Abstract

Cardiovascular diseases (CVD) such as coronary heart, cerebrovascular, peripheral arterial, rheumatic heart, myocardial infarction, and stroke are non-communicable diseases and major causes of mortality worldwide. A blood clot (thrombus) formed in the circulatory system due to the failure of hemostasis can cause vascular blockage, possible atherothrombotic diseases, and even death. Urokinase (UK), streptokinase (SK), and/or tissue plasminogen activators (t-PA) are widely used as clinical cost-effective thrombolytic agents for the treatment of severe or massive deep venous thrombosis, myocardial infarction, and other cardiac diseases. Warfarin and heparin, the two highest selling anticoagulant drugs in the market exert anticoagulant action by acting as a vitamin K1 antagonist and indirect inhibitor of thrombin, respectively. Nevertheless, these drugs are also associated with adverse reactions such as gastrointestinal bleeding, hypertension and allergic reactions. Moreover, argatroban, a synthetic direct inhibitor of thrombin cannot be administered to patients with hepatic impairments or hemorrhagic complications. Thus, alternative sources of medicine are in demand to treat cardiovascular diseases.

The increasing risk of CVDs has created a niche for the development of a safe, potent, natural, stable anticoagulant drug by the pharmaceutical and/or neutraceutical industries. Herbal remedies are often preferred in primary health care because they are cost-effective, culturally acceptable, and potent with fewer side-effects. The plant *Leucas indica*, belonging to the Lamiaceae family is mostly used in folk medicine for treating asthma and as decoctions in traditional medicine to reduce nasal congestion. Interestingly, studies from our laboratory have discovered the presence of anticoagulant fibrinogenolytic enzyme(s) in the aqueous leaf extracts of this plant. The fruit of the bitter melon (*Momordica charantia*), locally known as *karela*, is an Indian household vegetable traditionally used in China, India, Africa, and the South-eastern US for treating various diseases. The folk literature of India considers the fruit of *M. charantia* to be useful in treating gout, rheumatism, and sub-acute cases of spleen and liver diseases, malaria, diabetes hepatitis infections and fever. To the best of our knowledge, this is the first report on the anticoagulant mechanism of active fractions/ components purified from the aqueous leaf extract of *L. indica* and whole fruit of *M. charantia*. Based on the ethnomedical information, chemical diversity, and current literature, Indian medicinal plants may serve as great source of new bioactive compounds. **Unfortunately, there is no scientific**

validation to prove the pharmacological effect of the many of the traditionally used plant(s) in treating cardiovascular disorders. Therefore, in the present investigation, an effort was given to investigate the thrombolytic, anticoagulant, fibrinogenolytic/fibrinolytic and anti-platelet properties of unexplored plants with an aim to develop a safer herbal drug to treat cardiovascular disorders.

For the ease of understanding, this thesis has been divided into eight chapters.

Chapter I- Introduction: The first chapter of the thesis provides a brief appraisal on significance of medicinal plants and the natural products in the process of drug discovery. This chapter also provides an insight into the functional attributes of *Leucas indica* and *Momordica charantia* with particular reference to thrombosis-associated cardiovascular diseases and the purpose including aim and objectives of the present investigation have also been highlighted.

Chapter II- Review of literature: The second chapter reviews the published data on fibrin(ogen)olytic and anticoagulant, thrombolytic and platelet aggregation inhibition properties of protein(s)/peptide(s)/active component(s) isolated from medicinal plants.

Chapter III- Materials and methods: This chapter includes the materials and methods employed for performing the various experiments which include the isolation and purification of different active fraction(s)/anticoagulant protein from *L. indica* and *M. charantia*. The techniques used for the biochemical and pharmacological characterization of the isolated active fraction(s), fibrin(ogen)olytic protease as well as for understanding the mechanism(s) of action of the isolated active fraction(s), fibrin(ogen)olytic protease have been described.

Chapters IV to Chapter VII- Results and discussion: These four chapters analyses the data of the experiments and discuss the results of this study. A brief description is mentioned below.

Chapter IV-This chapter describes the screening of selected medicinal plants of Assam for assessment of their anticoagulant property and preparation of an active anticoagulant fraction from the selected medicinal plants.

Chapter V- This chapter describes characterization and mechanism of anticoagulant action of a fibrin(ogen)olytic serine protease (lunathrombase) purified from active fraction of *L. Indica* (AFLI).

Chapter VI- This chapter describes the characterization and mechanism of anticoagulant action of β-sitosterol - a major component ofthe active anticoagulant fraction of *L. Indica* (AFLI).

Chapter VII-This chapter describes the mechanism of anticoagulant action of an active anticoagulant fraction (AAF) from the fruits of *M. charantia.*

Chapter VIII- Conclusion: This chapter describes the conclusion on the major findings and visualizes the future prospects of the research findings of this study.

In this study, among the 6 plants tested, the aqueous shade dried leaves extract of *L. indica* and whole fruits extract of *M. charantia* showed best anticoagulant activity and were used for further studies. The optimized condition for best anticoagulant activity for *L. indica* is 4 h stirring at room temperature, pH 7.4 and for *M. charantia* is 1 h stirring at room temperature followed by 10 min sonication, pH 7.4. The active fraction prepared from *L. indica* was named as AFLI. The AFLI in combination with β- sitosterol (one of the major components of AFLI) at the ratio 5:1, exhibits significantly enhanced ($p<0.01$) anticoagulant and fibrin(ogen)olytic activities as compared to the crude plant extract or AFLI. This comprises the active anticoagulant preparation (AAP) of present study. The active anticoagulant fraction prepared from *M. charantia* was named as AAF. AAF surpasses the anticoagulant activity of crude extract of *M. charantia.*

Biochemical and SDS-PAGE analyses revealed the presence of αβ-fibrinogenase enzymes in AFLI/ AAF albeit LC-MS/MS analysis failed to identify the presence of such protease in these extracts suggesting that AFLI/AAF contains novel/previously uncharacterized protease enzyme(s). The GC-MS analysis revealed that β-sitosterol, methyl palmitate, octamethyl- tetracyclosiloxane are the major ingredients of active fraction of *L. indica* (AFLI). The most abundant phytochemicals of active anticoagulant fraction of *M. charantia* (AAF) are decanoic acid, 1,2,3-propanetriyl ester (22.3%), dodecanoic acid, 1,2,3-propanetriyl ester (17.3%) dodecenoic acid, 1,2,3- propanetriyl ester (12.5%), and 4-B-methylandrostane 2,3-diol-1,17-dione (11.4%). None of the above phytochemicals of AFLI/AAF have shown anticoagulant or antiplatelet activity.

The *in vitro* anticoagulant activity of AAP was found to be superior to heparin, warfarin and nattokinase ($p<0.05$) but slightly less than argatroban; however, the *in vitro* anticoagulant activity of AAF was found to be comparable to heparin and warfarin and nattokinase. The *in*

vitro thrombolytic potential of AAP/AAF is comparable to streptokinase, plasmin and nattokinase. AAP showed superior antiplatelet activity than aspirin ($p<0.05$)

A 35 kDa (m/z 34747.5230) serine protease (lunathrombase) showing fibrin(ogen)olytic activity and devoid of N- and O- linked oligosaccharides was purified from an extract of aqueous leaves from *L. indica* . The LC-MS/MS analysis, *de novo* sequencing, secondary structure, and amino acid composition determination suggested the enzyme's novel characteristic. Lunathrombase is an αβ-fibrinogenase, demonstrating anticoagulant activity with dual inhibition of thrombin (Ki: 26.90 ± 0.9 nM) and FXa (*Ki:* 10.35 ± 1.38 nM) by a non-enzymatic mechanism. The spectrofluorometric and isothermal calorimetric analyses revealed the binding of lunathrombase to fibrinogen, thrombin, and/or FXa with the generation of endothermic heat. It inhibited collagen/ADP/arachidonic acid-induced mammalian platelet aggregation, and demonstrated antiplatelet activity via COX-1 inhibition (*Ki*: 5.9 ± 0.9 µM) and the upregulation of the cAMP level. Lunathrombase showed *in vitro* thrombolytic activity and was not inhibited by endogenous protease inhibitors α_2 macroglobulin and antiplasmin.

β-sitosterol (BSS) demonstrated anticoagulant activity by the dose-dependent inhibition of thrombin with a *Ki* value 0.267 µM. Its activity was also demonstrated by partial inhibition of thrombin-catalyzed platelet aggregation with an IC_{50} value of 10.45 ± 2.88 µM. The *in silico* study indicated the binding of BSS to thrombin, which was experimentally verified by spectrofluorometric and isothermal calorimetric analyses. Under *in vitro* conditions, BSS demonstrated thrombolytic activity by activating plasminogen. BSS was non-cytotoxic to mammalian cells, non-hemolytic, demonstrated its *in vivo* anticoagulant activity when administered orally, and inhibited the k-carrageen-induced thrombus formation in the tails of mice. Our results suggest that dietary supplementation of BSS may help prevent thrombosis-associated cardiovascular disorders.

The anticoagulant mechanism of AAF is correlated to its defibrinogenation and partial antiplatelet activity. The spectrofluorometric analysis revealed the binding of AAF to fibrinogen but not to thrombin or FXa. AAF inhibited collagen/ADP-induced mammalian platelet aggregation, showed *in vitro* thrombolytic activity, and was non-cytotoxic to mammalian cells. . AAF demonstrated a dose-dependent *in vivo* plasma defibrinogenating and anticoagulant activities and inhibited k-carrageen-induced thrombus formation in the tails of mice.

In a nutshell, the potent *in vitro* and *in vivo* anticoagulant effects of lunathrombase/BSS and active fractions of *L. indica* and *M. charantia* suggest their pharmacological significance as anticoagulant and antithrombotic herbal drugs. In summary, the future therapeutic applications of the above mentioned herbal drugs for preventing and/or treating hyperfibrinogenemia- and thrombosis-associated cardiovascular disorders seem promising.