ethylene diaminetetra acetic acid
ECL	enhanced chemiluminescence
ED	effective dose

EE	ethanol extract
ELT	euglobulin lysis time
eV	electron volt
FDP	fibrin degradation products
FITC	Fluorescein isothiocyanate
FSC	forward scatter
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
gm	gram
GP	glycoprotein
HDL-cholesterol	high-density lipoprotein cholesterol
HeNe	helium-neon
HIV	human immunodeficiency virus
Hrs	hours
HPA-1a	human platelet antigen antibodies
^{125}I-FDPs	^{125}I-fibrinogen degradation
IgG	Immunoglobulin G
INR	international normalization ratio
IU	International unit
K time	clot kinetics
LD	lethal dose
LDH	lactate dehydrogenase
LY30	clot lysis at 30 minutes
M	molar
mA	milliampere

MA	maximum amplitude
MARDI	Malaysian Agricultural Research and Development Institute
min	minute
MC-540	merocyanine 540 fluorescent
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
MS	mass spectrometry
MW	molecular weight
NaCI	sodium cloride
NaHPO	sodium hydrogen phosphate
$NaHCO_3$	sodium bicarbonate
Na_2HPO_4	disodium hydrogen phosphate
NCCLS	National Committee for Clinical Laboratory Standards
nm	nanomolar
PA	plasminogen activators
PAC-1	procaspase-activating compound 1
PAI-1	plasminogen activator inhibitor type-1
PBS	phosphate-buffered saline
PRP	platelet rich plasma
PerCP	peridinin chlorophyll
PKC	protein kinase C
Poplar propolis	Romanian *Apis mellifera* propolis

PPP	platelet poor plasma
PAR	protease-activated receptor
PL	phosphoipid
PT	prothrombin time
PVDF	polyvinylidene fluoride
PE	phycoerythrin
R	reaction time
RBCs	red blood cells
RIPA	radioimmuno-precipitation assay
Risto	ristocetin
RGDS	Arg-Gly-Asp-Ser
Rpm	revolutions per minute
RT	retention time
rt-PA	recombinant tissue plasminogen activators
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SK	streptokinase
SSC	side scatter
Sp	species
TA2	thromboxane A2
TF	tissue factor
T.itama	*Tetratrigona itama*
TEG	thromboelastography
TMA	time to maximum amplitude
t-PA	tissue-plasminogen activator

TBS	tris-buffered saline
u-PA	urokinase-type plasminogen activator
V	volt
v/v	volume/volume
VASP	vasodilator-stimulated phosphoprotein
VTE	venous thromboembolism
VWF	von Willebrand factor
WB	whole blood
W.blot	Western blot
WBCs	white blood cells
w/v	weight/volume

LIST OF SYMBOLS

α	alpha
Δm	fractional clot mass loss
g	gravity
Ω	ohm
µg	microgram
µM	micromolar

AKTIVITI- AKTIVITI FIBRINOLISIS DAN HEMOSTATIK ASID KAFEIK ESTER FENETIL (CAPE) DAN PROPOLIS DARI LEBAH KELULUT MALAYSIA DAN POPLAR ROMANIA *IN VITRO*

ABSTRAK

Ester fenetil asid kafeik (CAPE) diambil daripada propolis dan tumbuh-tumbuhan. Tujuan kajian ini termasuk: (1) membangunkan dan validasi prosedur lisis bekuan darah utuh (DU) *in vitro* untuk kajian-kajian fibrinolitik, (2) Kajian aktiviti fibrinolitik, antiplatlet dan antikoagulasi CAPE, *Tetratrigona itama* (*T.itama*) Malaysia dan propolis poplar dan (3) Mengenalpasti dan menentukan kandungan CAPE propolis *T.itama* Malaysia.

Prosedur lisis bekuan DU dibangunkan menggunakan kaedah bekuan DU yang mengecut dan dieram dalam takungan platlet yang kekurangan plasma (PKP) mengikut pelbagai masa pengeraman. Prosedur ini berjaya divalidasi menggunakan streptokinase (SK). Aktiviti fibrinolik dinilai menggunakan D-Dimer (DD), morfologi fibrin menggunakan mikroskopi konfokal dan berat bekuan DU. DD diukur secara fotometrik melalui kaedah imun-turbidometrik. Aktiviti fibrinolitik CAPE, *T.itama* Malaysia dan propolis poplar dinilai menggunakan prosedur lisis bekuan DU pada kepekatan yang berlainan. Kajian aktiviti platlet CAPE dijalankan dengan esei *in vitro* yang berbeza termasuklah: 1) pengukuran aggregasi platlet oleh agregometri menggunakan pelbagai agonis termasuk adenosine difosfat (ADP), asid arakidonik (AA) dan ristosetin.

2) Pengukuran penanda pengaktifan platlet (PAC-1 dan P-selectin) oleh sitometri aliran. 3) Penentuan reseptor P2Y12 oleh teknik blot Barat. Kajian platlet *T. Itama* Malaysia dan propolis poplar dibuat dengan agregometri platlet. Parameter TEG direkodkan selepas eraman (inkubasi) CAPE, *T.itama* Malaysia dan propolis poplar dalam DU. Pengukuran kandungan CAPE dalam propolis dilakukan oleh analisis GC-MS menggunakan lekukan kalibrasi yang dibina di atas kawasan puncak berlawanan dengan kepekatan CAPE yang berbeza.

Untuk kajian aktiviti fibrinolitik, terdapat perbezaan purata tahap DD (ug/ml) berbeza secara signifikan ($p< 0.05$) merentasi sampel yang dieram dengan CAPE dalam pelbagai kepekatan. Juga terdapat perbezaan DD untuk kedua-dua jenis propolis dibandingkan dengan kawalan normal (PKP). Jisim median sebelum dan selepas inkubasi bekuan DU (gm) CAPE menurun, begitu juga untuk *T.itama* Malaysia dan propolis poplar. Morfologi fibrin dibuat secara mikroskopik dan menunjukkan kesan kebergantungan dos CAPE. Lima puluh peratus keberkesanan dos (ED50) CAPE (menggunakan DD) adalah 1.99 mg/ml.

Terdapat kesan anti-platlet dengan teknik yang digunakan dalam kaedah ini. CAPE, *T.itama* Malaysia dan propolis poplar ED50 (berdasarkan agregasi platlet) adalah masing-masing 7.31 µg/ml, 0.79 mg/ml dan 0.86 mg/ml. Keputusan TEG menunjukkan parameter fibrinolitik (LY30) berbeza ($p<0.17$) daripada kawalan normal sampel yang dieram dalam kepekatan CAPE *T.itama* Malaysia dan propolis poplar yang berbeza. Kesan anti-platlet CAPE mungkin menyebabkan pengurangan nilai MA pada esei TEG. Kuantiti CAPE yang diukur pada *T.itama* Malaysia dan propolis poplar adalah : 0.6 (0.1) dan 29.7 (1.2) mg/g.

Kajian ini mencadangkan CAPE, *T.itama* Malaysia dan propolis poplar mempunyai aktiviti fibrinolitik dan anti-platlet melalui kaedah *in vitro*. Walaubagaimanapun tiada kesan antikoagulasi seperti yang dikesan oleh esei TEG. Kajian seterusnya untuk CAPE dan propolis sebagai potensi ejen alternative hemostatik perlu diadakan pada masa hadapan.

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ABSTRACT

Caffeic acid phenethyl ester (CAPE) is a phenolic derivative from propolis and plants. The aims of this study include: (1) Development and validation of an *in vitro* whole blood (WB) clot lysis procedure for fibrinolytic activity study, (2) Investigation of fibrinolytic, antiplatelet and anticoagulant properties of CAPE, Malaysian *Tetratrigona itama* (*T.itama)* and poplar propolis and (3) Determination and quantification of CAPE compound in Malaysian *T.itama* propolis.

The WB clot lysis procedure was developed using a standardized unresected retracted WB clot incubated in pooled platelet poor plasma (PPP) for varying incubation times and successfully validated using streptokinase (SK). The fibrinolyic activity was assessed by D-Dimer (DD), fibrin morphology by confocal microscopy and WB clot weight. DD was measured photometrically by immuno-turbidometric method. Fibrinolytic activity of CAPE, Malaysian *T.itama* and poplar propolis was assessed by the new WB clot lysis procedure at different concentrations and different times. Platelet activity study of CAPE was performed using different *in vitro* assays including: 1) Platelet aggregation measurement by platelet aggregometry with different types of agonists, adenosine diphosphate (ADP), arachidonic acid (AA) and ristocetin. 2) Platelet activation markers (PAC-1 and P-selectin) expression by flow cytometry. 3) P2Y12 receptor determination by Western blot (W.blot) technique. The

platelet study of Malaysian *T.itama* and poplar propolis was done by platelet aggregometry. Thromboelastgraphy (TEG) parameters were recorded following WB incubation with CAPE, Malaysian *T.itama* and poplar propolis. Quantitation of CAPE in propolis was performed by Gas Chromatograph-Mass Spectrometer (GC-MS) analysis using constructed calibration curves on peak area versus various concentrations of CAPE.

The mean differences of DD (µg/ml) levels were significantly different ($p<0.05$) across samples incubated with different CAPE concentrations and both types of propolis compared with normal control (PPP). The median pre and post-incubation WB clot weights (gm) were significantly decreased for CAPE, Malaysian *T.itama* and poplar propolis. Fibrin removal was observed microscopically and indicated dose-dependent effects of CAPE compared with that of normal control at different time. The 50% effective dose (ED_{50}) of CAPE (based on DD) was 1.99 mg/ml.

The anti-platelet effect was observed by the techniques used in this study for CAPE and both propolis. The ED50 of CAPE, Malaysian *T.itama* and poplar propolis (based on platelet aggregation) was 7.31 µg/ml, 0.79 mg/ml and 0.86 mg/ml respectively. TEG results showed fibrinolytic parameter (LY30), was significantly different ($p<0.17$) from normal control samples incubated in different concentrations of CAPE, Malaysian *T.*itama and poplar propolis. The antiplatelet effect of CAPE may have contributed to the reduced maximum amplitude (MA) value of TEG assay. The quantity of CAPE measured in Malaysian *T.itama* and poplar propolis were: 0.6 (0.1) and 29.7 (1.2) mg/g respectively.

This study suggests that CAPE, Malaysian *T.itama* and poplar propolis possess fibrinolytic and antiplatelet activity by *in vitro* methods. However there was no anticoagulant effect as detected by TEG assay. Further investigation of CAPE and propolis as a potential alternative haemostatic agent should be conducted in future.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 The Haemostatic system

The term 'haemostasis' refers to the normal response of a blood vessel to injury by forming a clot that serves to limit haemorrhage (Rasche, 2001). Haemostasis is a complex and efficient system that depends upon a complicated interplay between plasma coagulation and fibrinolytic factors, blood cells, vessel walls, extracellular matrix, as well as haemorheological properties, such as blood viscosity and blood flow (Takada *et al.*, 1994b). The haemostatic system comprises of four compartments: the blood vessels, platelets, coagulation factors, and the fibrinolytic system (Chee, 2014). Normal haemostasis is divided into primary and secondary haemostasis.

1.1.1 Primary haemostasis

Primary haemostasis occurs when a blood vessel is damaged. The blood is exposed to collagen fibers in the basement membrane of the vessel (Figure 1.1). Platelets adhere to collagen and become activated. Activated platelets release adenosine diphosphate (ADP) and thromboxane which cause the aggregation of more platelets to the site of injury. This process of platelet aggregation at the site of injury is called primary haemostasis which results in the formation of a platelet plug which attempts to stop the flow of blood from the broken vessel. Furthermore, healthy vessels

secrete an enzyme called prostacyclin that functions in inhibiting platelet activation and aggregation through a number of platelet activation pathways that contribute to platelet adhesion, aggregation and activation (Hoffman *et al.*, 2005; Jennings, 2009).

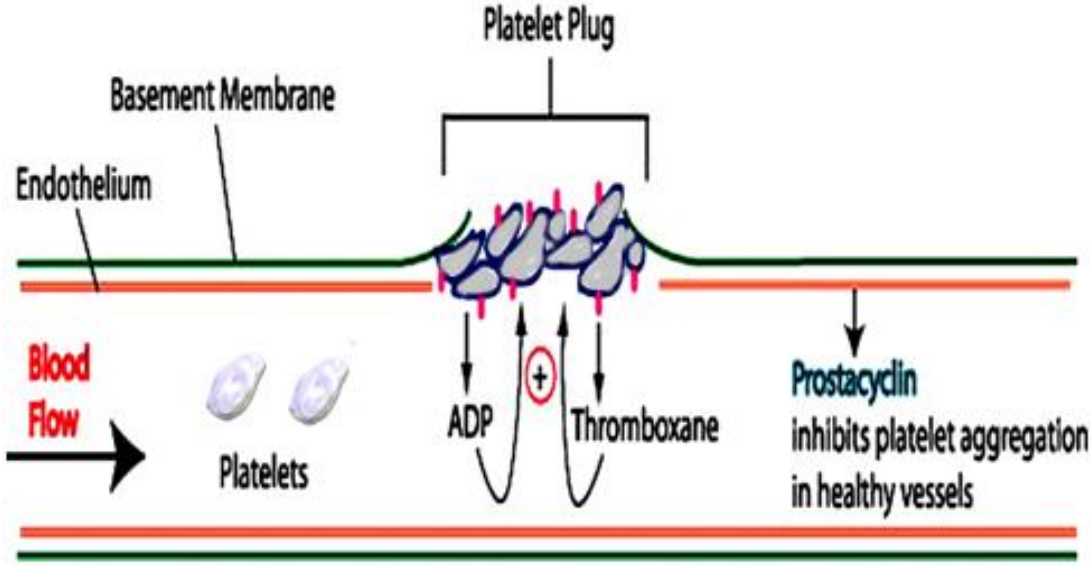

Figure 1.1 Formation of platelet plug

ADP: adenosine diphosphate.
[Modiefied from (http://www.biosbcc.net/doohan/sample/htm/Hemostasis.htm)].

1.1.1.1 Platelet activation pathways

Platelet activation occurs through multiple pathways which include collagen, ADP, thromboxane A2, epinephrine, serotonin and thrombin (Figure 1.2 and Table 1.1) (Scarano *et al.*, 1997; Offermanns, 2006; Davì and Patrono, 2007; Varga-Szabo *et al.*, 2008; Rivera *et al.*, 2009). ADP is stored in platelets dense granules and released from adherent platelets during platelet activation. It participates in platelet activation occurring both during primary haemostatic plug (monolayer of platelet) and during formation of occlusive platelet-rich thrombus. ADP also enhances platelet procoagulant activity through contact with P2Y1 and P2Y12 receptors.

Thromboxane A2 is released from adherent platelets and enhances recruitment and aggregation for primary plug formation and activates platelets during both protective haemostasis and pathologic thrombus formation (Rivera *et al*., 2009).

Collagen is a strong thrombogenic substrate. Under high-shear conditions, platelet adhesion is mediated by the binding of Von Willebrand factor (vWF) located on collagen or on the surface of activated platelets to GPIb. Subsequently, this interaction leads to activation of GPIIb/IIIa and to stabilize vWF-mediated platelet aggregates (Varga-Szabo *et al.*, 2008). However, the binding between GPIb and vWF is inadequate for stable adhesion and GPVI is the main platelet-collagen receptor that mediates platelet activation, which is necessary for adhesion, aggregation, degranulation and coagulant activity on the matrix (Varga-Szabo *et al.*, 2008).

Thrombin is the most effective platelet activator of platelets at very low concentrations, lower than those required for activating of the coagulation cascade (Brummel *et al.*, 2002; Mann *et al.*, 2003). Thrombin binds the protease-activated receptor (PAR)-1 on the platelet surface, leading to cleavage of the receptor, and exposing a tethered ligand, which binds and activates the receptor (Vu *et al.*, 1991). Platelets also express PAR-4, which requires higher concentrations of thrombin for activation (Han *et al.*, 2011). Activated platelets promote the catalysis of two sequential reactions of the blood coagulation cascade: the activation of factor X into factor Xa by a complex of factor IXa, factor VIlla, and Ca^{2+} and the conversion of prothrombin into thrombin by a complex of factor Xa, factor Va, and Ca^{2+} (Rosing *et al*., 1985).

Antiplatelet agents, including aspirin, thienopyridines and platelet glycoprotein GPIIb/IIIa receptor inhibitors, have been developed for the treatment and prevention of cardiovascular diseases. However, these agents have several clinical disadvantages including gastrointestinal side-effects and haemorrhagic events (Gum *et al.*, 2003; Rezkalla and Benz, 2003).

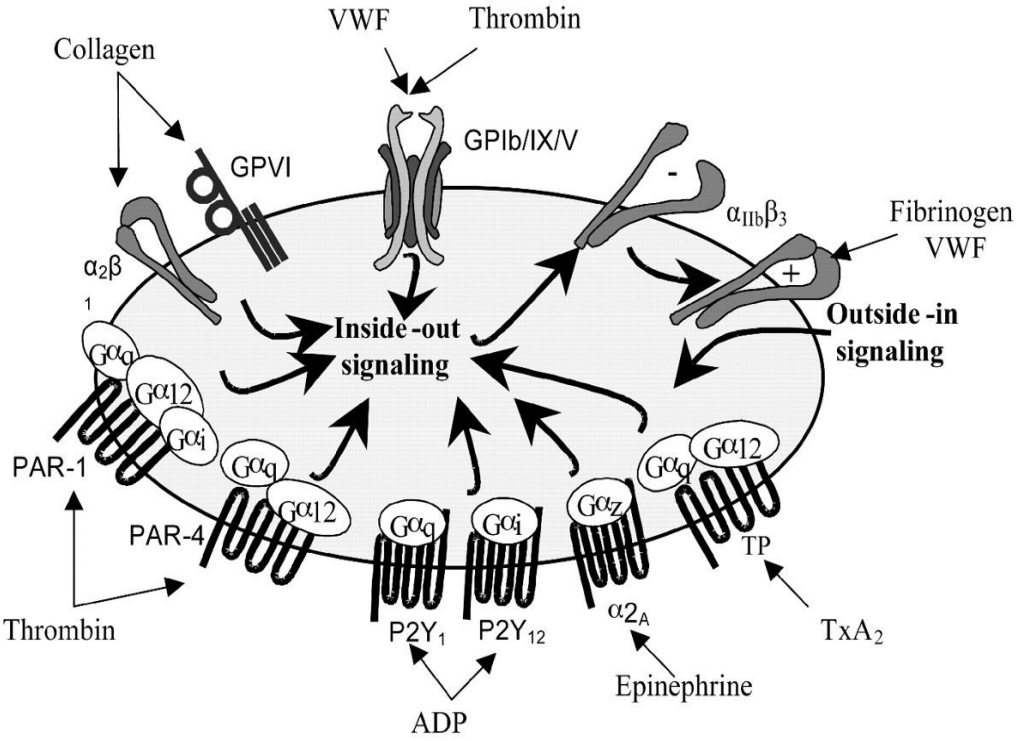

Figure 1.2 Major platelet receptor-ligand interactions

ADP: adenosine diphosphate; **GP:** glycoprotein; **PAR:** protease-activated receptor; **TxA_2:** Thromboxane A2; **vWF:** von Willebrand factor, [adapted from (Rivera *et al.*, 2009)].

Table 1.1 Agonists - enhanced platelet activation and their effects on platelets

Platelet activator	Receptor(s)	Effect on platelets
ADP	P2Y1 P2Y12	- Platelet shape change (P2Y1) - Transient aggregation (P2Y1) - Sustained irreversible aggregation (P2Y12). - Expression of P-selectin (P2Y12) - Release of thromboxane A2 (P2Y1 and P2Y12) - Platelet recruitment to sites of injury (P2Y12) - Induction of procoagulant activity and aggregation (P2Y12)
Thromboxane A2	TPα TPβ	- Platelet recruitment and aggregation to a primary platelet plug (TPα)
Serotonin	5HT-2A	- Platelet recruitment to sites of injury - Induction of procoagulant activity via retention of fibrinogen and thrombo spondin on platelet surface
Epinephrine	α2a	- Supplementary role overlapping P2Y12 receptor signaling
Collagen	GPIb (high shear via vWF) GPIIb/IIIa (high shear via vWF) GPIa/IIa (low shear) GPVI (low shear)	- Activation of GPIIb/IIIa - Release of ADP and thromboxane A2 - Platelet spreading - Platelet aggregation - Induction of procoagulant activity via release of Ca^{2+}
Thrombin	PAR- 1 PAR-4	- Platelet aggregation (PAR-1) - Release of ADP, thromboxane A2 (PAR-4), serotonin (PAR-1) and epinephrine (PAR-1) - Activation/mobilisation of P-selectin and CD40 ligand (PAR-1) - Induction of platelet procoagulant activity (PAR-1)

Receptors primarily responsible for activation in platelets are indicated in bold. **ADP**: adenosine diphosphate; **GP**: glycoprotein; **PAR**: protease-activated receptor; **vWF**: von Willebrand factor, [modified from (Jennings, 2009)].

1.1.2 Secondry haemostasis

Secondary haemostasis comprises three phases of coagulation which are the initiation phase (exposure of tissue factor to coagulation factors), amplification phase (conversion from extrinsic to intrinsic thrombin generation) and propagation phase (thrombin generation with fibrin deposition) (Figure 1.3). Secondary haemostasis usually starts when a tissue factor (TF) is exposed to blood following endothelial damage (Key *et al.*, 2007; Wintrobe and Greer, 2009). Initially, TF forms a complex with coagulation factor VII which in turn activates factor IX and factor X. After that activated factor X (FXa) forms the prothrombinase complex together with activated factor V (FVa) on a phospholipid membrane surface, which is provided by activated platelets and microparticles that convert prothrombin into thrombin.

Thrombin can increase its own generation through activating factor XI (FXI) to factor XIa (FXIa) which further activates factor IX (FIX) (Butenas and Mann, 2002). Factor IX together with activated factor VIII enhances the formation of factor X. Thrombin cleaves fibrinogen into fibrin monomers that polymerize and the resultant network is stabilized by factor XIIIa-catalyzed cross-linking. The site of vascular injury is occluded by a firm fibrin network along with activated platelets. Following that, the clot retracts and this retraction may improve the mechanical stability of the clots. The speed and degree of clot retraction are proportional to the number of platelets (Hoffman *et al.*, 2005). Table 1.2 shows mean plasma factors concentrations and properties of coagulation proteins and inhibitors.

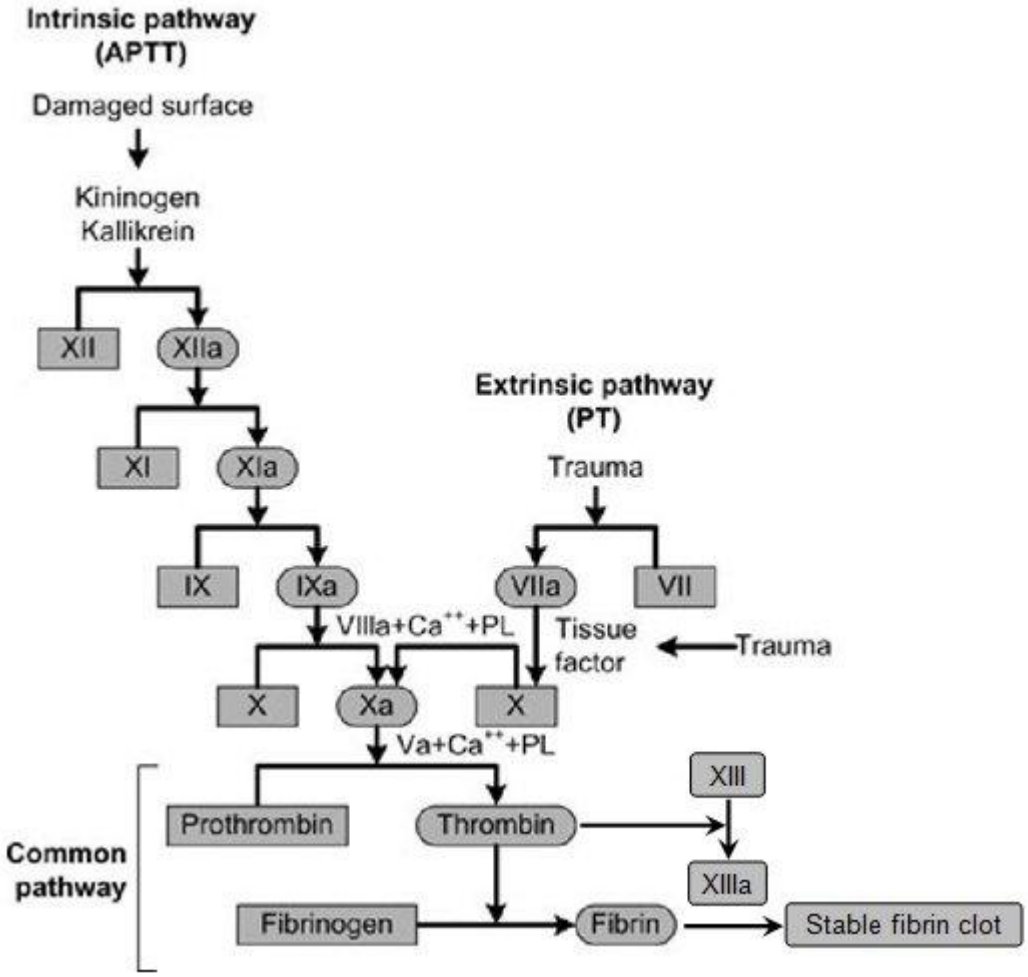

Figure 1.3 The coagulation cascade. The intrinsic, extrinsic and common pathway of coagulation

APTT: activated partial thromboplastin; **Ca^{++}:** calcium**; PL**: phosphoipid; **PT**: prothrombin time, [modified from (Adams and Bird, 2009)].

Table 1.2 Mean plasma concentrations and properties of coagulation proteins and inhibitors

Protein	Mean plasma concentration		Source	Function
	nM	µg/ml		
Prothrombin	1400	100	plasma	zymogen
Factor X	170	10	plasma	zymogen
Factor IX	90	5.1	plasma	zymogen
Factor XI	30	4.8	plasma	zymogen
Factor XIII	90	30	plasma	zymogen
Factor VII	10	0.5	plasma	zymogen
Protein C	60	3.7	plasma	zymogen
Factor V	20	6.6	plasma	procofactor
Factor VIII	0.7	0.2	plasma	procofactor
Factor VIIa	0.1	0.005	endothelium	enzyme
Tissue factor	NA	NA	endothelium	cofactor
Thrombomodulin	NA	NA	plasma	cofactor
Antithrombin III	3400	200	plasma	inhibitor
Protein S	300	21	plasma	inhibitor/cofactor
TFPI	2.5	0.1	plasma	inhibitor
Fibrinogen	7600	2600	plasma	precursor

Note: **NA:** not applicable (membrane protein).
TFPI: tissue factor pathway inhibitor, [modified from (Butenas and Mann, 2002)].

1.1.3 The Fibrinolytic system

Fibrinolysis is an essential process for the degradation of the solid-phase fibrin network formed by activation due to haemostasic mechanisms. The fibrin clot is formed either in the situation of appropriate activation in response to vascular trauma, or in pathological thrombosis (arterial or venous) and atherosclerosis (Romanic *et al.*, 1998). The essential mediator of fibrinolysis is plasmin, which cleaves fibrin at specific lysine and arginine residues, leading to the production of fibrin degradation products such as X, Y, D, and E (Ittyerah *et al.*, 1979; Wintrobe and Greer, 2009). Fibrinolysis is initiated by either one of the two plasminogen activators (PA),

urokinase-type plasminogen activator (u-PA) or tissue-plasminogen activator (t-PA). Figure 1.4 shows fibrin formation and fibrinolysis.

D-dimer (DD) is a clinically useful marker for exclusion of venous thromboembolism (VTE) and evaluating the risk of its recurrence in selected patients' populations. It is formed by the action of three enzymes which are thrombin, factor XIIIa and plasmin. Firstly, the fibrinogen is cleaved by thrombin to produce fibrin monomers. Following that, thrombin activates factor XIII to the active transglutaminase, factor XIIIa which leads to fibrin polymerization. Finally plasmin degrades the crosslinked fibrin to release fibrin degradation products (FDP) and expose the DD antigen (Weisel, 2005; Adam *et al.*, 2009). DD is normally not detectable, or is detectable in very low levels in human blood or plasma, unless the coagulation system has been activated (Adam *et al*., 2009). DD has a positive predictive value and is helpful in imaging investigation triage in acute venous syndromes (Ranasinghe and Bonser, 2010).

This fibrinolytic process is mainly controlled by two homologous proteins i.e. plasminogen activator inhibitor type-1 (PAI-1), which rapidly complexes with tissue t-PA, and α2-antiplasmin, which acts as a specific and rapid inhibitor of plasmin (Abdullah *et al.*, 2009). Another natural inhibitor thrombin activated fibrinolysis inhibitor (TAFI) is a non-serpin fibrinolysis inhibitor which is activated by thrombomodulin-associated thrombin (Broze and Higuchi, 1996; Mosnier *et al.*, 2001; Chapin and Hajjar, 2014).

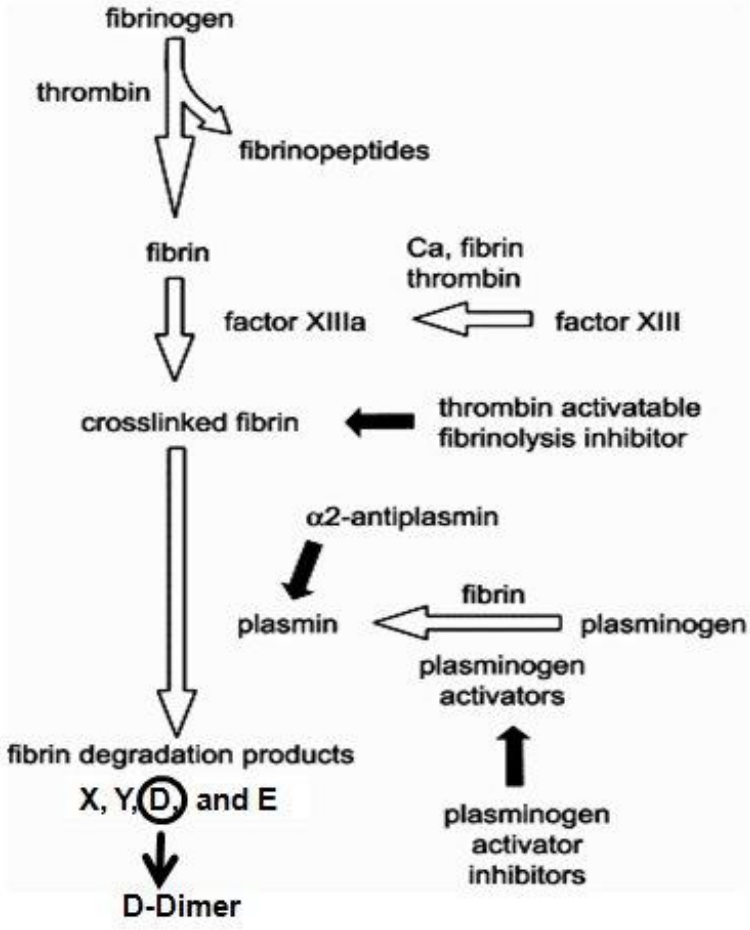

Figure 1.4 Basic scheme of fibrin clot polymerization and fibrinolysis

[Modified from (Weisel, 2005)]

The disturbances in hemostatic balance may result in inappropriate clot formation inside the blood vessels (thrombosis), or defect in the coagulation activity which may lead to bleeding (Takada *et al.*, 1994b; Wolberg *et al.*, 2012). Fibrin and platelets form the basic structure of a thrombus/blood clot, and red cells can be entrapped in the fibrin clot (Furie and Furie, 2008; Mackman, 2012). The fibrin clot will be lysed by the fibrinolytic process and this will allow the vascular lumen patency (Hoylaerts *et al.*, 1982). In acute myocardial infarction, the time for antithrombotic therapy or fibrinolytic agent administration is very critical to restore the organ function. The

fibrinolytic therapy appears to be beneficial for at least 12 hrs following the onset of symptoms. However, fibrinolytic therapy for stroke has proven beneficial only when used within 3 hrs (Mackman, 2008).

In order to detect the effect of thrombolytic drugs, certain products of fibrinolytic activity can be measured, such as DD (Wells *et al.*, 2003a; van Belle *et al.*, 2006), plasmin-antiplasmin complex (Bayes-Genis *et al.*, 1999; Lindholt *et al.*, 2001), fibrin monomers (Horan and Francis, 2001), fibrin polymerization and clot morphology (Parise *et al.*, 1993), t-PA and PAI-1 (Boudjeltia et al., 2002) and thromboelastographic (TEG) parameters e.g. LY30. (Zmuda and Neofotistos, 2000; Coppell *et al.*, 2006; Marschner *et al.*, 2010; Thakur and Ahmed, 2012). Figure 1.5 demonstrates the degradation of cross-linked fibrin specifically into DD derivatives.

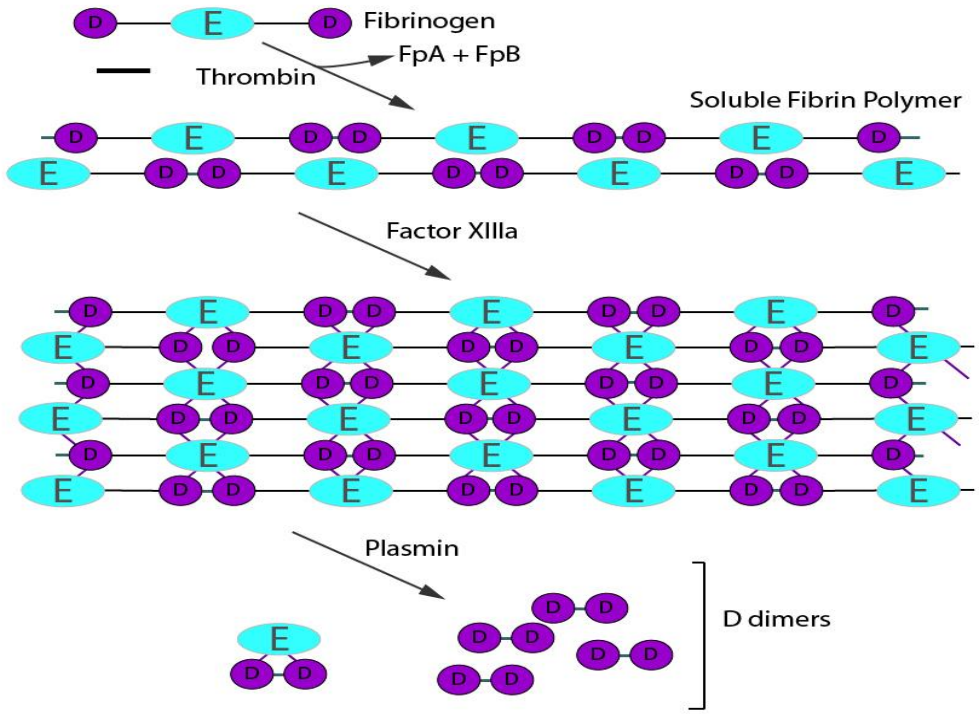

Figure 1.5 Degradation of cross-linked fibrin

Digestion/degradation of cross-linked fibrin by plasmin enzyme gives rise to a variety of fragments including D dimmers, [modified from (http://www.practical haemostasis.com/Fibrinolysis/d_dimers. html)].

1.1.3.1 Methods used for assessment of fibrinolysis

Different methods have been used to measure the clot lysis activity of thrombolytic drugs. Most of these methods had been tested on artificial clots and used ultrasound methods, complicated mathematical or computing skills to measure the thrombolytic activity of thrombolytic drugs. These methods are costly, and hence not suitable for routine clinical practice (Prasad *et al.*, 2006b).

Alternatively, study was conducted using the diluted blood clot lysis time, which represented the interaction between the activity of t-PA and PAI-1 to allow the measurement of both baseline and stimulated fibrin dissolution. This test is

inexpensive and adapted to a single test system. However, it has disadvantages since it has a very long subjective procedure and requires continuous monitoring by an observer so as to record the clot lysis activity (Urano *et al*., 1990).

The euglobulin lysis time (ELT) is an old method which was used to measure the overall fibrinolysis by mixing the citrated platelet-poor plasma with acid. Certain clotting factors are precipited in the form of complexes which is called the euglobulin fraction. This fraction contains fibrinogen, PAI-1, t-PA, plasminogen, $alpha_2$-antiplasmin and factor VIII which are measured by spectrophotmetry (Kowalski et al., 1959). It was reported that the ELT is used in ranking the streptokinase activity of five commercial clinical preparations. However, major drawbacks of this method include laborious and time consuming procedure for the preparation of the euglobulin fraction in addition to instability of the materials used (Couto *et al*., 2004).

Moreover, an *in vitro* perfusion model was designed to mimic arterial flow conditions using mechanical means. A less complete dissolution of a thrombus in conjunction with a greater amount of embolic debris was achieved with this approach, though the size of these embolic particles was reduced by the addition of a thrombolytic agent (Greenberg *et al.*, 2000). Another study has proposed an *in vitro* clot lytic model for assessment of fibrinolytic activity (Prasad *et al.*, 2006b). As this model is rather simple, it has a significant limitation because the fibrinolytic activity is calculated from clot weight. Many factors were not controlled in the preparation of whole blood (WB) clot, which may give false results.

Another method utilised ultrasound to enhance thrombolysis. In these studies clot mass loss (Δm) was used as an indicator for thrombolysis. It was found that 120 kHz ultrasound is effective in enhancing the thrombolytic efficacy of rt-PA at a temperature lower than 37^{o}C (Shaw *et al.*, 2006b). Similarly, another study used fractional clot mass loss (Δm) arising from *in vitro* human clot model exposed to tPA or plasminogen and hypothermia. It also concluded that the temperature changes contributed to the improved lytic efficacy of ultrasound enhanced thrombolysis (Shaw *et al.*, 2007b). In addition, haemoglobin was used as a fibrinolytic marker to detect the fibrinolytic effect of Lonomia achelous caterpillar venom at different concentrations *in vitro* using human WB. The venom and its active fractions degrade the clots at a slower rate than the enzymes urokinase plasminogen activator (uPA), t-PA and plasmin. It is minimally affected by physiological plasma protease inhibitors (Coll-Sangrona and Arocha-Piñango, 1998).

Several *in vitro* studies of thrombolysis using WB clots enriched with^{125}I-fibrinogen (^{125}I-Fg) suspended in phosphate-buffered saline (PBS) or serum containing the fibrinolytic inducers have been performed. The rate of release of ^{125}I-fibrinogen degradation products (^{125}I-FDPs) in the supernatant was taken as a measure of the plasmin-dependent degradation of the clot (Carroll *et al.*, 1981; Sabovic *et al.*, 1989; Kunitada *et al.*, 1992; Parise *et al.*, 1993). A comparison between two studies using different methods for fibrinolytic assessment (haemoglobin relase and ^{125}I-Fg) revealed that the haemoglobin release method gave higher values of clot lysis. Moreover, the release curves were similar to those obtained using the radioactive method and showed good correlation when using the same plasminogen activator (Lopez *et al.*, 2000).

1.2 Natural products and application in health

For thousands of years, natural products have played a very important role in health care and prevention of diseases. The ancient civilizations of the Chinese, Indians and North Africans provide written evidences for the use of natural resources for curing various diseases (Phillipson, 2001). The use of natural products in treatment of various diseases is increasing due to their broader pharmacological activities with less adverse effects. Bee products including honey, bees wax, propolis, pollen, bee bread, royal jelly, venom and larvae are reported to have various pharmacological activities (Krell, 1996). The medical benefit of honey and other bee products was mentioned in Verse 69, Surah An-Nahl in Holy Quraan as "Then eat of all the fruits and walk in the ways of your Lord submissively. There comes forth from within it a beverage of many colours, in which there is healing for men; most surely there is a sign in this for a people who reflect". Phenolic compounds in honey act as natural antioxidants and are popular because of their potential roles in human health (Khalil and Sulaiman, 2010).

1.2.1 Bee products

Bee products can be classified into two large groups: the products of animal and plant origin. Products of plant origin are: honey, bee pollen/breads, propolis, gathered by a bee from flowers and stored in the beehive. Products of animal origin are: royal jelly, beeswax and bee venom, produced by the bee in the hives. All these ingredients are essential to bees, without which bees and the entire bee-hives could not sustain (Owayss *et al.*, 2004).

Honey was used since ancient times in traditional medicine. It has many biological functions such as antibacterial, antioxidant, antitumor, anti-inflamatory, antibrowning, and antiviral effects. It is composed of more than 5000 substances and is basically a solution supersaturated in sugars, the fructose (38%) and glucose (31%) are the most abundant (Gheldof *et al*., 2002). The moisture content is about 24.30 to 27%, total acidity 3.1 to 4.1 and ashes constitute 0.06 to 0.08% (Viuda-Martos *et al.*, 2010). The composition of honey varies, depending on different factors such as pollen source, climate, environmental conditions, and the method used for processing (Gheldof *et al.*, 2002; Gómez-Caravaca *et al.*, 2006; Viuda-Martos *et al.*, 2008).

Royal jelly was demonstrated to have various functional properties such as antibacterial activity, anti-inflammatory activity, vasodilative and hypotensive activities, disinfectant action, antioxidant activity, antihypercholesterolemic activity, and antitumor activity. It is the exclusive food of the queen honeybee and the larvae. Royal jelly contains water (50% to 60%), proteins (18%), carbohydrates (15%), lipids (3% to 6%), mineral (1.5%) and vitamins together with a large number of bioactive substances (Nagai and Inoue, 2004; Viuda-Martos *et al.*, 2008).

Propolis is one of the most important bee products and of interest to many, particulary in this study. Propolis has a higher content of bioactive chemical compounds when compared to honey as it contains less sugar and water. Honey is more palatable compared to propolis, however propolis has higher potential to be processed as a nutraceutical agent by adding to honey or to be developed into pharmaceutical agent (De Castro, 2001).

1.2.1.1 Propolis

The word propolis was probably coined by Aristotle from the Greek words “pro” meaning “in front of” and “polis” meaning “city”. The combined meaning then becomes “In front of the City” or “Defender of the City (or Beehive)” and this is how bees use propolis (Haile and Dekebo, 2013). It is a gum that is gathered by bees from various plants which varies in color from light yellow to dark brown, and may cause staining of the comb or frame and can found in extracted honey (Burdock, 1998a).

Due to the popularity of using propolis in medicine and other domains, thousands of studies were developed from all over the world, studying the chemical composition, functions, or different properties of propolis extracts. More than 2700 articles and 250 books were published in Elsevier journals or publishing house (http://www.elsevier.com/), more than 2000 articles in Medline (http://www.ncbi.nih.gov/), and more than 1500 studies in Wiley-Blackwell database (http://www.onlinelibrary.wiley.com/) (Marghitas *et al.*, 2013). Most of the current literature concerning propolis is focused on determining the chemical composition and biological activity of propolis, besides determining the botanical and geographical origins by analyzing comparatively the resins from which propolis is derived (Miguel and Antunes, 2011).

1.2.1.1.1 Physical properties of propolis

Propolis colour and aroma differ according to geographical zones. It is a resin being yellowish-green, dark green, or brown in colour depending on its plant source and

age with a pleasant flavor of poplar buds, honey, wax and vanilla but it can also have a bitter taste. It is hard and brittle when cold, but becomes soft and very sticky when warm (Naama et al., 2010; Wagh and Borkar, 2012).

1.2.1.1.2 Chemical composition of propolis

Propolis was found to contain many polyphenolic compounds, flavones, flavonones, phenolic acid and esters. It contains about 55 % resins and balsams, 30 % waxes, 10 % etheric oils and 5% pollen. The components are rich in vitamins and mineral elements (Nikolaev, 1978; Khalil, 2006; Haile and Dekebo, 2013). The materials necessary for bees to produce of propolis are substances actively secreted by plants as well as substances exuded from wounds in plants such as lipophilic substances on leaves and leaf buds, resins, mucilages, gums, lattices, etc. The composition of a plant source determines the chemical composition of bee glue (Crane, 1990). The most important biologically active chemical constituents of propolis from different geographic areas and the corresponding plant sources are shown in Table 1.3 (Bankova, 2005b).

Table 1.3 Propolis types according to their plant origin and their active chemical composition

Propolis type	Geographic origin	Plant source	Main biologically active substances	References
Poplar propolis	Europe, North America, non-tropic regions of Asia	Populous spp. of section Aigeiros, most often P. nigra L	Flavones, flavanones, cinnamic acids and their esters	(Nagy *et al.*, 1986), (Greenaway *et al.*, 1990), (Bankova *et al.*, 2000),
Birch propolis	Russia	Betula verrucosa Ehrh.	Flavones and flavonols (not the same as in poplar propolis)	(Popravko and Sokolov, 1980)
Green (alecrim) propolis	Brazil	Baccharis spp., predominantly B. dracunculifolia DC.	Prenylated p-coumaric acids, diterpenic acids	(Marcucci and Bankova, 1999)
Red (Clusia) propolis	Cuba, Venezuela	Clusia spp.	Polyprenylated benzophenones	(Cuesta-Rubio *et al.*, 2002), (Trusheva *et al.*, 2004)
"Pacific" propolis	Pacific region (Okinawa, Taiwan)	Unknown	C-prenylflavanones	(Chen *et al.*, 2012), (Kumazawa *et al.*, 2004)
"Canarian" propolis	Canary Islands	Unknown	Furofuran lignans	(Christov *et al.*, 1999)

[Modified from (Bankova, 2005a)].

1.2.1.1.3 Clinical implications and biological properties of propolis

The use of propolis has been reported in 300 BC for health improvement and disease prevention and also reported for its broader spectrum of pharmacological activities (Toreti et al., 2013). The broader biological and pharmacological properties of propolis have attracted researchers' interest as an alternative medicine for various pathological conditions. The Indian stingless bee propolis has been reported to have a complex nature with 24 chemical compounds (Choudhari *et al.*, 2012). Data from the same study also revealed an antimicrobial activity. The crude methanol extract of Thai *Apis mellifera* propolis showed antibacterial activity for *Staphylococcus aureus* and *Escherichia coli* (Boonsai *et al.*, 2014). The Ethiopian crude ethanol extracted Haramaya propolis (EEHP) exhibited antifungal activities against *Fusarium* sp., *Aspergillus* niger and *Colletotrichum* sp. (Haile and Dekebo, 2013).

The chemical and biological properties of polar extracts of cerumen or propolis from the Australian stingless bee *Tetragonula carbonariain* were investigated using gas chromatography-mass spectrometry (GC-MS) analyses and *in vitro* 5-lipoxygenase (5-LOX) cell-free assays. *In vitro* inhibition of 5-Lox activity by this extract was documented (Massaro *et al*., 2011). Propolis-mediated apoptosis of cancer cells has also been previously reported (Szliszka et al., 2009; Szliszka *et al.*, 2011; Szliszka and Krol, 2013) and the ethanol extract of propolis is reported to have cytotoxic effect (Russo *et al.*, 2002; Markiewicz-Zukowska *et al.*, 2013) and anticancer properties (Naama *et al.*, 2010).

Propolis has been reported to have less toxic and fewer adverse effects than caffeic acid phenethyl ester (CAPE). Also it has a low order of acute oral toxicity in mice with minimum lethal dose (LD_{50}) ranging from 2000 to 7300 mg/kg (Burdock, 1998b). The safe concentration for humans was found to be 1.4 mg/kg, or about 70 mg/day. Another study investigated the treatment of rats with different concentrations of propolis from different extracts such as water or ethanol and different times of administration. Insignificant alterations in triglycerides, total lipids, high-density lipoprotein cholesterol (HDL-cholesterol) concentrations, aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) specific activities, were observed (Mani *et al.*, 2006). Additionally, the use of propolis as an additive in food and beverages is rapidly increasing in Japan and also in Western Countries (Nagaoka *et al.*, 2003; Markiewicz-Żukowska *et al.*, 2012). The same studies have reported that propolis improves health and prevents inflammation and diseases such as heart disease, diabetes and cancer.

Furthermore, propolis was used broadly in cosmetics (Wagh and Borkar, 2012). It was reported as apitherapeutic agent which can generate appropriate biochemical environment supporting reepithelization (Olczyk *et al.*, 2013b). Moreover, it accelerates chondroitin/dermatan sulfates structure modification which is responsible for binding growth factors that play a crucial role in tissue repair (Olczyk *et al.*, 2013a). Various research studies suggested that propolis can be used in medicine (Table1.4).

Table 1.4 Recent studies on application of propolis in medicine

Geographic origin of propolis	Activity attributed	Test performed	Reference
Brazil (southern)	Anti-HIV activity	*In vitro* (H9 Lymphocytes)	(Ito *et al.*, 2001)
Brazil	Anticancer activity	*In vivo*-mice (pulmonary tumors)	(Kimoto *et al.*, 2001)
Brazil	Anticancer activity	*In vitro* (human tumor cell lines)	(Akao *et al.*, 2003)
Brazil (group 3 and group 12)	Suppression of dioxin	*In vitro*	(Park *et al.*, 2004)
Chile	Antioxidant and anticancer	*In vitro* (KB cells-human mouth epidermoid carcinoma cells; Caco-2 cells-human colon adenocarcinoma cells)	(Russo *et al.*, 2004)
Brazil	Anticancer activity	*I n vitro* and *in vivo* (retinal damage)	(Inokuchi *et al.*, 2006)
Brazil (group 3, group 12, and bud resins of botanical origin)	Anticancer activity	*In vitro* (human prostate epithelial cells	(Li *et al.*, 2007)
Brazil	Antiinfluenza virus activity	*In vivo* -mice (influenza virus	(Shimizu *et al.*, 2008)
Jordanian	Antibacteria	*In vitro*	(Darwish *et al.*, 2013)
Tunisia	Anticancer activity	*In vitro* (cancer cell lines-HT29, A549, Hep-2, raw264.7, and Vero)	(Kouidhi *et al.*, 2010)
Brazil (group 12 and artepellin C)	Immunosuppressant	*In vitro* (CD4 T cell)	(Cheung *et al.*, 2011)
Portugal	Anticancer activity	*In vitro* (human renal cancer)	(Valente *et al.*, 2011)
Israel (Kibbutz Yad Mordecai and CAPE)	Anticancer activity	*In vitro* (human T-cell lines)	(Shvarzbeyn and Huleihel, 2011)

Table 1.4. Continued

Brazil	Anticancer activity	*In vitro* (human breast cancer MCF-7 cells)	(Kamiya *et al.*, 2012)
Brazil (group 12 and group 13)	Anticancer activity	*In vitro* (human cell lins of leukemia)	(Franchi *et al.*, 2011)
CAPE (derived from honeybee hive propolis)	Anticancer activity	*In vitro* (human prostate cancer cells)	(Chuu *et al.*, 2012)
Brazil	Anticancer activity	*In vivo* mice (skin carcinogenesis)	(Yasukawa *et al.*, 2012)
Poland	Anticancer activity	*In vitro* (U87MG human glioblastoma)	(Markiewicz-Żukowska *et al.*, 2013)

[Modified from (Toreti *et al.*, 2013)

1.2.1.1.4 Types of propolis

Stingless bees diverged since the Cretaceous times, have 50 times more species than *Apis* and are both distinctive and diverse (David, 2006). Various types of propolis are present and depend on geographical and climate zones. Among them are propolis derived from Malaysian *Tetratrigona (T.itam)* which is found in tropical regions and contains different types of plant species. Poplar *Apis mellifera (A.mellifera)* propolis is found in Europe, North America, and the non-tropical regions of Asia, for which the plant source is *Populu*s sp. of section Aigeiros, most often *P. nigra* (Marinescu and Tamas, 1980).

1.2.1.1.4.1 Malaysian *Tetratrigona itama* propolis

Propolis is a resinous mixture that is collected from tree buds, sap flows, or other botanical sources (Choudhari *et al.*, 2012). Malaysian *T.itama* belongs to the stingless bees group, which are of eusocial insects. It has five different genera, namely *Melipona, Tetrarigona, Meliponula, Dectylurina* and *Lestrimelitta.* They play an important role in pollination (Heard, 1999). *Trigona* is the largest genus of stingless bees found exclusively in the Neotropics, from Mexico to Argentina and in the Indo Australian region from India, Sri Lanka to Taiwan, the Solomon Islands, South Indonesia, New Guinea and Australia (Michener, 2000). Previous studies reported five species of *Trigona* in India (Gupta, 2003) and more than 23 species reported in Malaysia by Malaysian Agricultural Research and Development Institute (MARDI).