Screening Of Natural Products For Their Effect On Kallikrein-Kinin System: Potential Implications In The Treatment Of Hereditary Angioedema

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SCREENING OF NATURAL PRODUCTS FOR THEIR EFFECT ON KALLIKREIN-KININ SYSTEM: POTENTIAL IMPLICATIONS IN THE TREATMENT OF HEREDITARY ANGIOEDEMA

A Dissertation presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of BioMolecular Sciences Division of Pharmacology The University of Mississippi

By
Hassan Abdu Madkhali
August 2015
ABSTRACT

Plasma prekallikrein (PK) activation exerts both physiological and pathological effects within the cardiovascular system and central nervous system (CNS). Activated PK (kallikrein) controls cytokine release from human mononuclear cells and controls both the intrinsic blood coagulation and the alternative complement cascades. The uncontrolled activation of these cascades results in the development of pathological pain and angioedema. Increased plasma kallikrein levels are associated with hereditary angioedema (HAE) and myocardial infarction, whereby kallikrein amplifies the generation of activated factor XII (FXIIa) and bradykinin (BK) release from high molecular weight kininogen (HMWK). The primary objective of this study was to screen natural products with anti-inflammatory properties for their effects on the components namely HMWK, PK, FXIIa and FXIa. *Terminalia chebula* fruit, *Terminalia bellirica* fruit, *Terminalia arjuna* fruit, *Terminalia brownii* bark, *Terminalia arjuna* bark and black tea (*Camellia sinensis*) extracts inhibited plasma kallikrein with IC\textsubscript{50} values of 30, 65, 220, 240, 280 and 220 mg/ml, respectively. *T. chebula* fruit, *T. arjuna* fruit, *T. bellirica* fruit, *T. brownii* bark, *T. arjuna* bark and black tea extracts blocked plasma kallikrein on endothelial cells with IC\textsubscript{50} values of 15, 20, 20, 80, 160 and 400 mg/ml, respectively. *T. chebula* fruit, *T. bellirica* fruit, *T. arjuna* fruit, *T. arjuna* bark and black tea extracts blocked FXIIa activity with IC\textsubscript{50} values of 30, 50, 190, 190 and 27 mg/ml, respectively. Arjunoglucosed II and arjunic acid blocked FXIIa activity with IC\textsubscript{50} values of 370 and 780 μM, respectively. *T. chebula* fruit, *T. arjuna* fruit and black tea at 50 mg/ml did not affect endothelial cells viability.
While red yeast rice (RYR) extract showed no inhibitory effects on plasma kallikrein, FXIIa, FXIa and rPRCP, three pure compounds blocked FXIa activity with IC$_{50}$ values of 260, 270 and 400 μM, respectively. Overall, T. chebula fruit, T. arjuna fruit and black tea extracts might be useful to improve symptoms of HAE, inflammation and thrombosis. The identified inhibitors of FXIIa and FXIa could be used as a lead compound to find potent inhibitors of these coagulation factors.
# LIST OF ABBREVIATION AND SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>Stimulation</td>
</tr>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees in Celsius</td>
</tr>
<tr>
<td>🔴</td>
<td>Inhibition</td>
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## A

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AT III</td>
<td>Antithrombin III</td>
</tr>
<tr>
<td>α2AP</td>
<td>Alpha 2-anti-plasmin</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
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## B

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>B1R</td>
<td>Bradykinin B1 receptor</td>
</tr>
<tr>
<td>B2R</td>
<td>Bradykinin B2 receptor</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
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## C

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>C1-INH</td>
<td>C1 inhibitor</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CTI</td>
<td>Corn trypsin inhibitor</td>
</tr>
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## D

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>DFP</td>
<td>Diisopropylfluorophosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
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## E

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>EC</td>
<td>Epicatechin</td>
</tr>
<tr>
<td>EGC</td>
<td>Epigallocatechin</td>
</tr>
<tr>
<td>ECG</td>
<td>Epicatechin gallate</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin gallate</td>
</tr>
</tbody>
</table>
F
FXI  Factor 11 (XI)
FXIa Activated factor 11 (XI)
FXII Factor 12 (XII)
FXIIa Activated factor 12 (XII)

G

gC1qR Complement C1q receptor
GPCR G-protein-coupled receptor

H
HAE Hereditary angioedema
HMWK High molecular weight kininogen
HKa Cleaved HK
HPAEC Human pulmonary artery endothelial cells
hCMEC Human cerebral microvascular endothelial cells
HDL High-density lipoprotein
HSP90 Heat Shock Protein-90

I
IL-1β Interleukin-1 beta
IL-6 Interleukin-6

K
KKS Kallikrein-kinin system
KLKB1 Gene encoding plasma kallikrein
kDa Kilodalton

L
LMWK Low molecular weight kininogen
LDL Low-density lipoprotein
LPS Lipopolysaccharide

M
MMP Matrix metalloproteinase
a2M  Alpha2-macroglobulin
MAPK  Mitogen-activated protein kinase

N
NO  Nitric oxide
NOS  Nitric oxide synthase
NSAID  Non-steroidal anti-inflammatory drug

O
OD  Optical density
OGD-R  Oxygen-glucose deprivation followed by reoxygenation

P
PAR  Protease activated receptor
PBS  Phosphate buffered saline
PC12  Rat pheochromocytoma cells
PGI2  Prostaglandin I2/Prostacyclin
PK  Prekallikrein
PRCP  Prolylcarboxypeptidase

R
rPRCP  Recombinant prolylcarboxypeptidase
rC1-inh  Recombinant C1-inhibitor
RYR  Red yeast rice

S
SBTI  Soybean trypsin inhibitor

T
TF  Tissue factor
TG  Triglyceride
TNF-α  Tumor necrosis factor-alpha

U  US FDA United States Food and Drug Administration
ACKNOWLEDGEMENTS

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TABLE OF CONTENT

ABSTRACT.................................................................................. ii
LIST OF ABBREVIATIONS............................................................ iv
ACKNOWLEDGEMENTS..................................................................... vii
LIST OF TABLES........................................................................... xii
LIST OF FIGURES............................................................................ xiii

I. INTRODUCTION........................................................................... 1
   A. Kallikrein-Kinin system overview........................................ 1
   B. Kallikrein-Kinin system: roles of plasma Kallikrein............... 1
   C. Kallikrein-Kinin system: roles of Factor XII (Hageman Factor)..... 5
   D. Hereditary angioedema (HAE).................................................. 6
      1. General overview of HAE................................................... 6
      2. HAE treatment options...................................................... 12
   E. Natural products as sources for new drugs............................... 13
   F. Objective............................................................................... 17

II. EXPERIMENTAL METHODS....................................................... 18
   A. Materials............................................................................... 18
      1) Reagents........................................................................... 18
      2) Cell Cultures.................................................................... 19
   B. Extraction............................................................................... 20
   C. Effects of natural product extracts and/or bioactive compounds on plasma kallikrein activity in fluid phase.......................... 20
   D. Effect on plasma kallikrein on endothelial cell.......................... 21
   E. Effect on FXIIa activity in fluid phase........................................ 21
   F. Effect on FXIIa-induce HPAEC migration................................. 22
G. Effect on rPRCP activity in fluid phase..................................................23

1- Transfected Schneider (S2) cell culture.................................................23

2- rPRCP purification from the transfected S2 cells...............................24

   2.1 S2 cells induction...........................................................................24
   2.2 Dialysis I........................................................................................24
   2.3 Anion exchange chromatography..................................................24
   2.4 Dialysis II.......................................................................................25
   2.5 Cation exchange chromatography................................................25

3- rPRCP activity assay............................................................................26

H. Effect on FXIa activity in fluid phase..................................................26

I. Effect on FXIa activity on HPAEC.........................................................26

J. MTT cell viability assay..........................................................................27

K. Statistical analysis..................................................................................28

III. RESULTS...................................................................................................29

   A. Effects of natural product extracts and/or their bioactive compounds
      on plasma kallikrein activity .............................................................29

   B. Effects of natural product extracts and/or their bioactive compounds
      on plasma kallikrein generation on HPAEC.................................35

   C. Effects of natural product extracts and/or their bioactive compounds
      on FXIIa activity .............................................................................40

   D. Effects of natural product extracts and/or their bioactive compounds
      on rPRCP activity............................................................................45

   E. Effects of natural product extracts and/or their bioactive compounds
      on HPAEC viability using the MTT assay..........................................48

   F. Effects of the aqueous extract of black tea on FXIIa-induced HPAEC
      migration..........................................................................................55
G. Effects of natural product extracts and/or their bioactive compounds on FXIa activity……………………………………………………………………56

IV. DISCUSSION………………………………………………………………………70

LIST OF REFERENCES………………………………………………………………..85

VITA……………………………………………………………………………………103
LIST OF TABLES

Table 1: Diseases associated with contact system (plasma KKS) activation...... 3

Table 2: Overview of Hereditary Angioedema (HAE) types and treatment options.............................................................. 9

Table 3: Enzymes and substrates used in the study............................... 19

Table 4: Screened natural products and their health benefits............... 29

Table 5: Summary of effects of Terminalia compounds on plasma kallikrein, FXIIa, FXIa and rPRCP........................................ 59

Table 6: Summary of effects of red yeast rice compounds on plasma kallikrein, FXIIa, FXIa and rPRCP........................................ 62

Table 7: Summary of effects of natural product extracts on Kallikrein formed on HPAECs................................................................ 74

Table 8: Summary of effects of natural product extracts on Kallikrein, FXIIa and rPRCP.......................................................... 75

Table 9: Summary of the identified pure inhibitors from our study......... 81
LIST OF FIGURES

Figure 1: Roles of plasma kallikrein in hereditary angioedema through (contact activation (plasma kallikrein-kinin), complement and fibrinolytic system) ........................................... 4

Figure 2: Bradykinin overproduction caused by C1-INH deficiency .................................. 10

Figure 3: Schematic representation of the mechanisms involved in the liberation of the bradykinin (BK) from high molecular weight kininogen (HK), and subsequently BK results in increase vascular permeability ..................................................... 11

Figure 4: Schematic representation the scientific classification of Terminalia species used in this study and their distributions .......................................................... 16

Figure 5: Effect of natural product extracts on plasma kallikrein activity ................. 31

Figure 6: Effect of T. arjuna fruit extract on plasma kallikrein activity ................... 32

Figure 7: Effect of T. arjuna bark extract on plasma kallikrein activity ................... 33

Figure 8: Effect of T. bellirica fruit extract on plasma kallikrein activity .................. 33

Figure 9: Effect of T. brownii bark extract on plasma kallikrein activity .................. 34

Figure 10: Effect of T. chebula fruit extract on plasma kallikrein activity .................. 34

Figure 11: Effect of black tea aqueous extract on plasma kallikrein activity ............ 35

Figure 12: Effect of T. arjuna fruit extract on plasma kallikrein formed on endothelial cells .................................................................................................................. 36

Figure 13: Effect of T. arjuna bark extract on plasma kallikrein formed on endothelial cells .................................................................................................................. 36

Figure 14: Effect of T. bellirica fruit extract on plasma kallikrein formed on endothelial cells .................................................................................................................. 37
Figure 15: Effect of *T. brownii* bark extract on plasma kallikrein formed on endothelial cells

Figure 16: Effect of *T. cheula* fruit extract on plasma kallikrein formed on endothelial cells

Figure 17: Effect of arjunoglucoside II on plasma kallikrein formed on endothelial cells

Figure 18: Effect of arjunic acid on plasma kallikrein formed on endothelial cells

Figure 19: Effect of black tea aqueous extract on plasma kallikrein formed on endothelial cells

Figure 20: Effect of *T. arjuna* fruit extract on FXIIa activity

Figure 21: Effect of *T. arjuna* bark extract on FXIIa activity

Figure 22: Effect of *T. bellirica* fruit extract on FXIIa activity

Figure 23: Effect of *T. chebula* fruit extract on FXIIa activity

Figure 24: Effect of *T. brownii* bark extract on FXIIa activity

Figure 25: Effect of arjunoglucoside II on FXIIa Activity

Figure 26: Effect of arjunic acid on FXIIa activity

Figure 27: Effect of black tea aqueous extract on FXIIa activity

Figure 28: Effect of *T. arjuna* fruit extract on rPRCP activity

Figure 29: Effect of *T. chebula* fruit extract on rPRCP activity

Figure 30: Effect of *T. brownii* bark extract on rPRCP activity

Figure 31: Effect of black tea aqueous extract on rPRCP activity

Figure 32: Effect of black tea compounds on plasma kallikrein, FXIIa and
rPRCP activities..............................................................................................................................................48

**Figure 33:** Effect of *T. arjuna* fruit extract on HPAEC viability using the MTT assay.........................................................................................................................49

**Figure 34:** Effect of *T. arjuna* bark extract on HPAEC viability using the MTT assay.................................................................49

**Figure 35:** Effect of *T. bellirica* fruit extract on HPAEC viability using the MTT assay..........................................................50

**Figure 36:** Effect of *T. brownii* bark extract on HPAEC viability using the MTT assay.............................................................51

**Figure 37:** Effect of *T. chebula* fruit extract on HPAEC viability using the MTT assay..........................................................52

**Figure 38:** Effect of arjunoglucoside II on HPAEC viability using the MTT assay.................................................................52

**Figure 39:** Effect of arjunic acid on HPAEC viability using the MTT assay.................................................................53

**Figure 40:** Effect of black tea aqueous extract (1hr & 24 hrs treatment) on HPAEC viability using the MTT assay.................................54

**Figure 41:** Effects of black tea aqueous extract on HPAEC migration............. 56

**Figure 42:** Effects of black tea aqueous extract on FXIa-induced HPAEC migration....................................................................................57

**Figure 43:** Effects of *T. chebula* fruit extract on FXIa activity on endothelial cells........................................................................58

**Figure 44:** Effect of red yeast rice extract on plasma kallikrein activity...........66
Figure 45: Effect of red yeast rice extract on FXIIa activity .................................66

Figure 46: Effect of red yeast rice extract on rPRCP activity.................................67

Figure 47: Effects of red yeast rice active compounds on FXIa activity............ 68

Figure 48: Effects of red yeast rice active compounds on FXIa activity on endothelial cells .................................................................69
I- INTRODUCTION

A. Kallikrein-Kinin system overview
Kallikrein-kinin system (KKS) is a complex endogenous multiprotein system. The components of this system include the precursor kininogen, tissue and plasma kallikreins and kinins (BK, des-Arg⁹ BK and BK¹⁻⁵). In human, there are two types of kininogens, high-molecular-weight kininogen (HMWK) and low-molecular-weight kininogen (LMWK). While HMWK is synthesized in the liver and released into the plasma, LMWK is produced locally in several tissues. Bradykinin, the major bioactive peptide in the KKS, plays an important role in the pathological conditions including inflammation, increased vascular permeability, vasodilation, smooth muscle contraction, edema, pain and cell proliferation.

B. Kallikrein-Kinin system: roles of plasma Kallikrein
Human plasma kallikrein is a serine protease enzyme encoded by a single KLKB1 gene located at the q34-q35 region on the long arm of chromosome 4. Plasma kallikrein is predominantly synthesized and secreted by the liver as a zymogen PK, which is also known as Fletcher factor (Beaubien et al., 1991). PK is a single chain γ-globulin polypeptide of 619 amino acids with a molecular weight of 85-88 kDa. (Fisher C.A et al. 1982; Chung et al. 1986). Normally, 70-90% of the PK zymogen circulates in the human blood as a complex with HMWK with stoichiometric molar ratio 1:1 and at a range of concentration between 35-50 μg/mL (Mandle et al.1976; Reddigari S and Kaplan AP.1989). PK can be activated into kallikrein via the actions
of activated factor XII (FXIIa) on negatively charged surfaces, FXIIa in fluid phase and PRCP on endothelial cell surface (Figure 1) (Webster, 1968; Mandle, Jr. and Kaplan, 1977; Shariat-Madar et al., 2002; Wuepper and Cochrane, 1972). PK can be activated to kallikrein by the cleavage of the Arg371-Ile372 bond. This cleavage forms a protein with two-subunits containing the N-terminal heavy chain and the C-terminal light chain linked by a single disulfide bond located between Cys364 and Cys484 (Wuepper and Cochrane, 1972). While the four Apple domains (A1–A4) are located in N-terminal heavy region, the protease domain is located in C-terminal light chain of PK. In Kallikrein, the catalytic triad is made from His415, Asp464 and Ser559 (van der et al., 1982; McMullen et al, 1991).

Plasma kallikrein plays crucial roles in various pathological processes of diseases associated with inflammation through different systems in the human body including plasma KKS (also known as contact system or intrinsic pathway) and complement system (Figure 1). Several studies linked the activation of the contact system in plasma to the pathogenesis of various diseases (Table 1). Plasma kallikrein cleaves HMWK at two sites between Lys362-Arg363 and Arg371-Ser372 to liberate BK (Webster and Pierce, 1963; Kerbiriou and Griffin, 1979) and releases the cleaved high molecular weight kininogen (HMWKa). HMWKa binds to monocytes and induces the production and release of the inflammatory cytokines and chemokines that maintain the inflammatory response (Irma M. Sainz et al., 2007). The cellular effects of kinins are mediated via two types of G-protein-coupled receptors (GPCRs), the B1 receptors (B1R) and B2 receptors (B2R) (Leeb-Lundberg LM et al., 2005; Marceau F et al., 1998).
### Table 1: Diseases associated with contact system (plasma KKS) activation:

<table>
<thead>
<tr>
<th>Diseases</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>A localized reaction associated with swelling, redness, warmth and pain.</td>
<td>Weiser P. et al., 2010</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>Systemic autoimmune disease in which the immune system produces antibodies in the body attacking healthy cells.</td>
<td>Weiser P. et al., 2010</td>
</tr>
<tr>
<td>Hereditary angioedema with C1-inhibitor deficiency</td>
<td>An inherited diseases characterized by recurrent episodes of severe swelling.</td>
<td>Kaufman N. et al., 1991; Berrettini M et al., 1986</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>Systemic inflammation affects joints.</td>
<td>Rahman M. M et al., 1995</td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>A common form of arthritis cause a mechanical abnormalities by affecting joints and cartilages.</td>
<td>Rahman M. M et al., 1995</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>An inflammatory bowel disease characterized by inflammation and ulcers in the digestive tract.</td>
<td>Stadnicki A. et al., 1997</td>
</tr>
<tr>
<td>Psoriasis</td>
<td>A chronic disease in which the skin cells are growing rapidly.</td>
<td>Weiser P. et al., 2010</td>
</tr>
<tr>
<td>Diabetic retinopathy</td>
<td>A diabetic eye disease caused by the damage to the retina.</td>
<td>Phipps J. A et al., 2008; Clermont A., 2011</td>
</tr>
<tr>
<td>Macular edema</td>
<td>A swelling of the macula of the eye.</td>
<td>Phipps J. A et al., 2008; Clermont A., 2011</td>
</tr>
<tr>
<td>Sepsis</td>
<td>A life-threatening blood infection that can affect the vital organs.</td>
<td>Coppola R et al., 1996</td>
</tr>
<tr>
<td>Systemic amyloidosis</td>
<td>A disease resulted from the accumulation of amyloid protein in the organs.</td>
<td>Maas C. et al., 2008</td>
</tr>
<tr>
<td>Sickle cell disease</td>
<td>An inherited form of anemia that affects hemoglobin.</td>
<td>Miller R. L et al., 1983</td>
</tr>
<tr>
<td>Alzheimer disease</td>
<td>A chronic brain disease affecting memory and other mental functions.</td>
<td>Bergamaschini L et al., 2001</td>
</tr>
<tr>
<td>Allergic reaction</td>
<td>An overreaction resulted from immune response to harmless substances in the body.</td>
<td>Oschatz C. et al., 2011</td>
</tr>
<tr>
<td>Brain ischemia</td>
<td>Low blood supply to the brain to meet metabolic demand.</td>
<td>Makevnina L.G et al., 1994</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>Scarring of liver as a result from many liver diseases.</td>
<td>Cugno M. et al., 1995</td>
</tr>
</tbody>
</table>
The B2 receptors are constitutively expressed, while the expression of B1 receptors are induced during inflammation (Marceau F, 1995). The BK binds and activates its constitutive B2R on endothelial cell membrane leading to an increase in the intracellular Ca^{2+} levels and subsequent production of the endogenous vasodilators nitric oxide (NO) and prostacyclin (PGI_{2}) (Zhao et al., 2001; Hong, 1980; Palmer et al.1987).

Plasma kallikrein is also implicated in the contact surface-mediated activation of the intrinsic coagulation pathway (Wuepper, 1973; Saito et al., 1974; Weiss et al.,
1974; Colman et al., 1975; Griffin & Cochrane, 1976), in which it drives a positive-feedback loop by activating more FXII. In the complement system, plasma kallikrein activates the alternative complement pathway (an innate component of the immune system) and catalyzes the secondary cleavage of α-FXIIa to yield β-FXIIa, which activates the classical complement pathway (a group of plasma proteins that mediate the specific antibody response) (Discipio R.G. 1982; Gebrehiwet et al. 1981). However, the activation effect produced through either of these two complement system pathways resulted ultimately in the generation of chemotactic mediators (C3a and C5a) and opsonins (C3b, a molecule that enhances phagocytosis). While chemotactic mediators recruit the inflammatory cells, opsonins initiate the membrane attack pathway to form membrane-attack complex C5b-9 (Müller-Eberhard. 1988). In addition to its complement activating properties, plasma kallikrein can also stimulates neutrophil chemotaxis, aggregation, oxidative metabolism and neutrophil elastase release (Kaplan et al., 1972; Schapira et al. 1982; Wachtfogel et al. 1983).

**C. Kallikrein-Kinin system: roles of Factor XII**

Coagulation FXII is a serine protease enzyme that is produced and released by the hepatocytes. It is encoded by the F12 gene, which has been mapped to chromosome 5 in humans (Citarella F et al. 1988). The FXII circulates in the plasma as a zymogen. FXII can be activated to its active form, FXIIa on the negatively charged surfaces (Moreau ME et al. 2005). C1-INH is a major inhibitor of FXIIa and kallikrein (Figure 1). However, insufficient C1-INH levels will cause FXIIa to initiate the contact system leading to the local production of bradykinin (Weiss R et al. 1986). FXIIa might be implicated in hereditary angioedema (HAE) disease through
two possible pathways. First, converting PK to kallikrein. On the surface of endothelial cells, FXIIa can activate PK in the presence of HMWK into kallikrein, which in turn results in the generation of BK (Kaplan AP et al. 2003; Fernando LP et al. 2003). Second, FXIIa is a self-activating protease. This means the increase in the FXIIa is responsible for the increased BK concentrations in HAE (Kaplan AP et al. 2003) (Figure 2). The HMWK is capable of binding and regulating several endothelial cell surface proteins. These include cytokeratin 1, gC1qR, and urokinase plasminogen activator receptor (uPAR). Furthermore, HMWK binds via its domain 3 and domain 5 (Joseph K et al. 1999). HMWK binding functions as a cofactor for the activation of factor XII as well as PK (Kaplan AP et al. 2003; Mahdi F et al. 2001).

D. Hereditary angioedema (HAE)

1. General overview of HAE

Angioedema can be defined as a localized swelling beneath the skin surface in different human body sites. It was first described by Milton in 1876. Hereditary angioedema (HAE) is an autosomal dominant inherited disease caused by a deficiency of the multifunctional enzyme called C1-esterase inhibitor (C1-INH). In 1882, Quincke published the first study about HAE. Later in 1888, William Osler, studied the clinical features of HAE. In 1962, it is detected that C1-INH in the plasma of patients with HAE (Landerman et al., 1962). In 1963, the biochemical abnormality in the plasma of HAE patients with a deficiency of C1-INH was observed (Donaldson and Evans, 1963). The deficiency of C1-INH results from mutations in the C1-INH gene (SERPING1). Over 200 different mutations were identified in patients with HAE located on chromosome 11 (11q11-q13-1). These mutations resulted either in C1-INH protein depletion (type I HAE) or synthesis of an abnormal C1-INH protein (type
II HAE). Angioedema was classified into different types based on underlying causes such as mutations in the cases of HAE, drugs in the cases of acquired angioedema or allergic triggers in the cases of allergic angioedema (Table 2). Commonly, two different types of HAE are described based on the abnormality in the levels and functions of C1-INH. Type I HAE is the most common type, which accounts for up to 85% of all HAE cases with deficiency in C1-INH plasma levels compared to the normal values. Type II HAE accounts for up to 15% of all HAE cases with deficiency in C1-INH plasma levels compared to the normal values.

Epidemiologic studies showed that the incidence of HAE vary from 1 in 10,000 to 1 in 150,000 individuals worldwide with no differences between races, ages and genders (Frank MM. 2000; Talavera A. 1995). However, HAE is characterized by intermittent attacks of localized edema and pain mainly in the extremities, face, larynx and gastrointestinal tract without concomitant pruritus. Known triggers of HAE attacks include anxiety, stress, trauma and illness such as the cold and flu. The most serious attack in an HAE patient is the laryngeal attack because the swelling in this site could resulting in airway obstruction causing a death from asphyxiation. Several plasma proteinase inhibitors were indicated as deactivators of plasma kallikrein including C1-INH, α2-macroglobulin (α2M), antithrombin III (AT III), α1-antitrypsin, and α2-antiplasmin (α2AP) (Ratnoff O. D et al., 1969; Vennerod A. M and Laake K., 1975).

C1-INH is a protease inhibitor that belongs to the serpin superfamily. It is the major endogenous inhibitor of the plasma KKS, in which it inhibits FXIIa and Kallikrein (Pixley et al., 1985). In 1956, Lepow was the first to find that human plasma contained a heat sensitive factor that inhibited C1-INH activity (Lepow et al.1956). In addition, C1-INH prevents the activation of the complements C1r and C1s (Ziccardi R. J. and Cooper N. R., 1979). The plasma from patients with HAE showed a
markedly decreased inhibition toward kallikrein esterolytic and amidolytic activity compared to plasma from normal individuals, suggesting C1-INH is one of the major endogenous inhibitors of kallikrein (Gigli I et al., 1975; Trumpi-Kalshoven et al., 1978; Gallimore et al., 1979). In the plasma, C1-INH exhibited an inhibitory ability to inactivate the kallikrein-mediated BK production via forming a 1:1 stoichometric complex with kallikrein (Schapira et al., 1983a). During the HAE attack, the depletion in the levels of functional C1-INH as a result of the mutations of its encoded gene lead to an impaired ability to inhibit its key targets. As results, robust elevation of plasma BK levels were generated from HMWK by kallikrein, leading to swelling, neutrophil influx, chest pain, increased clotting, shortness of breath, and neurological symptoms (Figure 1).

BK is responsible for most of the symptoms associated with edema in patients experiencing acute HAE attacks. The reasons for this are the significant increase in the levels of plasma BK, the increase in the venous blood BK from the arms with localized HAE, and the elevations in the levels of the activated kallikrein in induced blister fluids (Nussberger et al., 1998; Cugno et al., 1997; Shoemaker et al., 1994; Curd et al., 1980; Nussberger et al. 2002) (Figure 2). Moreover, the levels of PK and HMWK in patients with acute HAE attacks were decreased (Schapira et al., 1983).
# Table 2: Overview of Hereditary Angioedema (HAE) types and treatment options

<table>
<thead>
<tr>
<th>Types</th>
<th>Description</th>
<th>Options</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Ref (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hereditary Angioedema Type I (HAE-I)</td>
<td>A) Similarity of HAE type I &amp; type II:</td>
<td>1) Androgens such as danazol, oxandrolone and stanozolol</td>
<td>FDA-approved</td>
<td>Liver toxicity, increase in cholesterol levels &amp; should not be used to treat children</td>
<td>Bork K. 2012</td>
</tr>
<tr>
<td></td>
<td>- Autosomal-dominant inherited (result from mutations in C1-inhibitor gene).</td>
<td>2) C1-inhibitor such as Cinryze™ and Berinert®</td>
<td>Self-administration</td>
<td>Breakthrough laryngeal or abdominal attacks &amp; serious thromboembolic events, hypersensitivity, transmitting infectious agents</td>
<td>Gandhi, P. K et al. 2008</td>
</tr>
<tr>
<td></td>
<td>- Occurring in all ages, genders &amp; races.</td>
<td>3) Bradykinin receptor antagonist such as Firazyr®</td>
<td>Treating acute abdominal, facial and laryngeal HAE attacks.</td>
<td></td>
<td>Bork K et al. 2008</td>
</tr>
<tr>
<td></td>
<td>- Decrease C4, normal C1q and C3 levels in both types.</td>
<td>4) Plasma kallikrein inhibitor such as Kalbitor®</td>
<td>FDA-approved</td>
<td></td>
<td>Banerji A et al. 2008</td>
</tr>
<tr>
<td></td>
<td>B) Dissimilarity:</td>
<td>5) Antifibrinolytics such as tranexamic acid</td>
<td>Self-administration</td>
<td></td>
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<tr>
<td></td>
<td>- Types I &amp; II represent: 80 to 85% and 15 to 20% of all HAE cases, respectively.</td>
<td></td>
<td>FDA-approved</td>
<td></td>
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<tr>
<td></td>
<td>- C1-inhibitor level is decrease in type I, while it is normal, but does not function properly in type II.</td>
<td></td>
<td>FDA-approved</td>
<td></td>
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</tr>
<tr>
<td>Hereditary Angioedema Type II (HAE-II)</td>
<td>- Unknown prevalence, but reported in women (mainly).</td>
<td>C1-INH concentrate and Icatibant can be used</td>
<td>For patients 16 years of age and older.</td>
<td>High cost, decrease effects of ACE inhibitors</td>
<td>Bork K et al. 2009</td>
</tr>
<tr>
<td></td>
<td>- Normal C1-inhibitor, C4, C1q and C3.</td>
<td></td>
<td></td>
<td>High cost, hypersensitivity</td>
<td>Bouillet L et al. 2009</td>
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<td></td>
<td>- FXII gene mutations.</td>
<td></td>
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<td></td>
<td>- Associated with pregnancy and the use of estrogen-containing oral contraceptives.</td>
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<tr>
<td>Hereditary Angioedema Type III (HAE-III)</td>
<td>- Very rare.</td>
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<tr>
<td>Acquired Angioedema Type I (AAE-I)</td>
<td>- Immune complexes that are usually linked to an underlying lymphoproliferative disorder destroy the function of C1-inhibitor.</td>
<td></td>
<td>Lymphoproliferative disorder must be targeted</td>
<td>Unapproved treatment options include antifibrinolytics, androgens or C1-INH concentrate</td>
<td>Zingale LC et al. 2006</td>
</tr>
<tr>
<td>Acquired Angioedema Type II (AAE-II)</td>
<td>A) Similarity of AAE type I &amp; type II:</td>
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<td></td>
<td>- Causes by ACE-Inhibitors such as captopril.</td>
<td></td>
<td>ACE-Inhibitors in use must be stopped or change and a bradykinin inhibitor, icatibant can be used</td>
<td></td>
<td>Weber MA et al. 2008</td>
</tr>
<tr>
<td></td>
<td>- Normal C1-inhibitor, C4, C1q and C3.</td>
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<td></td>
<td>- Swelling may commence anywhere from a few hours to years after first starting medication</td>
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<tr>
<td>Idiopathic</td>
<td>- Normal C1-inhibitor, C4, C1q and C3.</td>
<td></td>
<td>Primarily antihistamines. DHEA, 1-thyroxine for thyroid dysfunction. Prednisone therapy</td>
<td></td>
<td>Frigas E et al. 2006</td>
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<tr>
<td>NSAID-associated</td>
<td>- Angioedema without urticaria</td>
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<td></td>
<td>- Not responsive to H1 antihistamine blockers.</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>- Normal C1-inhibitor, C4, C1q and C3.</td>
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<tr>
<td>Allergic</td>
<td>- Swelling results from triggers such as food, latex, venom, bee sting, cold, heat, or drug.</td>
<td></td>
<td>Antihistamines, adrenaline (epinephrine) and avoid allergic reaction triggers,</td>
<td></td>
<td>Douglas T. Johnston. 2011</td>
</tr>
</tbody>
</table>
Figure 2: Bradykinin overproduction caused by C1-INH deficiency. Plasma BK levels in: 1) six patients with angioedema due to Hereditary C1-Inhibitor Deficiency, 2) four patients with angioedema related to angiotensin converting enzyme (ACE) inhibitors, 3) four patients with urticaria and angioedema that responded to antihistamines (histaminergic angioedema). Each circle represents one patient. Solid circles denote bradykinin levels during acute attacks of angioedema. Open circles denote levels during the remission. The shaded area indicates the normal range of venous plasma BK levels (0.2 to 7.1 pM). [Reproduced with permission from (Nussberger et al. 2002), Copyright Massachusetts Medical Society].
*Figure 3:* Schematic representation of the mechanisms involved in the liberation of the bradykinin (BK) from high molecular weight kininogen (HK), and subsequently BK results in increase vascular permeability. FXI: factor XI; FXIa: activated FXI; FXII: factor XII; FXIIa: activated factor XII; CTI: corn trypsin inhibitor; SBTI: soybean trypsin inhibitor; DFP: Diisopropylfluorophosphate; Long polyp: long-chain polyphosphate; rC1-inh: recombinant C1-inhibitor; IL-1β: Interleukin 1 beta; TNFa: tumor necrosis factor alpha; MMP2: matrix metalloproteinase-2; MAPK: Mitogen-activated protein kinases; COX-2: Cyclooxygenase 2; NOS: nitric oxide synthase. Red circles refer to enzymes involved mainly in the liberation of the bradykinin (BK).

BK induced the expression of several inflammatory genes including COX-2, MMPs, interleukins (IL-1β, IL-6) as well as chemokines (Zaczynska E et al. 2003; Terzuoli E et al. 2014) (*Figure 3*). The overexpression effects of these genes via BK are mediated through the activation of the NF-kB pathway (Terzuoli E et al. 2014). In addition, in the airway smooth muscle cells, BK stimulates MMP-2, the enzyme that is involved in the inflammatory processes (Zaczynska E et al. 2003; Corbel M et al.)
(Figure 3). Overall, plasma kallikrein is the major BK liberator, thus it plays a crucial role in the pathogenesis of BK-mediated diseases such as hereditary angioedema and inflammation.

2. HAE treatment options

While plasma kallikrein is the major enzyme for liberation of BK from HMWK, four other enzymes indirectly play roles in the liberation of BK from HMWK including: FXIIa, activated FXI (FXIa), plasmin, and recombinant prolylcarboxypeptidase (rPRCP) (Shariat-Madar Z et al., 2002; Kaplan AP et al., 1997; Merlini PA et al., 2004; Motta G et al., 1998; Nishikawa K et al., 1992; Molinaro G et al., 2002).

Because plasma KKS is deeply involved in the pathogenesis HAE, the therapeutic options were approved or designed based on their ability to inhibit this pathway. Different options were suggested for the treatment and anaphylaxis of HAE according to the duration of therapy including acute, short-term and long-term therapies (Table 2). Acute treatment strategies include C1-INH (human or recombinant) replacement therapy, plasma kallikrein inhibitor (ecallantide), or specific bradykinin B2 receptor antagonist (icatibant). While it is not approved for the treatment of acute attacks of HAE, the C1-INH agent Cinryze® was the first drug to be approved by the US FDA in 2008 for chronic replacement or prophylaxis of C1-INH in HAE (Cocchio and Marzella, 2009). It works by increasing C1-INH and C4 to their normal levels. However, the bradykinin peptidomimetic Icatibant (Firazyr®), was approved by the US FDA in 2011 as the first and only self-administered subcutaneous drug for treating acute HAE attacks (Cicardi et al. 2010; Aberer et al. 2014). Icatibant works by decreasing the vascular permeability via inhibiting the binding of bradykinin to its B2 receptors, which consequently resulted in the
reduction of vasodilation effects due to the reduction of NO and PGI\textsubscript{2} formation. In 2009, C1-INH Berinert\textsuperscript{®} and Kalbitor\textsuperscript{®} were approved by the US FDA for the treatment of acute attacks of HAE in adults, but not in children (Keating, 2009; Zuraw et al., 2010). Ecallantide works by directly inhibiting plasma kallikrein. Several limitations associated with Cinryze\textsuperscript{™}, Berinert\textsuperscript{®} and Kalbitor\textsuperscript{®} therapies are reported including high cost, risk of type I hypersensitivity reaction or anaphylaxis as well as the risk of transmitting plasma derived infections (Lunn et al., 2010; Riedl, 2010).

Antifibrinolytics such as tranexamic acid can also be used only if other therapies are not appropriate. Tranexamic acid works by blocking the conversion of plasminogen to plasmin thus abolishes plasmin formation. The use of antifibrinolytics is limited now due to their risks of thromboembolism, hypotension, and cardiac arrhythmias as well as their low efficacy (Zuraw et al. 2008; Agostoni et al. 2004; Cicardi et al. 2010; Dagen et al. 2010). Although fresh plasma was successfully used for the acute treatment and prophylaxis, it is still limited due to the potential threat of worsening HAE symptoms by its additional kinins and complement factor contents (Nzeako et al. 2001; Agostoni et al. 1992; Galan et al. 1996; McGlinchey et al. 2000; Pekdemir et al. 2007). Short-term prophylaxis strategies include C1-INH replacement, oral androgens, icatibant, antifibrinolytics, and fresh plasma (Zuraw et al. 2008; Bowen et al. 2008; Bowen et al. 2010; Marque´s et al. 2010) (Table 2).

E. Natural products as sources for new drugs

Natural products can be simply defined as any substance extracted or isolated from nature or living organisms. Broadly, natural products can be classified according to their sources such as plants, microbial agents, marine organisms and animals. Natural product sources from plants have been routinely used in the
treatment of various human diseases for thousands of years ago (Phillipson, J.D et al, 2001). More than 70,000 plant species have been screened for their biological activities as medicinally agents (Farnsworth, N.R et al, 1991).

Black tea (*Camellia sinensis*) is a species of plant in the Theaceae family. While black tea originated in Southeast Asia, it is now cultivated worldwide to produce commercial beverages, making it the second most popular after water (Graham H.N et al. 1991). The major chemical constituents of black tea are polyphenols and include catechins, theaflavins and thearubigins. The major catechins in black tea are epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC) (Li S et al. 2012). Black tea extract, and its major constituents such as EGCG, exhibited anti-inflammatory effects by reducing the lipopolysaccharide (LPS)-induced expression of the inflammatory cytokine production such as IL-1β and TNF-α in human cerebral microvascular endothelial cells (hCMECs), carrageenin-induced paw edema in rats as well as formaldehyde-induced arthritis in rats (Nag Chaudhuri A.K. et al. 2005; Jieliang Li et al. 2012). In a recent study, black tea extract decreased the rate of blood pressure variation by approximately 10% during the nighttime hours, suggesting its cardiovascular benefits (Hodgson et al. 2013).

*Terminalia* belongs to the family Combretaceae, native to Asia includes India and China. There are different species of Terminalia incuding *T. chebula*, *T. bellirica*, *T. arjuna* and *T. brownii* (Figure 4). *T. chebula* is a deciduous tree growing up to 30 m tall with a trunk up to 1 m in diameter. It is a popular folk medicine plant that has been traditionally used for several purposes such as an anti-inflammatory dietary sources, antiplatelet, antioxidant, and for its cardiovascular effects (Zia-Ul-Haq M. et al. 2012, Dolly Singh et al. 2012, Hyun-Ho Lee et al. 2015, Gautam M. K. et al.)
2013). In Ayurvedic Indian traditional medicine, *T.chebula*, as well as *T. bellirica* are two components of triphala, a popular Ayurvedic formulation that is used for several purposes including eye diseases, diabetes and improvement in digestion (Ayurvedic pharmacopoeia committee, 2003). *T.chebula* was indicated to have a strong anti-inflammatory activity in many studies. For example, the polyherbal formulation called Aller-7, which is the combination of seven medicinal plant extracts including *T. chebula* and *T. bellirica* extracts showed a strong anti-inflammatory effect (Pratibha et al. 2004). In an acetic acid-induced colitis model, it was noted that *T. chebula* could increase the antioxidant effects and decrease free radical formation (Gautam M. K et al. 2013).
Red yeast rice (RYR) is the fermented product obtained after red yeast (Monascus purpureus) is grown on rice. It had been used in China for centuries as a food flavoring as well as a medicinal product (Wang J et al. 1997). RYR has been used as a food colorant and preservative and in a traditional Chinese medicine. It was described by the Pharmacopeia of the People’s Republic of China as a body invigorative, helping in digestion, and revitalizing blood circulation (Erdogru O et al. 2004). Several preparations of RYR are commercially available including Cholestin, Xuezhikang and Zhibituo. These preparations were found to exhibit improvements
on lipid profile including significant reductions of serum total cholesterol, LDL-cholesterol, triglyceride (TG), and provide an increase in HDL-cholesterol (Liu JP et al. 2006). Xuezhikang is a partially purified extract of fermented red yeast rice (*Monascus purpureus*). It is composed of 13 natural statins, unsaturated fatty acids, ergosterol, amino acids, flavonoids, alkaloid and trace elements. Xuezhikang also exhibited lipid lowering and anti-inflammatory properties (Heber D et al. 1999).

**F. Objective**

Kallikrein levels are indicated to be associated with hereditary angioedema and myocardial infarction, whereby kallikrein amplifies the generation of activated factor XII (FXIIa) and bradykinin (BK). BK is responsible for most of the symptoms associated with edema in patients with acute HAE attacks. Thus, BK plays a crucial role in the pathophysiology and clinical manifestations of HAE. We hypothesized that kallikrein inhibitors represent a novel class of drugs for reducing inflammation with a broad spectrum of activity.

The primary objective of this study was to screen natural products known for their anti-inflammatory and cardiovascular properties for their effects on kallikrein-kinin system using amidolytic kallikrein/FXIIa assays to explore their potential implications in the treatment of Hereditary Angioedema.
II- EXPERIMENTAL METHODS

A. Materials

1) Reagents

Human pulmonary artery endothelial cells (HPAECs), Medium 200, low serum growth supplement, trypsin-EDTA (ethylenediaminetetraacetic acid), Trypsin-neutralizing solution (TNS), S2 cells and Hygromycin B were purchased from Invitrogen Life Technologies (Grand Island, NY, USA). SFX-insect serum free insect cell culture medium and DPBS (Dulbecco's phosphate-buffered saline) were purchased from Thermo Scientific (Logan, UT, USA). Single-chain HK, human PK, human plasma α-kallikrein and human FXIIa were purchased from Enzyme Research Laboratories (South Bend, IN, USA). S2302; BIOPHEN CS-31 (02) was purchased from Aniara (Mason, OH, USA). Ala-Pro-paranitroaniline (APpNA) was purchased from Bachem (Torrance, CA, USA). Diethylaminoethyl (DEAE) cellulose was purchased from Whatman (Fairfield, NJ, USA). Spectra/Por® Dialysis Membrane (MWCO 6-8 kDa) was purchased from Spectrum Laboratories, Inc (Rancho Dominguez, CA, USA), SP Sephadex 50-120 and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA).
Table 3: Enzymes and substrates used in the study:

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Name</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kallikrein and FXIIa</td>
<td>S2302</td>
<td>H-D-Pro-Phe-Arg-p-Nitroanilide,</td>
</tr>
<tr>
<td>FXIa</td>
<td>S2366</td>
<td>Giu-Pro-Arg-p-Nitroanilide</td>
</tr>
<tr>
<td>rPRCP</td>
<td>APpNA</td>
<td>H-Ala-Pro-pNA</td>
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2) Cell Cultures

Using the endothelial cell culture, HPAEC was obtained and cultured in growth medium 200 supplemented with low serum growth supplement according to the manufacturer’s protocol (Invitrogen, NY) into 96 well culture plates (BRAND; Wertheim, Germany) and incubated in a CO₂ controlled incubator (5% CO₂) at 37°C overnight.

Using the Schneider (S2) cell culture, S2 cells were derived from Drosophila melanogaster embryo. PRCP-transfected S2 cells with a plasmid were cultured in 20 mL of HyQ SFX–Insect cell culture medium (Hyclone, Utah) supplemented with 1mM Hygromycin (Invitrogen, Carlsbad, CA) in 75 cm² tissue culture flasks (Fisher Scientific, PA) in a temperature controlled incubator (23 °C) (Chajkowski et al. 2011). Once the cells were confluent, 1 flask of cells was subcultured to 3 flasks and from 3 to 10 flasks, and incubated overnight.
B. Extraction

Black tea was extracted with deionized (DI) water (Tea was soaked in boiling water for 15 minutes, 4 g/10 ml DI water). After 15 min it was filtered and the filtrate was lyophilized and kept in -20 °C freezer until fractioned and analyzed. *Terminalias* extracts, red yeast rice extract and their isolated compounds were prepared and processed by the National Center for Natural Products Research (NCNPR) at the University of Mississippi. All other natural product extracts were prepared according to the method of Radwan MM et al. 2014.

C. Effects of natural product extracts and/or bioactive compounds on plasma kallikrein activity in fluid phase

In this experiment the effect of the extracts or the pure compounds on the rate of S2302 hydrolysis by kallikrein was assessed. Kallikrein (2 nM) was incubated with increasing concentrations of the extracts (20 mg/ml to 500 mg/ml) or the pure compounds (1 μM to 1000 μM) in the presence of 0.4 mM of the substrate (H-D-Pro-Phe-Arg- p-Nitroanilide, S2302) in HEPES-carbonated buffer (137 mM NaCl, 3 mM KCl, 12 mM NaHCO₃, 14.7 mM HEPES, 5.5 mM Glucose, 0.1% Gelatin, 2 mM CaCl₂, 1 mM MgCl₂, 7.1 pH) for 1 hour at 37 °C. The negative control has the same reaction mixture except kallikrein. The positive control contains the same reaction mixture component except the extract or the pure compound. The same increasing concentrations of the extracts plus the substrate in HEPES-carbonated buffer without kallikrein was used as a color control. However, the substrate hydrolysis was assessed spectrophotometrically at 405 nm. Kallistop™ (100 μM, equivalent to 77.4
μg/ml) was used as a reference kallikrein inhibitor and HEPES-carbonated buffer was used for the negative control.

**D. Effects of natural product extracts and/or bioactive compounds on plasma kallikrein on endothelial cells**

To determine the biological action of plant extracts or the pure compounds to inhibit kallikrein activity on endothelial cell surface, HPAECs were grown in medium 200 supplemented with low serum growth supplement (contains fetal bovine serum, basic fibroblast growth factor, heparin, hydrocortisone, and epidermal growth factor) overnight in 96 well plates. Then, the cells were incubated with 1% gelatin buffer to block the surface of the plate and the remaining cell surface to prevent nonspecific binding of HMWK during subsequent steps. After one hour incubation period, the cells were incubated with 30 nM HMWK for 1 hour at 37 ºC, and followed by 30 nM of PK with increasing concentrations of the extracts (20 mg/ml to 500 mg/ml) or the pure compounds (1 µM to 1000 µM) for 1 hour at 37 ºC. Finally, cells were incubated with 0.5 mM of S2302 in HEPES-carbonated buffer for 1 hour at 37 ºC. Cells were washed gently with HEPES-carbonated buffer three times between each incubation step and the substrate hydrolysis was assessed spectrophotometrically at 405 nm.

**E- Effects of natural product extracts and/or bioactive compounds on FXIIa activity in fluid phase**

Next, investigations were performed to determine the biological action of the plant extracts and pure compounds on FXIIa. Increasing concentrations of the extracts (20 mg/ml to 500 mg/ml) or the pure compounds (1 µM to 1000 µM) were incubated with 17 nM of FXIIa and 0.4 mM of the substrate (H-D-Pro-Phe-Arg- p-Nitroanilide,
S2302) in HEPES-carbonated buffer at 37 °C for 1 hour. The negative control had 0.4 mM of S2302 in HEPES-carbonated buffer. The positive control had 0.4 mM of S2302 plus 17 nM of FXIIa without the extract or the pure compound. The color controls have the same increasing concentrations of the extracts plus 0.4 mM of S2302 in HEPES-carbonated buffer without FXIIa enzyme. Then the hydrolysis was measured at wavelength 405 nm using absorbance plate reader. Corn trypsin inhibitor (9 μM, equivalent to 126 μg/ml) was used as a reference FXIIa inhibitor.

**F- Effects of natural product extracts and/or bioactive compounds on FXIIa-induce HPAEC migration**

After plating 5,000 of HPAECs per well, the cells were incubated overnight to achieve 90 % confluence. HPAEC monolayers were scratched using 200 μL pipette tip, and serum-containing media was added to each well. Reference points near the scratch were marked using the marker. Wells were washed with 100 μL of DPBS (Dulbecco's phosphate-buffered saline) buffer two times to remove the floating cells resulted from the scratch. First, to determine the magnitude of the pharmacological effect, cells were incubated with various concentrations of the plant extracts. However, 100 μL of growth medium in the presence of increasing concentrations of the plant extract was added to the wells. Other types of treatment include the following: 1) 100 μl of growth medium per well were used as positive control, 2) 240 nM of FXIIa in 100 μl total volume of growth medium per well were used to induced HPAECs migration, 3) selected plant extract concentrations were added to 100 μl total volume of growth medium per well in the absence of 240 nM FXIIa, 4) selected plant extract concentrations were added to 100 μl total volume of growth medium per well in the presence of 240 nM FXIIa. Images of the wells were taken at 0 hr with a
phase contrast microscope. After 24 hrs, growth medium of all the wells was decanted and wells were washed with 100 µL DBPS two times, 100 µL of cold methanol were added to each well and incubated at 4 °C for 5 mins. Next, the cold methanol was decanted and 70 µL of diluted crystal violet dye (0.1%) in DBPS was added to each well and incubated for 30 minutes at room temperature. The loose from the cells dye was washed by the water and the wells allowed to dry for 1 hr at room temperature. Serial images of the reference points at the beginning and during the migration close to the scratch (wound) area were taken. The effects of the plant extract on the migration rate were quantified by comparing the images using imageJ. Each group had wells in triplicates, and each experiment was repeated 3 times.

G- Effects of natural product extracts and/or bioactive compounds on rPRCP activity in fluid phase

1- Transfected Drosophila Schneider (S2) cell culture

The enzyme rPRCP was purified from Schneider (S2) cells in which S2 cells were derived from Drosophila melanogaster embryo. S2 cells were transfected previously with a plasmid carrying the human PRCP gene, as described previously (Shariat-Madar Z et al. 2004). S2 cells were cultured in 20 mL of HyQ SFX–Insect cell culture medium (Hyclone, UT) and hygromycin B (Invitrogen, CA) were added in a concentration of 1 mM to facilitate the selective growth of cells with the rPRCP1 plasmid in 75 cm² tissue culture flasks (Fisher Scientific, PA) at 23 °C in atmospheric air. Once the cells were confluent, 1 flask of cells was subcultured to 3 flasks and from 3 flasks to 10 flasks and incubated at 23 °C in atmospheric air.
2- rPRCP purification from transfected S2 cells

2.1 S2 cells induction

In the induction step, the suspended S2 cells from 12 flasks were transferred to several 50 mL polypropylene centrifuge tubes and centrifuged for 10 minutes at 350x g at 23 °C. The cell pellet was resuspended in the induction medium, HyQ SFX-Insect cell culture medium (Hyclone, UT) in which 1 mM hygromycin B (Invitrogen, CA) and 0.66 mM CuSO₄ (CuSO₄ induced human PRCP gene through metallothionein gene to express rPRCP (Lys⁴⁶-His⁴⁹⁶) protein into the supernatant) was added and incubated in dark for 48 hours at 23 °C on a shaker with circular or rocker shaking motion for gentle mixing and oxygenating the cells.

2.2 Dialysis I

Following 48 hours of incubation, the supernatant was collected from the cell suspensions via centrifugation for 10 min at 350x g at 23 °C. Then the supernatant was transferred to spectra/por 1 dialysis membrane (Spectrum Laboratories, CA). This tubing membrane has 6-8 kDa molecular weights cut off. The membrane was prepared for dialysis according to the manufacturer's instructions. The supernatant was dialyzed in 4 liters of 1x Tris Buffer (10 mM Tris, 25 mM NaCl, 0.5 mM EDTA, 0.5 mM β-mercaptoethanol; pH 7.1) overnight at 4 °C with two times buffer changes after 4 to 6 hrs.

2.3 Anion exchange chromatography

In anion exchange chromatography, ten grams of diethylaminoethyl (DEAE) cellulose resin (Whatman, AZ) was soaked in 40 ml of 1x Tris Buffer (10 mM Tris, 25 mM NaCl, 0.5 mM EDTA, 0.5 mM β-mercaptoethanol; pH 7.1) and the buffer was
changed once after 30 mins. A DEAE-cellulose solution was added to column (3.5 cm inner diameter X 25 cm length) and then packed and equilibrated with 3 times the bed volume (20 to 22 mL) of 1x Tris buffer. The dialysate was loaded into the column. The column was subsequently washed with 3 times the bed volume of 1x Tris buffer.

2.4 Dialysis II

The flow-through from the sample and washing steps were pooled and transferred into dialysis tubing membrane. The sample was dialyzed in 1x CH$_3$COONa buffer (10 mM CH$_3$COONa, 0.5 mM EDTA, 0.5 mM β-Mercaptoethanol; pH 4.8) overnight in 4°C with two times buffer changes after 4 to 6 hrs.

2.5 Cation exchange chromatography

In the cation exchange chromatography, 0.45 g of sephadex-SP 150-20 column resin (Sigma-Aldrich Co., MO) was soaked in 40 ml and equilibrated in 1x CH$_3$COONa buffer (10 mM sodium acetate trihydrate, 0.5 mM EDTA, 0.5 mM β-mercaptoethanol, 4.8 pH) for one hour. The buffer was changed once after 30 minutes. The sephadex-SP solution was added to a column (1.5 cm inner diameter X 25 cm length) and then packed and equilibrated with 3 times the bed volume (30 to 35 mL) of 1x CH$_3$COONa buffer at 4°C using a cold room. The dialysate was loaded into the column and the column was washed with 1x CH$_3$COONa buffer. rPRCP adsorbs to the column. The bound rPRCP protein was recovered from the column by eluting with 0.2 M KCl (in 1x CH$_3$COONa) (in a volume equivalent to 3 times the bed volume). The rPRCP obtained was stored at 4 °C to be used in the activity assay.
3- rPRCP activity assay

To determine the inhibitory effects, increasing concentrations of the extracts (20 mg/ml to 500 mg/ml) or the pure compounds (1 µM to 1000 µM) were incubated with the purified rPRCP (30 µg/ml) and 0.5 mM of the substrate H-Ala-Pro-pNA (APpNA) in HEPES-carbonated buffer for 1 hour at 37 ºC. The negative control has only 0.5 mM of APpNA in HEPES-carbonated buffer. The positive control has 0.5 mM of APpNA plus an appropriate volume of the purified rPRCP without the extract. The color controls have the same increasing concentrations of the extract plus 0.5 mM of APpNA in HEPES-carbonated buffer without rPRCP. The substrate hydrolysis was assessed spectrophotometrically at 405 nm. Z-Pro-Prolinal (10 mM, equivalent to 3.3 mg/ml) was used as a reference rPRCP inhibitor.

H. Effects of natural product extracts and/or bioactive compounds on FXIa activity in fluid phase

To determine the pharmacological effects of the plant extracts and the pure compounds to inhibit FXIa, increasing concentrations of the extracts (20 mg/ml to 500 mg/ml) or the pure compounds (1 µM to 1000 µM) were incubated with 2 nM of FXIa and 0.291 mM of the substrate (Glu-Pro-Arg-p-Nitroanilide, S2366) in HEPES-carbonated buffer for 1 hour at 37 ºC. Then the substrate hydrolysis was assessed spectrophotometrically at 405 nm.

I. Effects of natural product extracts and/or bioactive compounds on FXIa activity on HPAEC

To determine the pharmacological effects of the plant extracts and the pure compounds to inhibit the production of FXIa on the surface of endothelial cells,
HPAECs were grown in medium 200 supplemented with low serum growth supplement overnight at 37 °C in 96 well plates. The cells were washed three times with HEPES-carbonated buffer and blocked with 1% gelatin for 1 hour at 37 °C to prevent non-specific binding. After three times washing with HEPES-carbonated buffer, HPAEC were incubated with 80 nM of HK for 1 hour at 37 °C. At the end of incubation, cells were washed three times with HEPES-carbonated buffer to remove the unbound HK and then treated with 40 nM of FXI. After incubation for one hour at 37 °C, cells were washed three times with HEPES-carbonated buffer and 0.5 mM S2366 (Glu-Pro-Arg-pNA·HCl) in HEPES-carbonated buffer was added to determine the effects on FXIa activity. Substrate hydrolysis was allowed to proceed for 1 hour at 37 °C. FXIa activity was measured as the change in UV absorbance at 405 nm, using BioTek ELx800 Absorbance Microplate Reader.

J. MTT cell viability assay

Cell viability was determined by thiazolyl blue tetrazolium bromide (MTT) colorimetric assay. First, 25,000 cells per well of HPAECs were seeded in 96-well culture plates cultivated and incubated overnight at 37 °C. Following the addition of arjunoglucoside-II for 1 hour, 20 μl of filtered MTT solution containing 5 mg/ml MTT in serum free medium was added to each well. After 4 h of incubation at 37 °C, the medium was aspirated and the cells were lysed by the addition of 150 μL per well MTT solvent containing 4 mM HCl and 0.1% Nondet P-40 (NP40) in 100% isopropanol. Plates were covered with aluminium foil and placed on an orbital shaker for 15 minutes at room temperature. The optical density (OP) of each sample was measured in a microplate reader using a reference wavelength of 570 nm with
background subtraction at 690 nm. Each group had three wells and each experiment was replicated 3 times.

**K. Statistical analysis**

The mean ± SEMs from the bioassays of plasma kallikrein, rPRCP, and FXIIa, FXIa and kallikrein formed on cells were analyzed with nonlinear regression in GraphPad Prism 6.0. GraphPad Software, Inc., USA. Experiments were performed at least three times in duplicate or triplicate. Data was analyzed using one-way analysis of variance (ANOVA). For all comparisons, statistical significance was defined as *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ or ****$P \leq 0.0001$. 
III-RESULTS

A) Effects of natural product extracts and/or their bioactive compounds on plasma kallikrein activity:

Plasma kallikrein plays an important role in the pathogenesis of hereditary angioedema in which the levels of plasma kallikrein-mediated bradykinin production is increased due to the depletion in the levels of its major endogenous, C1-INH. So, in order to develop a safe and effective plasma kallikrein inhibitor that could inhibit kallikrein-mediated generation of BK from HMWK, we have screened several natural plant extracts listed in (Table 4) for their effects on enzymes involved either directly such as plasma kallikrein or indirectly such as FXIIa, and rPRCP in the liberation of BK from HMWK. These natural products were selected based on their anti-inflammatory and cardiovascular effects shown in the literature as well as their safety and availability (Table 3).

Table 4: Screened natural products and their reported health benefits.

<table>
<thead>
<tr>
<th>Plants</th>
<th>Species</th>
<th>Part used</th>
<th>Health benefits</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anise</td>
<td>Pimpinella anisum</td>
<td>Fruit</td>
<td>Antiinflammatory and antioxidant</td>
<td>Al-Ismail KM et al. 2004, Monika M. 2010</td>
</tr>
<tr>
<td>Basil</td>
<td>Ocimum basilicum</td>
<td>Herb</td>
<td>Antiinflammatory and antioxidant</td>
<td>Gülçin I et al. 2007, Monika M. 2010</td>
</tr>
<tr>
<td>Plant</td>
<td>Scientific Name</td>
<td>Part</td>
<td>Function</td>
<td>References</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------------</td>
<td>------------</td>
<td>---------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Cayenne pepper</td>
<td>Capsicum annuum</td>
<td>Fruit</td>
<td>Antiinflammatory, decrease cholesterol levels and Antioxidant</td>
<td>Liang Y. et al. 2013, Wang Q et al. 2014, Monika M. 2010</td>
</tr>
<tr>
<td>Clove</td>
<td>Syzygium aromaticum</td>
<td>Fruit</td>
<td>Antioxidant, anti-inflammatory, hypolipidemic effect and antihypertensive</td>
<td>Peng Z et al. 2013, Mnafigui K et al. 2013, Monika M. 2010</td>
</tr>
<tr>
<td>Poppy seed</td>
<td>Papaver somniferum</td>
<td>Seed</td>
<td>Antioxidant</td>
<td>Cevik-Demirkan A et al. 2012</td>
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<tr>
<td>Dill</td>
<td>Anethum graveolens</td>
<td>Fruit</td>
<td>Antiinflammatory and antioxidant</td>
<td>Al-Ismail KM et al. 2004, Monika M. 2010</td>
</tr>
<tr>
<td>Fennel</td>
<td>Foeniculum vulgare</td>
<td>Fruit</td>
<td>Antiinflammatory and antioxidant</td>
<td>Choi EM et al. 2004</td>
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<td>Mentha</td>
<td>Mentha spicata</td>
<td>Herb</td>
<td>Antiinflammatory</td>
<td>P. Arumugam et al. 2008</td>
</tr>
<tr>
<td>Nutmeg</td>
<td>Myristica fragrans</td>
<td>Fruit</td>
<td>Antiinflammatory and antioxidant</td>
<td>Kareem, M. A et al. 2013, Monika M. 2010</td>
</tr>
<tr>
<td>Paprika</td>
<td>Papaver somniferum</td>
<td>Seed</td>
<td>Antioxidant</td>
<td>Monika M. 2010</td>
</tr>
<tr>
<td>Thyme</td>
<td>Thymus vulgaris</td>
<td>Herb</td>
<td>Antiinflammatory and antioxidant</td>
<td>Monika M. 2010, Eli-Nekeety AA et al. 2011</td>
</tr>
<tr>
<td>Terminalia arjuna</td>
<td>Terminalia arjuna</td>
<td>Fruit and bark</td>
<td>Antihypertensive, Antioxidant, effective in heart diseases</td>
<td>Maulik SK et al. 2012, Chander R et al. 2004</td>
</tr>
<tr>
<td>Terminalia brownii</td>
<td>Terminalia brownii</td>
<td>Bark</td>
<td>Anti-inflammatory of arjunic acid (terminalia brownii constituent)</td>
<td>Yang MH et al. 2014</td>
</tr>
</tbody>
</table>
To determine their pharmacological effects on plasma kallikrein activity, we have screened thirty plant extracts on kallikrein activity at a selected concentration 200 mg/ml and accordingly continue our study on the extracts showing promising effects (Figure 5).

<table>
<thead>
<tr>
<th>Extract</th>
<th>% Kallikrein Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Black pepper</td>
<td>75</td>
</tr>
<tr>
<td>Cardamon</td>
<td>50</td>
</tr>
<tr>
<td>Cayenne pepper</td>
<td>25</td>
</tr>
<tr>
<td>Colodium</td>
<td>10</td>
</tr>
<tr>
<td>Cumin</td>
<td>5</td>
</tr>
<tr>
<td>Papry seed</td>
<td>2</td>
</tr>
<tr>
<td>Dill</td>
<td>1</td>
</tr>
<tr>
<td>Fennel</td>
<td>0.5</td>
</tr>
<tr>
<td>Garlic</td>
<td>0.25</td>
</tr>
<tr>
<td>Marigold</td>
<td>0.1</td>
</tr>
<tr>
<td>Oregano</td>
<td>0.05</td>
</tr>
<tr>
<td>Paprika</td>
<td>0.01</td>
</tr>
<tr>
<td>Rosemary</td>
<td>0.005</td>
</tr>
<tr>
<td>Thyme</td>
<td>0.001</td>
</tr>
<tr>
<td>Cilantro</td>
<td>0.0005</td>
</tr>
<tr>
<td>Black tea</td>
<td>0</td>
</tr>
<tr>
<td>T. arjuna fruit</td>
<td>75</td>
</tr>
<tr>
<td>T. arjuna bark</td>
<td>50</td>
</tr>
<tr>
<td>T. bellirica fruit</td>
<td>45</td>
</tr>
<tr>
<td>T. brownii bark</td>
<td>85</td>
</tr>
<tr>
<td>Red yeast extract</td>
<td>65</td>
</tr>
<tr>
<td>Pomegranate</td>
<td>45</td>
</tr>
<tr>
<td>Black tea aqueous extract</td>
<td>80</td>
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</tbody>
</table>

**Figure 5: Effect of natural product extracts on plasma kallikrein activity.** 200 mg/ml of thirty natural product extracts were incubated with Kallikrein (2 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments. [*P ≤ 0.05, **P ≤ 0.001, ***P ≤ 0.0001 compare to control]*

*T. arjuna* fruit and *T. arjuna* bark were tested at 200 mg/ml. While *T. arjuna* fruit exhibited a significant inhibitory effect on plasma kallikrein by 45%, its bark extract showed no effect (Figure 5). *T. bellirica* fruit, *T. brownii* bark and *T. chebula* fruit methanol extracts significantly blocked the liberation of paranitroaniline from S2302 by plasma kallikrein by 65%, 45% and 85%, respectively (Figure 5). Black tea aqueous extract was found to have a significant anti-inflammatory effect (Chatterjee P et al. 2012). Additionally, black tea is one of the most common beverage worldwide (Graham H.N et al. 1991; Monika Mueller. 2010). Investigations indicated that black tea aqueous extract significantly inhibited the hydrolysis of
S2302 (0.4 mM) by plasma kallikrein (2 nM) by 55%. At 200 mg/ml, methanol extracts of some other natural products also significantly inhibited plasma kallikrein activity; black pepper (35%) and cardamom (30%), cayenne pepper (15%), clove (15%), cumin (25%), poppy seed (50%), dill (20%), garlic (15%), ginger (17%) and rosemary (17%) (Figure 5). Red yeast rice extract was also tested on plasma kallikrein activity and showed no effect (Figure 5). Although the majority of the plant extracts inhibited kallikrein to various extents, we decided to continue evaluating the methanol extracts of all *Terminalia* species as well as the aqueous extract of black tea.

The methanol extracts of *T. arjuna* fruit and *T. arjuna* bark inhibited the hydrolysis of S2302 by plasma kallikrein with IC$_{50}$ values of 220 mg/ml and 280 mg/ml, respectively (Figures 6 & 7).

![Graph: Effect of Terminalia arjuna fruit extract on plasma kallikrein activity. Increasing concentrations of *T. arjuna* fruit extract were incubated with Kallikrein (2 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.](image)
Figure 7: Effect of *Terminalia arjuna* bark extract on plasma kallikrein activity. Increasing concentrations of *T. arjuna* bark extract were incubated with Kallikrein (2 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

The methanol extracts of *T. bellirica* fruit (Figure 8), *T. brownii* bark (Figure 9) and *T. chebula* fruit (Figure 10) showed inhibitory effects on the hydrolysis of S2302 by plasma kallikrein with IC$_{50}$ values of 65 mg/ml, 240 mg/ml and 30 mg/ml, respectively.

Figure 8: Effect of *Terminalia bellirica* fruit extract on plasma kallikrein activity. Increasing concentrations of *T. bellirica* fruit extract were incubated with Kallikrein (2 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.
Figure 9: Effect of *Terminalia brownii* bark extract on plasma kallikrein activity. Increasing concentrations of *T. brownii* bark extract were incubated with Kallikrein (2 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

![Graph showing the effect of *Terminalia brownii* bark extract on plasma kallikrein activity.](image)

Figure 10: Effect of *Terminalia chebula* fruit extract on plasma kallikrein activity. Increasing concentrations of *T. chebula* fruit extract were incubated with Kallikrein (2 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

![Graph showing the effect of *Terminalia chebula* fruit extract on plasma kallikrein activity.](image)

Black tea aqueous extract blocked the activity of plasma kallikrein in fluid phase with an IC$_{50}$ value of 220 mg/ml (Figure 11).
Figure 1: Effect of black tea aqueous extract on plasma kallikrein activity. Increasing concentrations of black tea aqueous extract were incubated with Kallikrein (2 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

We have also tested black tea major compounds including epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), caffeine and quercetin on plasma kallikrein activity in fluid phase, but all showed no effect (Figure 32).

B) Effects of natural product extracts and/or their bioactive compounds on plasma kallikrein generation on HPAEC:

Plasma kallikrein can be generated from the activation of PK on the endothelial cell surface via two stimuli, activated factor XII (FXIIa) on negatively charged surfaces and prolylcarboxypeptidase (PRCP) (Figure 1) (Webster, 1968; Mandle, Jr. and Kaplan, 1977; Shariat-Madar et al., 2002; Wuepper and Cochrane, 1972). Thus, we aimed to test the effects of the plant extracts and the pure compounds on kallikrein produced as a result of PK activation on endothelial cell
surface. HPAECs were treated with 30 nM HMWK followed by 30 nM PK in the presence of increasing concentrations of the extracts or the compounds.

Both *T. arjuna* fruit and *T. arjuna* bark inhibited the hydrolysis of S2302 by kallikrein produced on HPAEC with IC$_{50}$ values of 20 and 160 mg/ml, respectively (Figures 12 & 13).

**Figure 12:** Effect of *Terminalia arjuna* fruit extract on plasma kallikrein formed on endothelial cells. HPAECs were incubated with HMWK (30 nM) for 1 hr at 37 °C. Then the cells were incubated with PK (30 nM) in the presence of increasing concentrations of *T. arjuna* fruit extract for 1 hr at 37 °C. Next, the cells were incubated with S2302 (0.5 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

**Figure 13:** Effect of *Terminalia arjuna* bark extract on plasma kallikrein formed on endothelial cells. HPAECs were incubated with HMWK (30 nM) for 1 hr at 37 °C. Then the cells were incubated with PK (30 nM) in the presence of increasing concentrations of *T. arjuna* bark extract for 1 hr at 37 °C. Next, the cells were incubated with S2302 (0.5 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.
*T. bellirica* fruit (Figure 14), *T. brownii* bark (Figure 15) and *T. chebula* fruit (Figure 16) extracts also inhibited the generation of kallikrein on the endothelial cells with IC$_{50}$ values of 20, 80 and 15 mg/ml, respectively.

**Figure 14**: Effect of *Terminalia bellirica* fruit extract on plasma kallikrein formed on endothelial cells. HPAECs were incubated with HMKW (30 nM) for 1 hr at 37 °C. Then the cells were incubated with PK (30 nM) in the presence of increasing concentrations of *T. bellirica* fruit extract for 1 hr at 37 °C. Next, the cells were incubated with S2302 (0.5 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

**Figure 15**: Effect of *Terminalia brownii* bark extract on plasma kallikrein formed on endothelial cells. HPAECs were incubated with HMKW (30 nM) for 1 hr at 37 °C. Then the cells were incubated with PK (30 nM) in the presence of increasing concentrations of *T. brownii* bark extract for 1 hr at 37 °C. Next, the cells were incubated with S2302 (0.5 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.
Figure 16: Effect of *Terminalia chebula* fruit extract on plasma kallikrein formed on endothelial cells. HPAECs were incubated with HMWK (30 nM) for 1 hr at 37 °C. Then the cells were incubated with PK (30 nM) in the presence of increasing concentrations of *T. chebula* fruit extract for 1 hr at 37 °C. Next, the cells were incubated with S2302 (0.5 mM) for 1 hr at 37 °C 1 hr. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

We have tested the activity of two *Terminalia* isolated compounds on PK activation on HMWK bound to HPAECs. Arjunoglucoside II and arjunic acid inhibited the generation of kallikrein in a concentration dependent manner with IC$_{50}$ values of 300 and 240 μM, respectively (*Figures 17 & 18*).
**Figure 17:** Effect of arjunoglucoside II on plasma kallikrein formed on endothelial cells. HPAECs were incubated with HMWK (30 nM) for 1 hr at 37 °C. Then the cells were incubated with PK (30 nM) in the presence of increasing concentrations of arjunoglucoside II for 1 hr at 37 °C. Next, the cells were incubated with S2302 (0.5 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

**Figure 18:** Effect of arjunic acid on plasma kallikrein formed on endothelial cells. HPAECs were incubated with HMWK (30 nM) at 37 °C for 1 hr. Then the cells were incubated with PK (30 nM) in the presence of increasing concentrations of arjunic acid for 1 hr at 37 °C. Next, the cells were incubated with S2302 (0.5 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.
We tested the effect of black tea aqueous extract on the generation of kallikrein on endothelial cells and indicated that black tea blocked kallikrein with an IC$_{50}$ value of 400 mg/ml (Figure 19).

Figure 19: Effect of black tea aqueous extract on plasma kallikrein formed on endothelial cells. HPAECs were incubated with HMWK (30 nM) for 1 hr at 37 °C. Then the cells were incubated with PK (30 nM) in the presence of increasing concentrations of black tea aqueous extract for 1 hr at 37 °C. Next, the cells were incubated with S2302 (0.5 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

C) Effects of natural product extracts and/or their bioactive compounds on FXIIa activity:

Because FXIIa can activate PK to kallikrein, which in turn results in the generation of BK (Kaplan AP et al. 2003; Fernando LP et al. 2003), we aimed to determine whether Terminalias and black tea extracts could inhibit FXIIa activity. The results indicated that *T. arjuna* fruit (Figure 20), *T. arjuna* bark (Figure 21), *T. bellirica* fruit (Figure 22) and *T. chebula* fruit (Figure 23) extracts blocked FXIIa
activity with IC$_{50}$ values of 190, 190, 50 and 30 mg/ml, respectively. *T. brownii* bark extract showed a minor inhibitory activity in amidolytic system. The extract from *T. brownii* bark had the least inhibitory effect on FXIIa activity. It inhibited FXIIa by 27% at highest concentration (Figure 24).

**Figure 20: Effect of Terminalia arjuna fruit extract on FXIIa activity.** Increasing concentrations of *T. arjuna* fruit extract were incubated with FXIIa (17 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

**Figure 21: Effect of Terminalia arjuna bark extract on FXIIa activity.** Increasing concentrations of *T. arjuna* bark extract were incubated with FXIIa (17 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.
Figure 22: Effect of Terminalia bellirica fruit extract on FXIIa activity. Increasing concentrations of *T*. *bellirica* fruit extract were incubated with FXIIa (17 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

Figure 23: Effect of Terminalia chebula fruit extract on FXIIa activity. Increasing concentrations of *T*. *chebula* fruit extract were incubated with FXIIa (17 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.
**Figure 24:** Effect of *Terminalia brownii* bark extract on FXIIa activity. Increasing concentrations of *T. brownii* bark extract were incubated with FXIIa (17 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

**Figure 25:** Effect of black tea aqueous extract on FXIIa activity. Increasing concentrations of black tea aqueous extract were incubated with FXIIa (17 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.
Black tea aqueous extract blocked the activity of FXIIa with an IC_{50} value of 27 mg/ml (Figure 25). Black tea major compounds including epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), caffeine and quercetin were ineffective in inhibiting FXIIa activity (Figure 32).

On the effects of Terminalia pure compounds, among all tested compounds, arjunoglucoside II and arjunic acid showed inhibitory effects with IC_{50} values of 370 and 780 µM, respectively (Figures 26 & 27).

Figure 26: Effect of arjunoglucoside II on FXIIa activity. Increasing concentrations of arjunoglucoside II were incubated with FXIIa (17 nM) and S2302 (0.4 mM) for 1 hr at 37 °C (structure is shown). The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.
**Figure 27:** Effect of arjunic acid on FXIIa activity. Increasing concentrations of arjunic acid were incubated with FXIIa (17 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

D) Effects of natural product extracts and/or their bioactive compounds on recombinant PRCP activity:

Because PRCP is also an activator of PK to kallikrein on the endothelial cells, we aimed to determine whether *Terminalia* and black tea extracts could inhibit PRCP activity. Using recombinant human PRCP (rPRCP), we tested the methanol extracts of all *Terminalia* species as well as the aqueous extract of black tea on rPRCP activity. As described above in methods, increasing concentrations of extracts were incubated with rPRCP (30 µg/ml) and the substrate APpNA (0.5 mM). *T. arjuna* fruit, *T. chebula* fruit and *T. brownii* bark extracts blocked the release of paranitroaniline from APpNA by rPRCP with an IC$_{50}$ values of 150, 120 and 280 mg/ml, respectively (Figures 28, 29 & 30). *T. arjuna* bark and *T. bellirica* fruit did not inhibit rPRCP activity.
Figure 28: Effect of *Terminalia arjuna* fruit extract on rPRCP activity. Increasing concentrations of *T. arjuna* fruit extract were incubated with rPRCP (30 µg/ml) and the substrate APpNA (0.3 mM) for 1 hr at 37 °C. The absorbance of paranitronilide was detected at 405 nm. Data represent the mean ± SEM of three experiments.

Figure 29: Effect of *Terminalia chebula* fruit extract on rPRCP activity. Increasing concentrations of *T. chebula* fruit extract were incubated with rPRCP (30 µg/ml) and the substrate APpNA (0.3 mM) for 1 hr at 37 °C. The absorbance of paranitronilide was detected at 405 nm. Data represent the mean ± SEM of three experiments.
Figure 30: Effect of Terminalia brownii bark extract on rPRCP activity. Increasing concentrations of *T. brownii* bark were incubated with rPRCP (30 µg/ml) and the substrate APPNA (0.3 mM) for 1 hr at 37 °C. The absorbance of paranitronilide was detected at 405 nm. Data represent the mean ± SEM of three experiments.

Black tea aqueous extract inhibited rPRCP with IC₅₀ value of 160 mg/ml (Figure 31). Black tea major compounds showed no effect on rPRCP activity (Figure 32).

Figure 31: Effect of black tea aqueous extract on rPRCP activity. Increasing concentrations of black tea aqueous extract were incubated with rPRCP (30 µg/ml) and the substrate APPNA (0.3 mM) for 1 hr at 37 °C. The absorbance of paranitronilide was detected at 405 nm. Data represent the mean ± SEM of three experiments.
Figure 32: Effect of black tea pure compounds on plasma kallikrein, FXIIa and rPRCP activities. Panel A Increasing concentrations of epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC) were incubated with Kallikrein (2 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments. Panel B Increasing concentrations of EGCG, EGC, ECG and EC were incubated with FXIIa (17 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments. Panel C Increasing concentrations of EGCG, EGC, ECG and EC were incubated with rPRCP (30 µg/ml) and the substrate APpNA (0.5 mM) for 1 hr at 37 °C. The absorbance of paranitronilide was detected at 405 nm. Data represent the mean ± SEM of three experiments.

E) Effects of natural product extracts and/or their bioactive compounds on HPAEC viability using the MTT assay:

To test whether the inhibitory effects of tested extracts on the generation of plasma kallikrein from PK are not from their effects on HPAEC viability, we determined if these extracts were directly toxic to the endothelial cells using the MTT assay in which the yellow tetrazole is reduced to purple formazan only in living cells. While T. arjuna fruit showed no significant effects on HPAECs viability (Figure 33), T. arjuna bark was significantly reduced HPAECs viability by 40% at concentrations
equal to or greater than 30 mg/ml (Figure 34).

**Figure 33:** Effect of *Terminalia arjuna* fruit extract on HPAEC viability using the MTT assay. Bar diagrams showing the results of HPAECs viability (% of control). HPAECs treated by *T. arjuna* fruit extract using the MTT assay. The wells containing HPAECs were treated *T. arjuna* fruit extract for its effect on cell viability, and the wells containing only a complete growth medium were used as a control. Data represent the mean ± SEM of three experiments.

**Figure 34:** Effect of *Terminalia arjuna* bark extract on HPAEC viability using the MTT assay. Bar diagrams showing the results of HPAECs viability (% of control). HPAECs treated by *T. arjuna* bark extract using the MTT assay. The wells containing HPAECs were treated *T. arjuna* bark extract for its effect on cell viability, and the wells containing only a complete growth medium were used as a control. Data represent the mean ± SEM of three experiments. [****P ≤ 0.0001 compare to % control].
*T. bellirica fruit* and *T. brownii bark* were significantly toxic to HPAECs in which *T. bellirica fruit* exhibited its cytotoxic effect in dose-dependent manner (*Figure 35*) and *T. brownii bark* reduced HPAECs viability at concentrations equal to or greater than 20 mg/ml by approximately 40% indicating its toxic effect on endothelial cells (*Figure 36*).

![Figure 35: Effect of Terminalia bellirica fruit extract on HPAEC viability using the MTT assay.](image)

*Figure 35: Effect of Terminalia bellirica fruit extract on HPAEC viability using the MTT assay.* Bar diagrams showing the results of HPAECs viability (% of control). HPAECs treated by *T. bellirica fruit* extract using the MTT assay. The wells containing HPAECs were treated *T. bellirica fruit* extract for its effect on cell viability, and the wells containing only a complete growth medium were used as a control. Data represent the mean ± SEM of three experiments. [*P ≤ 0.05, ***P ≤ 0.001, ****P ≤ 0.0001 compare to % control*]
**Figure 36:** Effect of *Terminalia brownii* bark extract on HPAEC viability using the MTT assay. Bar diagrams showing the results of HPAECs viability (% of control). HPAECs treated by *T. brownii bark* extract using the MTT assay. The wells containing HPAECs were treated *T. brownii bark* extract for its effect on cell viability, and the wells containing only a complete growth medium were used as a control. Data represent the mean ± SEM of three experiments. [**P ≤ 0.01, ***P ≤ 0.001 compare to % control]

Interestingly, *T. chebula fruit* extract exhibited no effects on HPAECs viability at concentrations up to 500 mg/ml (*Figure 37*). Among all *Terminalias*, the results of *T. chebula fruit* extract suggested that the inhibitory effect of the methanol extract of *T. chebula* fruit on kallikrein produced on endothelial cell surface is not due to the cytotoxicity to endothelial cells. Thus, methanol extract of *T. chebula* fruit might be considered a safe natural supplement inhibitor of kallikrein.

We have tested the two compounds from *Terminalia* that showed effects on the generation of kalikrein on HPAECs to explore their effects on HPAECs viability. We indicated that arjunoglucoside II significantly deceased the viability of cells only at high dose up to 1 mM (*Figure 38*), but arjunic acid induced cellular toxicity at concentrations equal to or greater than 300 μM (*Figure 39*).
Figure 37: Effect of *Terminalia chebula* fruit extract on HPAEC viability using the MTT assay. Bar diagrams showing the results of HPAECs viability (% of control). HPAECs treated by *T. chebula* fruit extract using the MTT assay. The wells containing HPAECs were treated *T. chebula* fruit extract for its effect on cell viability, and the wells containing only a complete growth medium were used as a control. Data represent the mean ± SEM of three experiments.

Figure 38: Effect of arjunoglucoside II on HPAEC viability using the MTT assay. Bar diagrams showing the results of HPAECs viability (% of control). HPAECs treated by arjunoglucoside II using the MTT assay. The wells containing HPAECs were treated arjunoglucoside II for its effect on cell viability, and the wells containing only a complete growth medium were used as a control. Data represent the mean ± SEM of three experiments. [*P ≤ 0.05 compare to % control*]
**Figure 39:** Effect of arjunic acid on HPAEC viability using the MTT assay. Bar diagrams showing the results of HPAECs viability (% of control). HPAECs treated by arjunic acid using the MTT assay. The wells containing HPAECs were treated arjunic acid for its effect on cell viability, and the wells containing only a complete growth medium were used as a control. Data represent the mean ± SEM of three experiments. [*P ≤ 0.05, ****P ≤ 0.0001 compare to % control*]

The aqueous extract of black tea had no significant effect on HPAECs viability after 1 hr treatment (**Figure 40A**). However, the aqueous extract of black tea induced a higher cytotoxicity on HPAECs compare to the control after 24 hours of incubation at 37 °C. The results indicated that 100 mg/ml black tea aqueous extract decreased cellular viability by 80% (**Figure 40B**).
Figure 40: Effect of black tea aqueous extract (1hr & 24 hrs treatments) on HPAEC viability using the MTT assay. Bar diagrams showing the results of HPAECs viability (% of control). HPAECs treated by black tea aqueous extract for 1 hr or 24 hrs using the MTT assay. The wells containing HPAECs were treated with black tea aqueous extract for its effect on cell viability, and the wells containing only a complete growth medium were used as a control. Data represent the mean ± SEM of three experiments. [****P ≤ 0.0001 compare to % control].
F) Effects of the aqueous extract of black tea on FXIIa-induced HPAEC migration:

To confirm the inhibitory effect exhibited by the aqueous extract of black tea on FXIIa activity, we tested the effect of the aqueous extract of black tea on FXIIa-induced HPAEC migration. Evidence suggests that FXII stimulates the proliferation of endothelial cell (Gretchen A. LaRusch et al. 2010). FXII was found to significantly affect cell migration in the low nanomolar range (60 to 240 nM). Contact of plasma with the negatively charged surfaces results in binding and autoactivation of FXII to FXIIa (Moreau ME et al. 2005).

To determine at which concentrations the aqueous extract of black tea itself can inhibit HPAEC migration, we investigated the concentration-dependent effects of the aqueous extract of black tea ranging from 5 to 100 mg/ml. The aqueous extract of black tea significantly inhibited HPAECs migration only at 100 mg/ml (Figure 41). Since the aqueous extract of black tea did not inhibit HPAECs migration at concentrations up to 50 mg/ml, we assessed the effect of 50 mg/ml black tea aqueous extract on FXIIa-induced HPAEC migration. We found that 50 mg/ml was significantly inhibited FXIIa-induce HPAEC migration confirming our previous finding on the effect of black tea aqueous extract on FXIIa activity in fluid phase (Figure 42).
Figure 41: Effects of black tea aqueous extract on HPAEC migration. HPAECs were seeded in a 96-well plate and incubated overnight at 37 °C to achieve confluence. Then a denuded area was created with a pipette tip. Next, the plate was incubated with increasing concentrations of black tea extract for 24 hrs and pictures were taken at 0 hr and 24 hrs. Data represent the mean ± SEM of three experiments. [****P ≤ 0.0001 compare to control]

G) Effects of natural product extracts and/or their bioactive compounds on FXIa activity:

Investigations were performed to determine selective inactivation of the serine protease FXI by the methanol extract of *Terminalia chebula fruit*. FXI is produced in the liver as a zymogen and circulates in human plasma in complex with HMWK at an approximate concentration of 3-7 μg/ml (Bouma, B.N et al. 1977; Roger C. Wiggins et al. 1977; Thompson, R. E et al. 1977). Thus, we tested the effect of *Terminalia* compounds on FXIa activity in fluid phase (Table 5).
Figure 42: Effects of black tea aqueous extract on FXIIa-induced HPAEC migration. HPAECs were seeded in a 96-well plate and incubated overnight at 37 °C to achieve confluence. Then a denuded area was created with a pipette tip. Next, the plate was incubated with FXIIa (240 nM) in the presence and absence of 10 or 50 mg/ml black tea extract and pictures were taken at 0 hr and 24 hrs (pictures are shown). Data represent the mean ± SEM of three experiments. [****p ≤ 0.0001 compared to Control, ####p ≤ 0.0001 compared to FXIIa (240 nM)].

We next studied the effects of the methanol extract of *T. chebula* fruit on FXI activation on endothelial cell surface. To determine the effect of the methanol extract of *T. chebula* fruit on FXIa activity on endothelial cells, HPAEC were treated with 80 nM HMWK, followed by slow increase in the concentrations of *T. chebula* fruit extract in the presence of 40 nM FXI. The activity of FXIa on HPAEC was assessed by the S2366 substrate hydrolysis. We found that *T. chebula* fruit extract blocked FXIa activity on endothelial cells with an IC\textsubscript{50} value of 35 mg/ml (Figure 43).
**Figure 43:** Effects of *Terminalia chebula* fruit extract on FXIa activity on endothelial cells. HPAECs were incubated with HMWK (80 nM) for 1 hr at 37 °C. Then the cells were incubated with FXI (40 nM) in the presence of increasing concentrations of *T. chebula* fruit extract for 1 hr at 37 °C. Next, the cells were incubated with S2366 (0.5 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2366 was detected at 405 nm. Data represent the mean ± SEM of three experiments.
Table 5: Summary of effects of *Terminalia* compounds on plasma kallikrein, FXIIa, FXIa and rPRCP

<table>
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<tr>
<th>Terminalia Compounds</th>
<th>Structure</th>
<th>Kallikrein</th>
<th>FXIIa</th>
<th>FXIa</th>
<th>rPRCP</th>
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<td>NE</td>
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<tr>
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<td>NE</td>
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<td>NE</td>
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<td>NE</td>
<td>NE</td>
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<td>NE</td>
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<td>NE</td>
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**NE:** no effect
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</thead>
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<td>(R)-6-(2-((1S, 2S)-2,6-dimethyl-1,2-dihydrophtalene-1-yl)ethyl)-5,6-dihydro-2H-pyran-2-one</td>
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<td>NE</td>
</tr>
<tr>
<td>α,β-Dehydrodihydro monacolin K</td>
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<td>NE</td>
</tr>
<tr>
<td>Dehydromonacolin K</td>
<td></td>
<td>NE</td>
</tr>
<tr>
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<tr>
<td>Compound</td>
<td>Structure</td>
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<td>------------------------------------------------</td>
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<tr>
<td>Monacolin K (Lovastatin)</td>
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<td></td>
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<tr>
<td>Dihydromonacolin K</td>
<td><img src="image2" alt="Dihydromonacolin K structure" /></td>
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</tr>
<tr>
<td>(6R)-6-((1R, 2R, 3S, 6R)-3-hydroxy-2,6-dimethyl-1,2,3,5,6,7,8,8a-octahydropyrene-1-yl)ethyl)-5,6-dihydro-2H-pyran-2-one</td>
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<tr>
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</tr>
<tr>
<td>-------------------------------------------------------------------------</td>
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<tr>
<td>2H-Pyran-2-one, 6-[2-(1,2-dihydro-2,6-dimethyl-1-naphthalenyl)ethyl]</td>
<td>tetrahydro-4-hydroxy-, ([4R-{4\alpha, 6\beta(1S^<em>,2S^</em>)}])</td>
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<td>(2S)-(3R, 4S, 6S, 7R, 8S)-4,6-dihydroxy-3,7-dimethyl-8-(2-((R)-6-oxo-3,6-dihydro-2H-pyran-2-yl)ethyl)-1,2,3,4,6,7,8,8a-octahydronaphthalen-1-yl 2-methylbutanoate</td>
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<td>(\beta, 8)-dihydroxy-7-[(1,2,3,5,6,7,8,8a-octahydro-3,5-dihydroxy-2,6-dimethyl-8-(2-methylbutyryloxy)naphthalen-1-yl]-heptanoic acid (\delta)-lactone</td>
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</table>
RYR extract has a cholesterol lowering properties. It is composed of 13 natural statins, unsaturated fatty acids, ergosterol, amino acids, flavonoids, alkaloid and trace element. The previous studies have indicated the lipid lowering and anti-inflammatory properties of xuezhiwang, a commercial RYR extract preparation (Heber D et al. 1999). We tested the effects of RYR extract as well as its pure compounds on plasma kallikrein, FXIa, FXIIa and rPRCP. The results indicated that RYR extract has no effects on plasma kallikrein (Figure 44), FXIIa (Figure 45) and FXIa, with a minor inhibitory effect on rPRCP 30% inhibition at 200 mg/ml (Figure 46).
Figure 44: Effect of red yeast rice extract on plasma kallikrein activity. Increasing concentrations of RYR extract was incubated with Kallikrein (2 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.

Figure 45: Effect of red yeast rice extract on FXIIa activity. Increasing concentrations of RYR extract were incubated with FXIIa (17 nM) and S2302 (0.4 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2302 was detected at 405 nm. Data represent the mean ± SEM of three experiments.
Figure 46: Effect of red yeast rice extract on rPRCP activity. Increasing concentrations of RYR extract was incubated with rPRCP (30 µg/ml) and the substrate APpNA (0.3 mM) for 1 hr at 37 °C. The absorbance of paranitronilide was detected at 405 nm. Data represent the mean ± SEM of three experiments.

During the course of screening of the RYR pure compounds on kallikrein, FXIa and FXIIa, three of them showed inhibitory effects on FXIa (Table 6). Accordingly, we decided to determine whether their effects are concentration dependent. The results indicated that dihydromonacolin K (Figure 47, Tables 6 & 9), 1-naphthalenepropanoic acid 1,2,4a,5,6,7,8,8a-octahydro-2,6-dimethyl-(1S,2S,4aR, 6R,8aS) (Figure 47, Tables 6 & 9) and α,β-dehydrodihydromonacolin K (Figure 47, Tables 6 & 9) have inhibitory effects with IC50 values of 260, 270 & 400, respectively.
Figure 47: Effects of RYR active compounds on FXIa activity. Increasing concentration of the RYR pure compounds (1 µM to 1000 µM) were incubated with 2 nM of FXIa and 0.291 mM of the substrate (Glu-Pro-Arg-p-Nitroanilide, S2366) in HEPES buffer for 1 hr at 37 ºC. Then the substrate hydrolysis was assessed spectrophotometrically at 405 nm. Data represent the mean ± SEM of three experiments.
Figure 48: Effects of RYR active compounds on FXIa activity on endothelial cells. HPAECs were incubated with HMWK (80 nM) for 1 hr at 37 °C. Then the cells were incubated with FXI (40 nM) in the presence of increasing concentrations of RYR compounds for 1 hr at 37 °C. Next, the cells were incubated with S2366 (0.5 mM) for 1 hr at 37 °C. The absorbance of hydrolyzed S2366 was detected at 405 nm. Data represent the mean ± SEM of three experiments.
IV-DISCUSSION

Investigations revealed for first time the effects of some important natural product extracts on kallikrein-kinin system. The major findings of this study are: (1) methanol extracts of different Terminalia species (T. arjuna fruit, T. arjuna bark, T. bellirica fruit, T. brownii bark and T. chebula) inhibited plasma kallikrein, PK activation on endothelial cell surfaces, FXIIa and rPRCP activities; (2) methanol extract of T. chebula fruit abolished the activation of FXI to FXIa on endothelial cell surfaces; (3) arjunoglucoside II and arjunic acid from Terminalias inhibited FXIIa activity; (4) aqueous extract of black tea inhibited the activities of plasma kallikrein, FXIIa, rPRCP and FXIIa-induced endothelial cell migration; (5) dihydromonacolin K, 1-naphthalenepropanoic acid 1,2,4a,5,6,7,8,8a-octahydro-2,6-dimethyl-(1S,2S,4aR, 6R,8aS) and α,β-dehydrodihydromonacolin K from red yeast rice inhibited the activity of FXIa.

Plasma kallikrein is implicated in the pathological processes inflammation and HAE through different systems plasma Kallikrein-Kinin, complement and fibrinolytic systems. These systems are regulated in plasma mainly by C1-INH. C1-INH inhibits FXIIa and kallikrein (Pixley et al., 1985). In addition, C1-INH inhibits the activation of the complements C1r and C1s in the complement system. In HAE, mutations of C1-INH gene cause a reduction in the levels of functional C1-INH protein, leading to an overproduction of BK.

Plasma kallikrein hydrolyzes HMWK to release the potent proinflammatory peptide BK. BK mediates its effects mainly via B2R (Leeb-Lundberg LM et al., 2005;
Marceau F et al., 1998). The binding of BK to B2R on endothelial cells results in an increase in the intracellular Ca^{2+} levels and subsequent production of endogenous vasodilators NO and PGI_{2} causing an increase in vascular permeability and edema (Zhao et al., 2001; Hong, 1980; Palmer et al. 1987). The deficiency of C1-INH results in reduced levels of plasma PK and HMWK (Gigli I et al., 1975; Trumpi-Kalshoven et al., 1978; Gallimore et al., 1979). BK is responsible for most of the symptoms associated with the edema in patients with acute HAE attacks (Nussberger et al. 2002). Undoubtedly, BK is deeply implicated in the pathophysiology and clinical manifestations of HAE.

We have screened several natural plant extracts listed in (Table 4) for their effects on FXIIa, kallikrein and rPRCP in the liberation of BK from HMWK that could be used for the purpose of HAE management. These natural products were selected based on their anti-inflammatory and cardiovascular effects reported in the literature as well as their safety and availability. Using a selected concentration 200 mg/ml, we explored the inhibitory effects of those extracts and accordingly continued our study on the extracts that showed the most promising effects. Methanol extracts of different species of Terminalia were tested for their inhibitory effects on plasma kallikrein activity in fluid phase using 200 mg/ml of each. T. arjuna fruit, T. bellirica fruit, T. brownii bark and T. chebula fruit significantly blocked the proteolytic activity of plasma kallikrein activity in fluid phase by 45%, 65%, 45% and 85%, respectively (Figure 5). In contrast, T. arjuna bark extract did not show a significant inhibitory effect on plasma kallikrein at the same concentration (Figure 5). The aqueous extract of black tea was selected for study for two main reasons, its significant anti-inflammatory effect (Chatterjee P et al. 2012) and the high consumption of black tea, in which it is considered one of the most common beverages worldwide (Graham
H.N et al. 1991; Monika Mueller. 2010). At 200 mg/ml, it was indicated that black tea aqueous extract in fluid phase significantly inhibited the hydrolysis of S2302 by plasma kallikrein by 55%. At the same selected concentration, methanol extracts of some other natural product extracts also significantly inhibited plasma kallikrein activity; black pepper (35%) and cardamom (30%), cayenne pepper (15%), clove (15%), cumin (25%), poppy seed (50%), dill (20%), garlic (15%), ginger (17%) and rosemary (17%) (Figure 5). The methanol extract of red yeast rice was also tested on plasma kallikrein activity and showed no effect (Figure 5). Initial studies showed that the methanol extracts of Terminalia species and the aqueous extract of black tea exhibited the highest inhibitory effects toward plasma kallikrein activity.

In the traditional Ayurvedic medicines, T. arjuna bark was used for different purposes including antidyslipidemic, anti-inflammatory, antioxidant and maintaining heart functions by strengthening and improving cardiac muscles (Chander R et al, 2004; Maulik SK et al. 2012). Although T. arjuna bark has no effects on kallikrein at highest concentrations, studies were done to compare it with other species.

The results indicated that T. arjuna fruit, T. arjuna bark, T. bellirica fruit, T. brownii bark and T. chebula fruit inhibited plasma kallikrein activity with IC$_{50}$ values of 220, 280, 65, 240 and 30 mg/ml, respectively (Figures 6, 7, 8, 9 & 10). T. arjuna bark showed stimulatory effects at lower concentrations, but at higher concentrations blocked plasma kallikrein activity. Among all the species tested, T. chebula fruit has shown the highest inhibitory effect on plasma kallikrein activity. We also determined the effects of Terminalias pure compounds. Compounds isolated from T. bellirica fruit were chebulagic acid, gallic acid, gallic acid methyl ester, (1, 2, 3, 6-tetra-O-galloyl-β-D-Glucose) and (1,2,3,4,6-penta-O-galloyl-β-D-Glucose). Compounds isolated from T. chebula fruit were arjunoglucoside II, arjunic acid, terminolic acid,
arjungenin, arjunglucoside I, chebuloside II, arjunolic acid, shikimic acid and ellagic acid. Three compounds were isolated from both *T. bellirica* fruit and *T. chebula* fruit were corilagin, chebulinic acid and (2, 3, 6-tri-O-galloyl-β-D-Glucose). At 300 μM, all tested compounds showed no inhibitory effects on plasma kallirein in fluid phase. Some compounds instead showed minor stimulatory effects on plasma kallikrein compared to the control. These include arjunolic acid and ellagic acid (from *T. chebula* fruit), 1,2,3,4,6-penta-O-galloyl-β-D-Glucose and gallic acid methyl ester (from *T. bellerica* fruit) with stimulatory effects of 19%, 26%, 16% and 13%, respectively. Ellagic acid showed the highest stimulatory effect on plasma kallikrein activity in fluid phase. Ellagic acid was also isolated from *T. bellerica* fruit (Pfundstein B et al. 2010). In addition, gallic acid and its derivatives such as (1,2,3,4,6-penta-O-galloyl-β-D-glucose) are predominant polyphenols in the methanol extract of *T. bellerica* fruit compare to *T. chebula* fruit. The greater presence of compounds with minor stimulatory effects on plasma kallikrein in *T. bellerica* than *T. chebula* probably explains the lower inhibitory effect of *T. bellerica* (IC₅₀ value of 65 mg/ml) compare to *T. chebula* (IC₅₀ value of 30 mg/ml) through their weak stimulatory effects. The presence of these stimulatory compounds in very low concentrations in the extracts probably allowed the inhibitory effects of the extracts to outweigh their minor plasma kallikrein stimulatory effects. The inhibitory effects exhibited by *Terminalias* extracts versus the isolated compounds could be due to the positive interactions between the components, in which the pharmacodynamic synergy might be crucial for their effects. The aqueous extract of black tea also blocked the activity of plasma kallikrein with an IC₅₀ value of 220 mg/ml (Figure 11).
Table 7: Summary of effects of natural product extracts on Kallikrein formed on HPAEC

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>Inhibition of Kallikrein formed on HPAEC surface</th>
<th>IC₅₀ values (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anise</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>Basil</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Black Pepper</td>
<td></td>
<td>500</td>
</tr>
<tr>
<td>Cinnamon</td>
<td></td>
<td>NE</td>
</tr>
<tr>
<td>Coriander</td>
<td></td>
<td>250</td>
</tr>
<tr>
<td>Fennel</td>
<td></td>
<td>NE</td>
</tr>
<tr>
<td>Mentha</td>
<td></td>
<td>300</td>
</tr>
<tr>
<td>Poppy seed</td>
<td></td>
<td>300</td>
</tr>
<tr>
<td>Thyme</td>
<td></td>
<td>175</td>
</tr>
<tr>
<td>Black tea</td>
<td></td>
<td>400</td>
</tr>
<tr>
<td>Terminalia T. arjuna fruit</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Terminalia T. arjuna bark</td>
<td></td>
<td>160</td>
</tr>
<tr>
<td>Terminalia T. bellirica fruit</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Terminalia T. brownii bark</td>
<td></td>
<td>80</td>
</tr>
<tr>
<td>Terminalia T. chebula fruit</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Red yeast rice</td>
<td></td>
<td>NE</td>
</tr>
</tbody>
</table>

NE: no effect
Table 8: Summary of effects of natural product extracts on Kallikrein, FXIIa and rPRCP

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>% kallikrein inhibition (200 mg/ml)</th>
<th>% rPRCP inhibition (200 mg/ml)</th>
<th>% FXIIa inhibition (200 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anise</td>
<td>NE</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>Basil</td>
<td>NE</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>Black Pepper</td>
<td>35</td>
<td>30</td>
<td>NE</td>
</tr>
<tr>
<td>Cinnamon</td>
<td>NE</td>
<td>70</td>
<td>65</td>
</tr>
<tr>
<td>Coriander</td>
<td>NE</td>
<td>25</td>
<td>NE</td>
</tr>
<tr>
<td>Fennel</td>
<td>NE</td>
<td>52</td>
<td>NE</td>
</tr>
<tr>
<td>Mentha</td>
<td>NE</td>
<td>45</td>
<td>30</td>
</tr>
<tr>
<td>Poppy seed</td>
<td>50</td>
<td>22</td>
<td>NE</td>
</tr>
<tr>
<td>Thyme</td>
<td>NE</td>
<td>23</td>
<td>NE</td>
</tr>
<tr>
<td>Black tea</td>
<td>55</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td><strong>Terminalia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. arjuna fruit</td>
<td>45</td>
<td>65</td>
<td>55</td>
</tr>
<tr>
<td>T. arjuna bark</td>
<td>NE</td>
<td>NE</td>
<td>55</td>
</tr>
<tr>
<td>T. bellirica fruit</td>
<td>65</td>
<td>NE</td>
<td>90</td>
</tr>
<tr>
<td>T. brownii bark</td>
<td>45</td>
<td>NE</td>
<td>20</td>
</tr>
<tr>
<td>T. chebula fruit</td>
<td>85</td>
<td>80</td>
<td>98</td>
</tr>
<tr>
<td>Red yeast rice</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
</tbody>
</table>

NE: no effect
The major black tea compounds included epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), caffeine and quercetin, which showed no inhibitory effects on plasma kallikrein activity (Figure 32). We have also tested black tea extract with more than 80% theaflavins (theaflavin and theaflavin gallates) and also showed no effect. In conclusion, our data suggest that *Terminalia* and black tea extracts have specific effects on the components of KKS. In addition, our data indicate the presence of uncharacterized inhibitor(s) of kallikrein. All *Terminalia* methanol extracts (except *T. arjuna* bark) as well as the black tea aqueous extract inhibited plasma kallikrein activity. The results indicated that *T. chebula* fruit extract showed the highest inhibitory effect on plasma kallikrein activity with an IC\textsubscript{50} value of 30 mg/ml (Figure 10).

Plasma kallikrein is produced and released from the liver as a zymogen (prekallikrein) that is circulated in plasma as a complex with HMWK, which functions as a cofactor for the activation PK (Kaplan AP et al. 2003). The assembly of the HMWK-PK complex on endothelial cell surfaces resulted in a rapidly activation of PK to kallikrein. This activation is mediated by the interaction of HMWK with a complex of several proteins including cytokeratin 1, gC1qR as well as urokinase plasminogen activator receptor (uPAR) (Joseph K et al. 1999; Mahdi F et al. 2001).

The results indicated that the methanol extracts of the *Terminalia* species and the aqueous extract of black tea blocked the activity of plasma kallikrein in fluid phase. Next, we determined the pharmacological effects of the methanol extracts of the *Terminalia* species and the aqueous extract of black tea on the formation of kallikrein on endothelial cells (HPAEC). The investigations indicated that *T. arjuna* fruit, *T. arjuna* bark, *T. bellirica* fruit, *T. brownii* bark and *T. chebula* fruit inhibited the kallikrein produced on HPAEC with IC\textsubscript{50} values of 20 and 160, 20, 80 and 15 mg/ml,
respectively (Figures 12, 13, 14, 15 & 16). We also indicated that the aqueous extract of black tea blocked kallikrein formed on endothelial cells (HPAEC) with an IC$_{50}$ value of 400 mg/ml (Figure 19). The inhibitory effects of these extracts might be due to either directly blocking effects on the activity of kallikrein produced by the activation of the HMWK-PK complex on HPAEC or due to interference with the assembly of this complex on HPAEC surfaces. In conclusion, all *Terminalias* methanol extracts as well as the black tea aqueous extract abolished kallikrein generation on HPAEC surfaces.

In HAE patients, the reduction in the C1-INH levels causes overproduction of FXIIa to initiate a contact system leading to a local production of BK (Moreau ME et al. 2005; Weiss R et al. 1986). Thus, studies indicated that FXIIa might be implicated in HAE disease either through converting PK to kallikrein or activating of its autoactivation cycle producing more FXIIa, and ultimately up-regulates kallikrein generation (Kaplan AP et al. 2003; Fernando LP et al. 2003). It was reported that PRCP and Heat Shock Protein-90 (HSP90) could also directly activate PK to kallikrein on endothelial cells independently from FXII-activation pathway (Shariat-Madar et al. 2002; Joseph K et al. 2009). Thus, PRCP-dependent PK activation might be considered as an additional BK-forming mechanism leading to additional NO and PGI$_2$ production. Several stimuli were found to activate PK to kallikrein on endothelial cells such as FXIIa and PRCP, we determined whether *Terminalias* methanol extracts as well as the black tea aqueous extract could inhibit FXIIa and rPRCP activities in fluid phases. Our results indicated that *T. chebula fruit* also showed the highest inhibitory effect on FXIIa activity in fluid phase with IC$_{50}$ value of 30 mg/ml compared to the effects of *T. arjuna fruit, T. arjuna bark and T. bellirica fruit* which blocked FXIIa activity with IC$_{50}$ values of 190, 190 and 50 mg/ml, respectively.
(Figures 20, 21, 22 & 23). The results also indicated that T. brownii bark showed the weakest inhibitory effect with only 27% reduction in the activity of FXIIa at concentration up to 300 mg/ml (Figure 24). Investigation of the Terminalias pure compounds revealed that the triterpene glucoside, arjunoglucoside II and an aglycone terpenoid, arjunic acid blocked the activity of FXIIa activity with IC\textsubscript{50} values of 370 and 780 µM, respectively (Figures 26 & 27). The study also indicated that ellagic acid and (1,2,3,4,6-penta-O-galloyl-β-D-glucose) increased FXIIa activity by 25% and 23%, respectively. Previously, it was reported that ellagic acid could activate FXII (Hageman factor) to FXIIa in a mechanism similar to the negatively charged surfaces such as glass or kaolin (Ratnoff OD and Saito H. 1982) and can be used as a model to induce Hageman factor activation experimentally (Ratnoff OD. 1981). The low concentration of ellagic acid among mixture of compounds in Terminalias extract was not enough to interfere with extract inhibitory properties (Table 5).

On the effect of the aqueous extract of black tea on FXIIa in fluid phase, we found that the aqueous extract of black tea also blocked the activity of FXIIa in a dose-dependent manner with an IC\textsubscript{50} value of 27 mg/ml (Figure 25). Additionally, we also found that black tea pure compounds including epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), caffeine and quercetin showed no inhibitory effects on FXIIa activity (Figure 32). The inhibitory effect of the aqueous extract of black tea on FXIIa-induced HPAEC migration was assessed. To determined which concentrations of black tea aqueous extract can inhibit HPAEC migration, a dose response was performed with increasing concentrations of tea 5-100 mg/ml and indicated that the aqueous extract of black tea significantly inhibited HPAEC migration only at concentration up to 100
mg/ml (Figure 41). Using the highest concentration of FXII 240 nM to induce HPAEC migration, it was found that the aqueous extract of black tea at 50 mg/ml was significantly inhibited FXIIa-induce HPAEC migration, confirming the inhibitory effect of black tea aqueous extract on FXIIa activity (Figure 42). On the effect of extracts on rPRCP activity, studies indicated that T. arjuna fruit, T. chebula fruit and T. brownii bark extracts blocked rPRCP activity with an IC50 values of 150, 120 and 280 mg/ml, respectively (Figures 28, 29 & 30). T. arjuna bark and T. bellirica fruit did not block rPRCP activity. Furthermore, all Terminalia pure compounds showed no effects on rPRCP activity. While black tea aqueous extract blocked rPRCP activity with IC50 values of 160 mg/ml, pure compounds epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), caffeine and quercetin showed no inhibitory effects on rPRCP activity in fluid phase (Figures 31 & 32). In addition to the evidence presented above, which demonstrates the inhibitory effects of the methanol extracts of Terminalias and the aqueous extract of black tea on kallikrein activity, we were interested in determining whether Terminalias and black tea aqueous extracts are capable of reducing cell viability. While T. arjuna fruit showed no significant effects on HPAECs viability (Figure 33), T. arjuna bark significantly reduced HPAECs viability by 40% at concentrations as low as 30 mg/ml using cell survival ratio using the MTT assay (Figure 34). A previous study indicated that T. arjuna fruit extract was capable of maintaining hepatocytes viability and reducing cadmium-induced hepatic oxidative stress using the MTT assay (J. Ghosh et al. 2010). In the present study, we confirmed the capability of T. arjuna fruit extract to maintain cell viability of HPAECs using the MTT assay. Our finding also confirmed that a component of T. arjuna fruit is an inhibitor of kallikrein. On the other hand, T. bellirica fruit and T. brownii bark
were significantly toxic to HPAECs in which *T. bellirica fruit* exhibited a cytotoxic effect in a dose-dependent manner and *T. brownii bark* reduced HPAECs viability at concentrations as low as 20 mg/ml by approximately 40% indicating its highly toxic effect on endothelial cells (*Figures 35 & 36*). Interestingly, *T. chebula fruit* extract exhibited no effects on HPAECs viability at concentrations up to 500 mg/ml (*Figure 37*). A previous study reported that *T. chebula fruit* extract significantly decreased rat pheochromocytoma cells (PC12) death induced by oxygen-glucose deprivation followed by reoxygenation (OGD-R), H2O2 and LPS suggesting a protective effect of *T. chebula fruit* extract through its antioxidant and antiinflammatory properties (Gaire BP et al. 2013). In this study, we confirmed that *T. chebula fruit* extract was capable of maintaining cell viability of HPAECs using the MTT assay (*Figure 37*). Among all *Terminalias*, the results of *T. chebula fruit* extract suggested that the inhibitory effect of the methanol extract of *T. chebula fruit* on kallikrein produced on endothelial cell surfaces is not due to a direct cytotoxic effect to endothelial cells.

While arjunoglucoside II significantly deceased the viability of cells only at high dose up to 1 mM (*Figure 38*), arjunic acid reduced HPAEC viability by 20% at concentration 300 μM and by approximately 100% at 1 mM (*Figure 39*). The investigation revealed that black tea aqueous extract has no significant effect on HPAECs viability after 1 hr treatment. On the contrary, HPAECs viability was significantly reduced by approximately 80% at concentrations starting from 100 mg/ml after 24 hours treatment (*Figure 40*). Taken all together, our data indicated that the extracts of *T. chebula fruit* and *T. arjuna* fruit were capable of inhibiting kinin-generating enzymes and did not contain cytotoxic factors for HPAEC at the effective concentrations.

In order to further characterize the selectivity of the most active extract *T.
chebula fruit extract to plasma kallikrein and also due to the structural similarities between plasma kallikrein and FXIa, we have determined the ability of T. chebula fruit extract to inhibit the activation of FXI to FXIa on HPAEC surfaces. The study indicated that T. chebula fruit extract blocked FXIa activity on HPAECs with an IC₅₀ value of 35 mg/ml (Figure 43). We also tested the effects of Terminalias compounds on FXIa activity in fluid phase.

**Table 9:** Summary of the pure secondary metabolites identified in our study

<table>
<thead>
<tr>
<th>Plant source</th>
<th>Compounds</th>
<th>Structure</th>
<th>Inhibitory effects with IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kallikrein</td>
</tr>
<tr>
<td>Terminalia</td>
<td>Arjunoglucoside II</td>
<td>[Structure Image]</td>
<td>NE</td>
</tr>
<tr>
<td>Terminalia</td>
<td>Ajunic acid</td>
<td>[Structure Image]</td>
<td>NE</td>
</tr>
<tr>
<td>Red yeast rice</td>
<td>α,β-Dehydrodihydromonacolin K</td>
<td>[Structure Image]</td>
<td>NE</td>
</tr>
<tr>
<td>Red yeast rice</td>
<td>Dihydromonacolin K</td>
<td>[Structure Image]</td>
<td>NE</td>
</tr>
<tr>
<td>Red yeast rice</td>
<td>1-Naphthalene propanoic acid, 1,2,4a,5,6,7,8,8a-octahydro-2,6-dimethyl-(1S,2S,4aR,6R,8aS)</td>
<td>[Structure Image]</td>
<td>NE</td>
</tr>
</tbody>
</table>
The results indicated that chebuloside II, corilagin, gallic acid, chebulagic acid, chebulinic acid, (2, 3, 6-tri-O-galloyl-β-D-Glucose) and (1, 2, 3, 6-tetra-O-galloyl-β-D-glucose) blocked FXIa activity by 30%, 30%, 45%, 25%, 50%, 45% and 52% at 300 μM, respectively (Table 6). In conclusion, our data demonstrated that T. chebula fruit also have an affinity for FXIa. Thus, this extract might be used safely as a supplement to improve the anticoagulant effects.

In conclusion, our data demonstrated that T. chebula fruit also have an affinity for FXIa. Thus, this extract might be used safely as a supplement to improve the anticoagulant effects.

The extract of RYR had a cholesterol lowering effect, and different RYR preparations are commercially available. The lipid lowering and anti-inflammatory properties of xuezhikang, a commercial RYR extract preparation, were confirmed (Heber D et al. 1999). The extract of RYR had pharmacologically active constituents including thirteen natural statins. Thus, we have tested the effects of RYR extract as well as its isolated pure compounds on all enzymes of interest that are involved in BK liberation. We determined for the first time that RYR extract had a stimulatory effect on plasma kallikrein up to 20% at 10 mg/ml and exceeded 43% at 300 mg/ml. In a previous study, it was indicated that lovastatin, also known as monacolin K (RYR component) upregulates the expression of cardioprotective B2R in endothelial cells (Liesmaa I et al. 2007). Our findings, explored the impact of RYR extract on the Kallikrein-Kinin system through plasm kallikrein, the major liberator of BK from HMWK in human body. We also indicated that RYR extract has no effects on FXIIa and FXIa, whereas it has a minor inhibitory effect on rPRCP 30% inhibition at 200 mg/ml (Figures 44, 45 & 46). The results indicated that RYR pure compounds showed no effects on plasma kallikrein, FXIIa and rPRCP. Interestingly, among all RYR compounds, three of them named dihydromonacolin K, [1-Naphthalene propanoic acid 1,2,4a,5,6,7,8,8a-octahydro-2,6-dimethyl-(1S,2S,4aR,6R,8aS)] and
α,β-dehydrodihydromonacolin K showed inhibitory effects on FXIa activity in fluid phase by 55%, 55% and 40% at 300 μM, respectively (Table 6). Thus, we determined their IC$_{50}$ values and found that dihydromonacolin K, 1-naphthalenepropanoic acid 1, 2,4a,5,6,7,8,8a-octahydro-2,6-dimethyl-(1S,2S,4aR,6R,8aS) and α,β-dehydrodihydromonacolin K blocked FXIa activity in fluid phase with IC$_{50}$ values of 260, 270 & 400, respectively (Figures 47 & 9). Recently, FXIa inhibitor named BMS-262084 demonstrated antithrombotic effect in rat models of arterial and venous thrombosis, suggesting FXIa an alternative anticoagulation target (Schumacher A et al. 2007). In conclusion, it was reported that RYR extract consumption is associated with an edema symptoms. Thus, our findings suggested that RYR extract is probably implicated in the appearance of edema symptoms through a plasma Kallikrein-Kinin mediated mechanism. In addition, we indicated novel FXIa inhibitors that could be used as a lead compounds for identifying more potent FXIa inhibitors that might be used for thrombosis management purposes.

In summary, the effects of all screened natural product extracts on plasma kallikrein, kallikrein formed on HPAEC, FXIIa, FXIa and rPRCP were summarized in Tables 7 & 8. The results indicated that among all screened natural product extracts, the methanol extract of *T. chebula fruit* and the aqueous extract black tea were indicated as the most promising extracts to develop inhibitors of plasma kallikrein and FXIIa activities. The results also suggested that the methanol extract of *T. chebula fruit* could be used as natural supplement for HAE as well as thrombosis managements. Additionally, five inhibitors were identified as pure inhibitors from our study. Two were isolated from *T. chebula* extract showing inhibitory effects on FXIIa activity and three were isolated from RYR extract showing inhibitory effects on FXIa activity.
activity. Thus, we suggested that the identified inhibitors in our study (Table 9) could be used as lead compounds for future chemical modification work to identify more potent inhibitors.
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