Characterization and mechanism of anticoagulant action of β-sitosterol - a major component of the active anticoagulant fraction of *L. Indica* (AFLI)

6.1 Brief introduction

The search for anticoagulant agents from natural sources that would be safe and easily absorbed is an area of renewed scientific interest with broad interdisciplinary research approaches [1,2]. Recent trends have clearly shown that plant-derived products and/or their synthetic counterparts will be among the most important sources of new drugs in the years to come. β-sitosterol (BSS), a natural phytosterol (Fig. 6.1), has shown various promising biological properties including cholesterol-lowering, immunomodulatory, and anti-cancer activities [3-5], and its safety has been assessed in pre-clinical studies [6,7]. Nevertheless, no direct evidence has been found for the anticoagulant or antithrombotic properties of BSS. In this chapter, the mechanism of the antithrombotic action of a plant-derived BSS and a structural analysis of the binding of BSS to thrombin has been presented. Furthermore, studies have shown that BSS can function as an oral anticoagulant, inhibiting *in vivo* thrombus formation in an experimental mouse model.

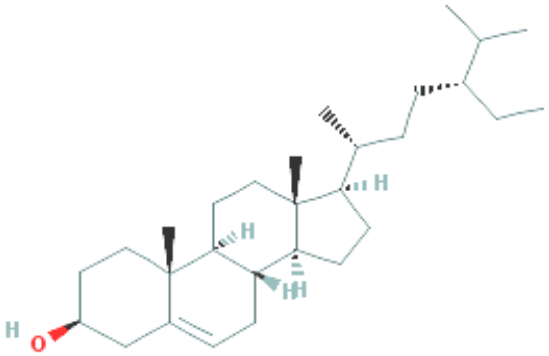

Fig. 6.1. Chemical structure of β- sitosterol**.**

6.2 Results

6.2.1 BSS demonstrated dose-dependent anticoagulant activity of PPP and whole blood

BSS dose-dependently prolonged the Ca^{2+} clotting time of PPP, and at a concentration of 2.5 µM of BSS saturated anticoagulant activity was observed (Fig. 6.2a). BSS (2.5 µM) demonstrated the optimum anticoagulant activity at 15 min of pre-incubation with PPP (Fig 6.2b).

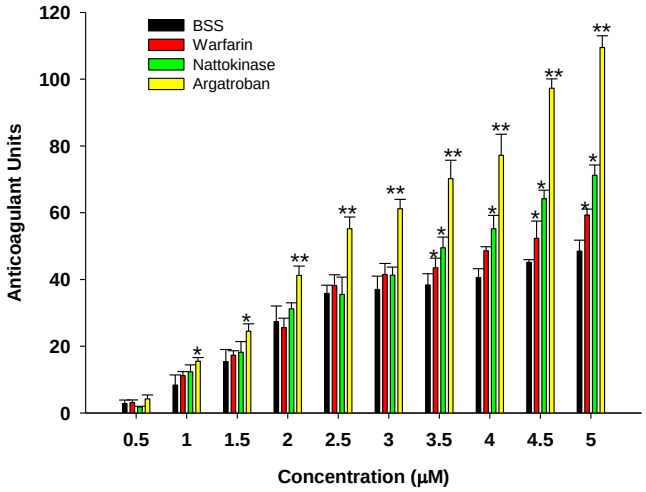

Fig. 6.2a. Dose-dependent *in vitro* anticoagulant activity of BSS, warfarin, nattokinase, and argatroban against human platelet-poor plasma (PPP). The values are means ± S.D. of three independent experiments. Significance of difference with respect to positive controls, *p <0.05; **p<0.01.

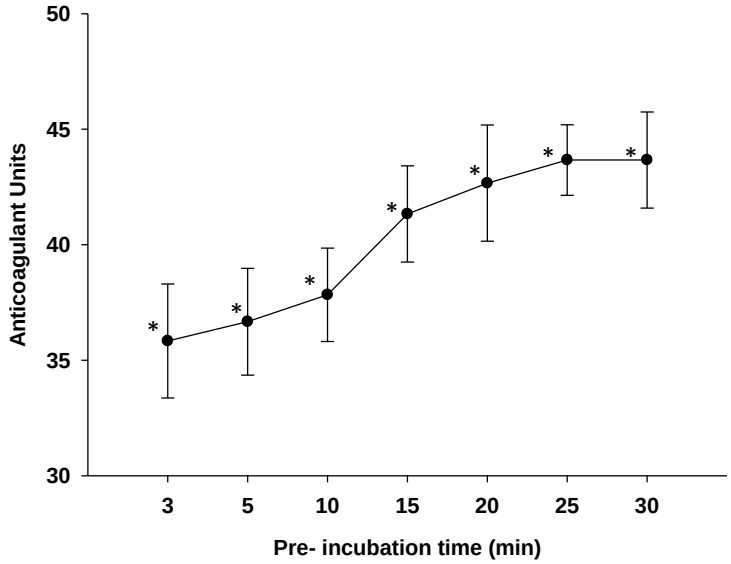

Fig. 6.2b. Time-dependent *in vitro* anticoagulant activity of BSS (2.5 μM) against human PPP. The values are means ± S.D. of three independent experiments. Significance of difference with respect to control, * p< 0.05.

The increasing BSS concentration was associated with a concomitant increase in the APTT of PPP, though the APTT concentration-response curve was curvilinear and flattened at higher concentration (10 μM) of BSS (Fig. 6.2c). BSS did not affect the PT

(INR) of PPP (Fig.6.2d). Pre-incubation of mammalian blood with BSS resulted in prolongation of clotting time (Fig. 6.2e).

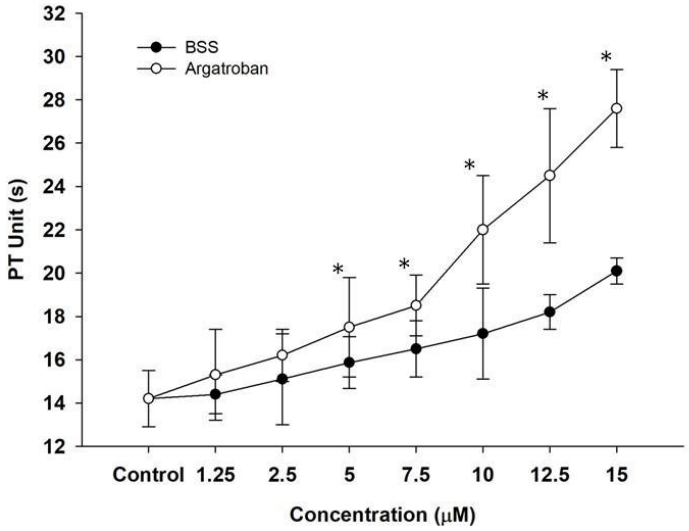

Fig.6.2c. Effect of BSS/argatroban (1.25-15.0 µM) on PT of human PPP. The values are means ± S.D. of three independent experiments. Control PT showing clotting time of 14.2 ± 1.3 s (mean ± S.D., n=3).Significance of difference with respect to control (without BSS), * p< 0.05.

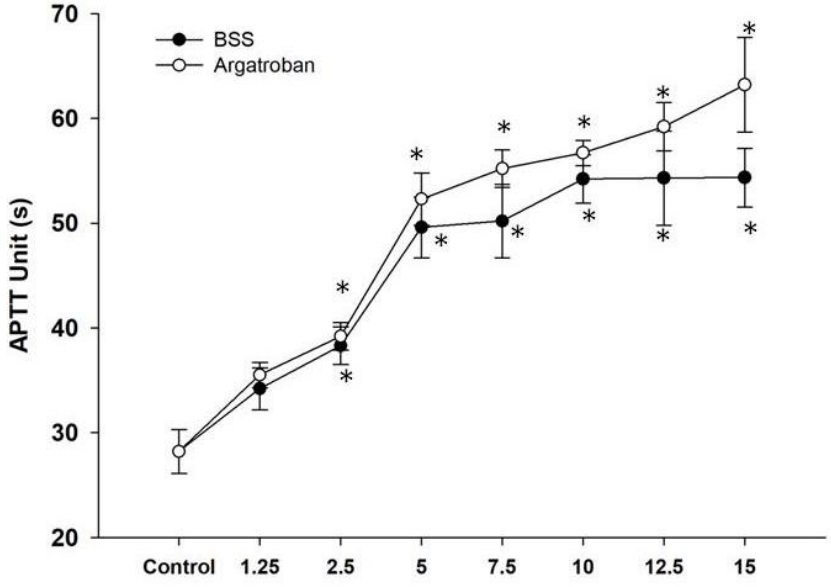

Fig. 6.2d. Effect of BSS/argatroban (1.25-15.0 µM) on APTT of human PPP. Control APTT showing clotting time of 28.2 ± 2.1 s (mean ± S.D., n=3).The values are means ± S.D. of three independent experiments. Significance of difference with respect to control (without BSS), * p< 0.05.

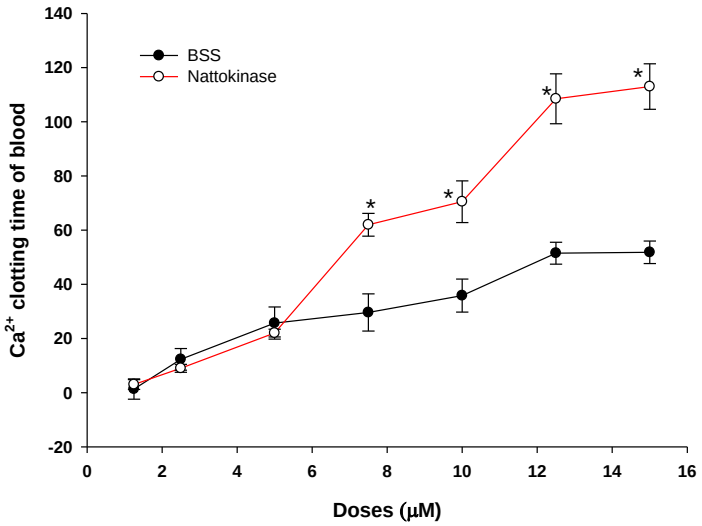

Fig. 6.2e. Dose-dependent *in vitro* whole blood clotting time of BSS and nattokinase against mammalian (goat) blood. The plots are means of three independent experiments. Significance of difference with respect to nattokinase * $p < 0.05$.

6.2.2 BSS inhibited the catalytic activity of thrombin but did not influence FXa activity

BSS dose-dependently inhibited the amidolytic activity of thrombin with a *Ki* value at 267.2 ± 34.3 nM (mean ± SD, n=3) (Fig. 6.3a). BSS also prolonged the fibrinogen clotting time of thrombin in a dose-dependent manner and at 7.5 µM concentration of BSS saturation in thrombin, inhibition was observed (Fig. 6.3b). At the optimum concentration of BSS, optimum thrombin inhibition was observed at 20 min of pre-incubation of BSS with thrombin (Fig. 6.3c). BSS also enhanced the thrombin inhibition produced by the heparin-AT-III complex (Fig. 6.3d).

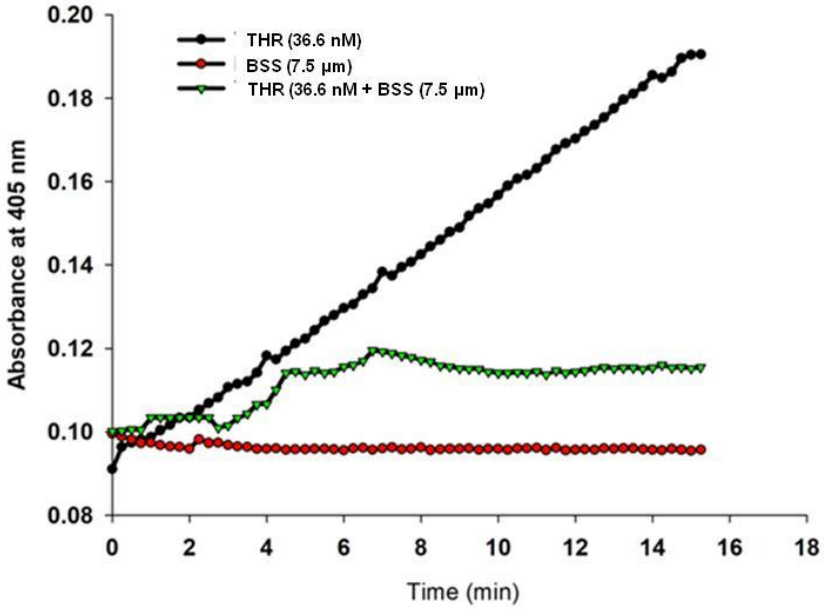

Fig. 6.3a. Effect of BSS (7.5 µM) on amidolytic activity of thrombin (36.6 nM) against its chromogenic substrate T1637 (0.2 mM). The plots are the means of three independent measurements.

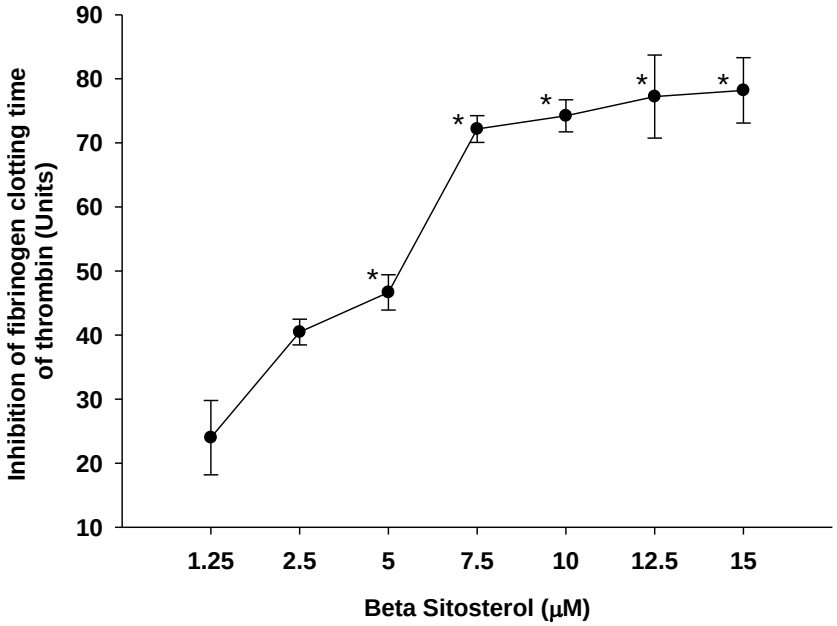

Fig. 6.3b. Inhibition of fibrinogen clotting activity of thrombin (36.6 nM) by BSS (1.25-15 µM) at 37°C, pH 7.4. The fibrinogen clotting time of thrombin under identical experimental conditions (control) was determined to be 39.17 ± 1.54 s. The values are means ± S.D. of three independent experiments. Significance of difference with respect to control (without BSS), *p< 0.05.

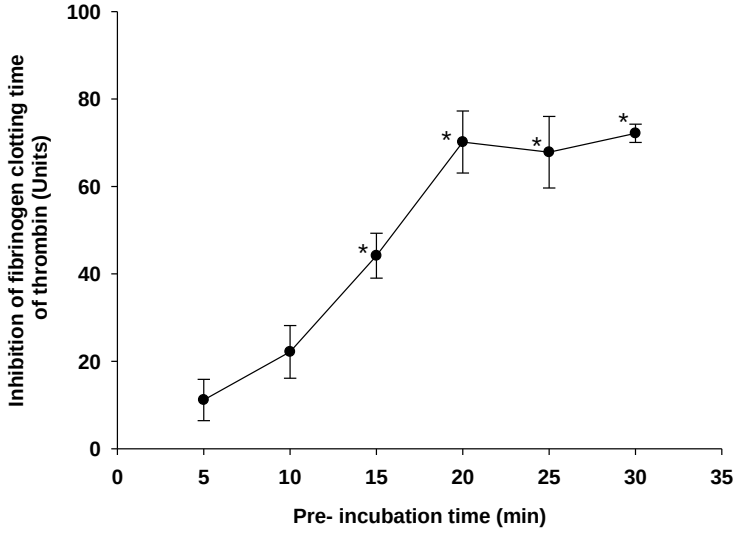

Fig. 6.3c. Time-dependent inhibition of fibrinogen clotting time of thrombin (36.6 nM) by BSS (7.5 µM) at 37°C, pH 7.4. The fibrinogen clotting time of thrombin under identical experimental conditions (control) was determined to be 39.17 ± 1.54 s. The values are means ± S.D. of three independent experiments. Significance of difference with respect to control (without BSS), *p< 0.05.

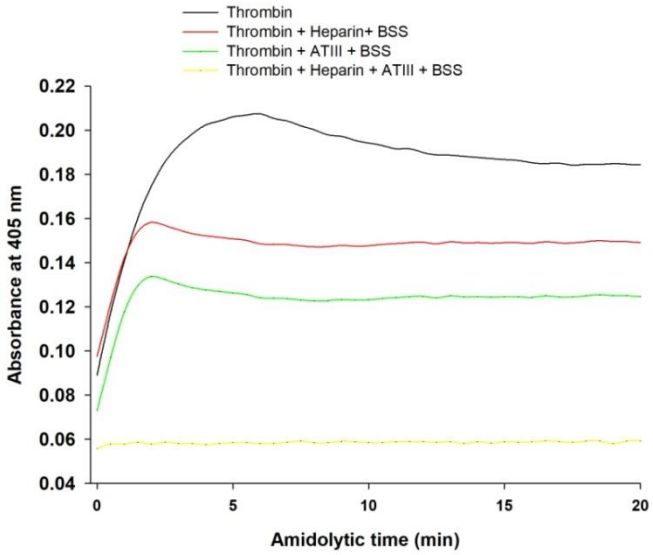

Fig. 6.3d. Inhibition of amidolytic activity of thrombin against its substrate T1637. Thrombin (36.6 nM) was pre-incubated with heparin/BSS (0.5 mIU/7.5 µM), AT III/BSS (2.5 µM/7.5 µM), and heparin/AT III/BSS (0.5 mIU/2.5 µM/7.5µM) and 1X PBS (control) for – min before initiating the reaction.

Nevertheless, BSS failed to inhibit the amidolytic activity of FXa (Fig. 6.4a) or the prothrombin activation by FXa (Fig. 6.4b) suggesting that BSS does not inhibit FXa.

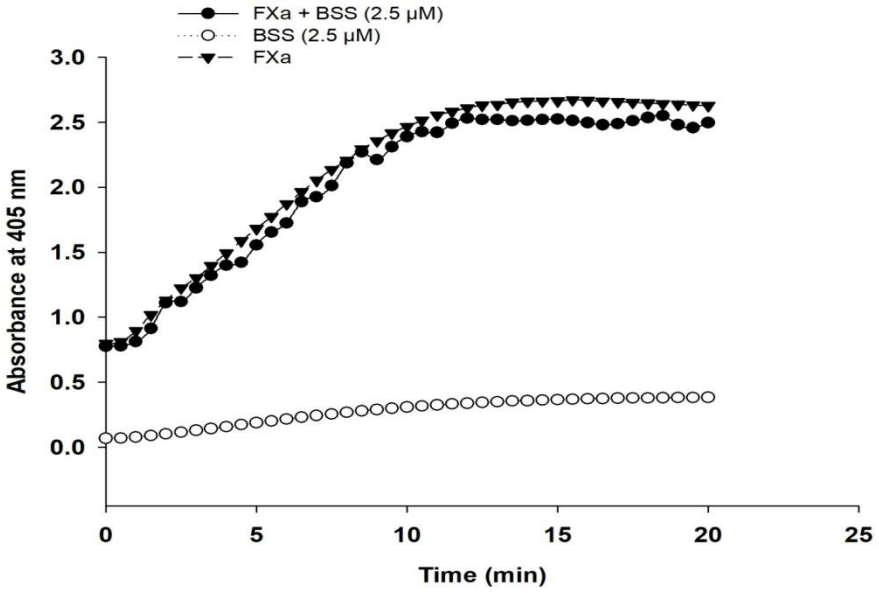

Fig. 6.4a. Effect of BSS (7.5 µM) on amidolytic activity of FXa (0.13 µM) against its chromogenic substrate F3301 (0.2 mM). The values are means of triplicate determinations.

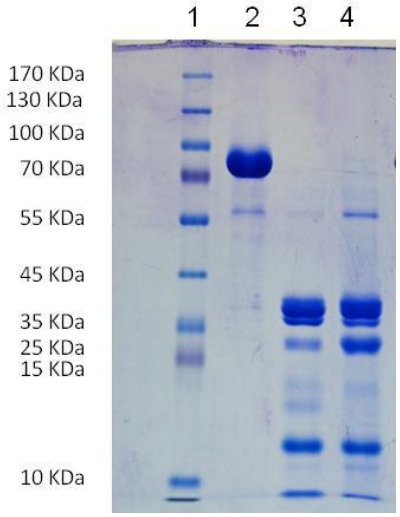

Fig. 6.4b. Inhibition of prothrombin activation property of FXa by BSS. After reduction with β-mercaptoethanol, degradation products were separated by 12.5% SDS-PAGE. Lane 1, protein molecular markers; lane 2, 1.4 µM PTH; lane 3, PTH (1.4 µM) incubated with FXa (0.13 µM) for 30 min at 37ºC, pH 7.4; lane 4, [FXa (0.13 µM) pre-incubated with BSS (7.5 µM) for 15 min] + PTH (1.4 µM).

6.2.3 BSS demonstrated antiplatelet activity and inhibited thrombin- induced platelet aggregation

A comparison of the dose-dependent platelet de-aggregation (antiplatelet) property of BSS and aspirin (positive control) is shown in Figure 6.5a. Aspirin showed slightly higher antiplatelet activity compared to BSS (Figure 6.5a). Further, BSS in a concentration-dependent manner significantly ($p<0.05$) inhibited the thrombin-induced aggregation of human PRP and washed platelets. From the regression equation, the IC_{50} value of thrombin inhibition (concentration at which BSS demonstrated 50% inhibition of thrombin-induced platelet aggregation) was determined to be 10.5 ± 2.9 µM and 9.2 ± 1.2 µM (mean ± SD, n=3) for PRP and washed platelets, respectively (Figure 6.5b).

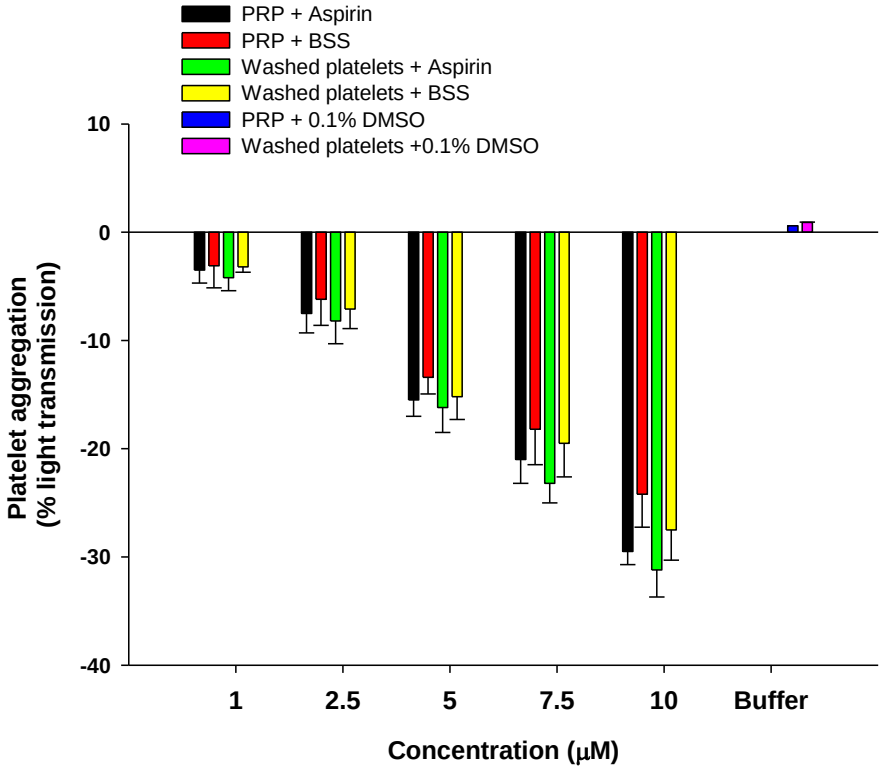

Fig. 6.5a. Dose-dependent platelet deaggregation by BSS/aspirin. Different concentrations (1-10 µM) of BSS or aspirin were incubated with platelet rich plasma or washed platelets at 37 °C and the absorbance was recorded at 540 nm.

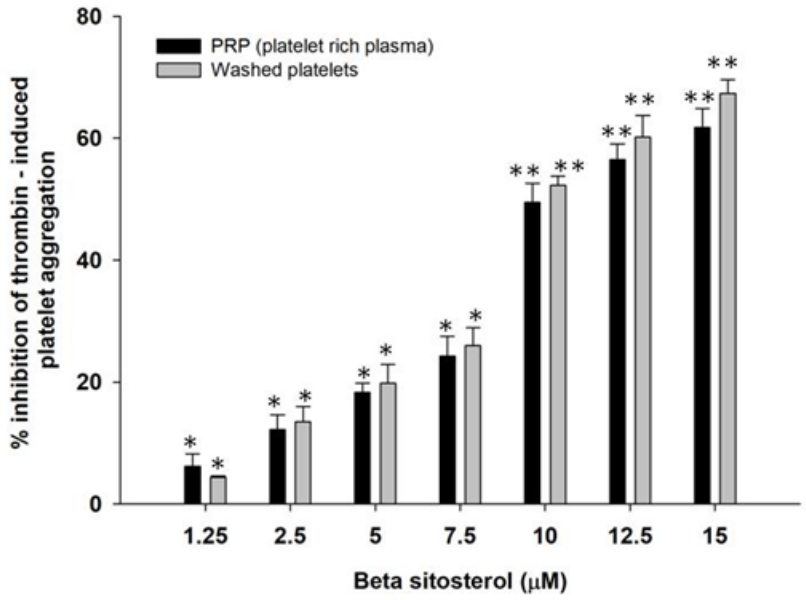

Fig. 6.5b. Effect of BSS on thrombin-induced platelet aggregation. Thrombin (0.03 U/ml) was incubated with BSS (1.25-15.0 μM) for 5 min at 37 ºC. PRP/washed platelets were added to the mixture and the decrease in absorbance was monitored at 540 nm for 5 min. The values are means ± S.D. of three independent experiments. Significance of difference with respect to control; * $p<0.05$; ** $p<0.01$.

6.2.4 The *in silico* binding study, spectrofluorometric analysis, and isothermal calorimetric titration suggest interaction of BSS with thrombin

The ligand-protein interaction study showed that the lowest binding energy and intermolecular energy for the binding of BSS with thrombin was -5.61 and -7.7 kcalmol^{-1}, respectively. Further, in the binding site of thrombin, the BSS ligand was shown to be in close proximity with protein residues PHE339, LEU346, ARG382, ARG388, THR389, ARG390, and TYR391. A prominent hydrogen bond interaction at a distance of 2.088 Å was demonstrated with the protein residue ARG388 (Fig. 6.6a).

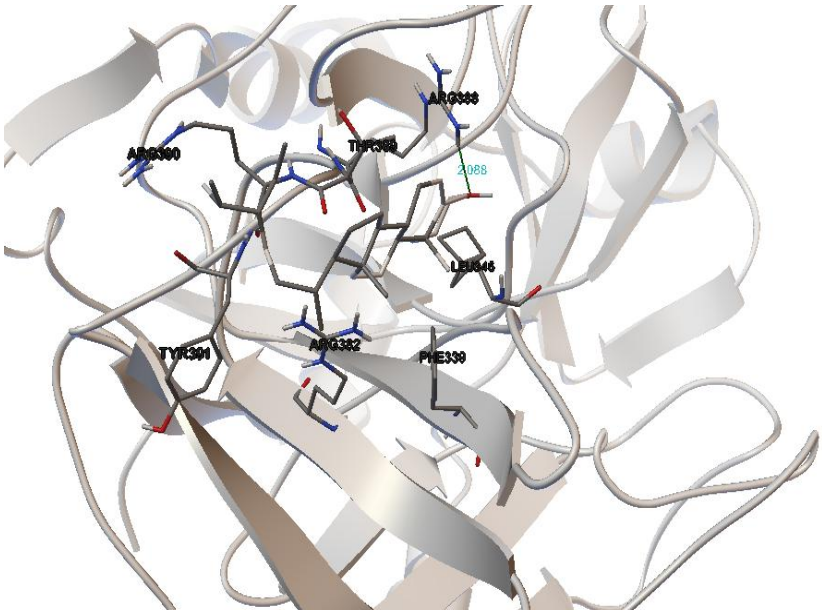

Fig. 6.6a. Interaction site of the BSS around the thrombin residues as determined by *in silico* study using AutoDock 4.0 software.

To verify the result of the *in silico* study, spectrofluorometric analysis was performed to study the interaction between BSS and thrombin. A steady increase in the fluorescence intensity of thrombin in the presence of BSS was seen in comparison to the fluorescence intensity of individual protein/ligand (Fig. 6.7a). The dissociation constant (*Kd*) for the binding of BSS to thrombin was determined at 1.21 ± 0.4 µM (Fig. 6.7a).No change in the fluorescence intensity of fibrinogen/FXa was observed in the presence of BSS, suggesting that it does not bind with fibrinogen or FXa (Figs. 6.7b, c).

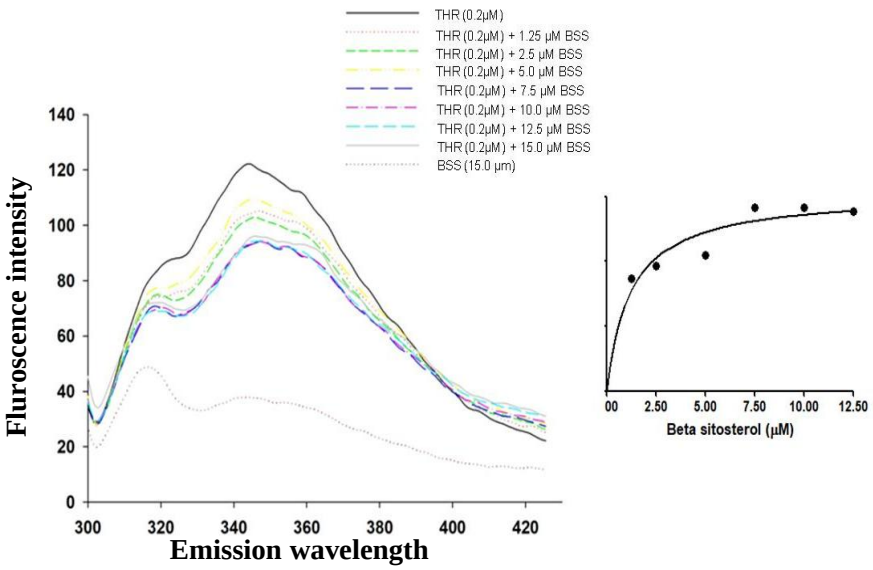

Fig. 6.7a. Fluorescence spectra showing interaction of thrombin (0.2 μM) with different concentrations of BSS (1.25-15.0 μM). Inset. One site saturation binding curve of BSS for thrombin.

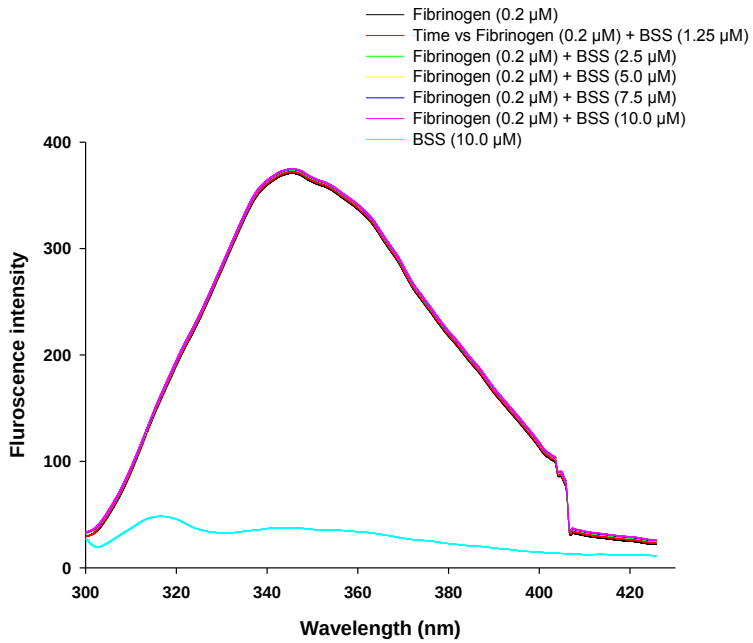

Fig. 6.7b. Fluorescence spectra showing interaction of fibrinogen (0.2 μM) with different concentrations of BSS (1.25-10.0 μM).

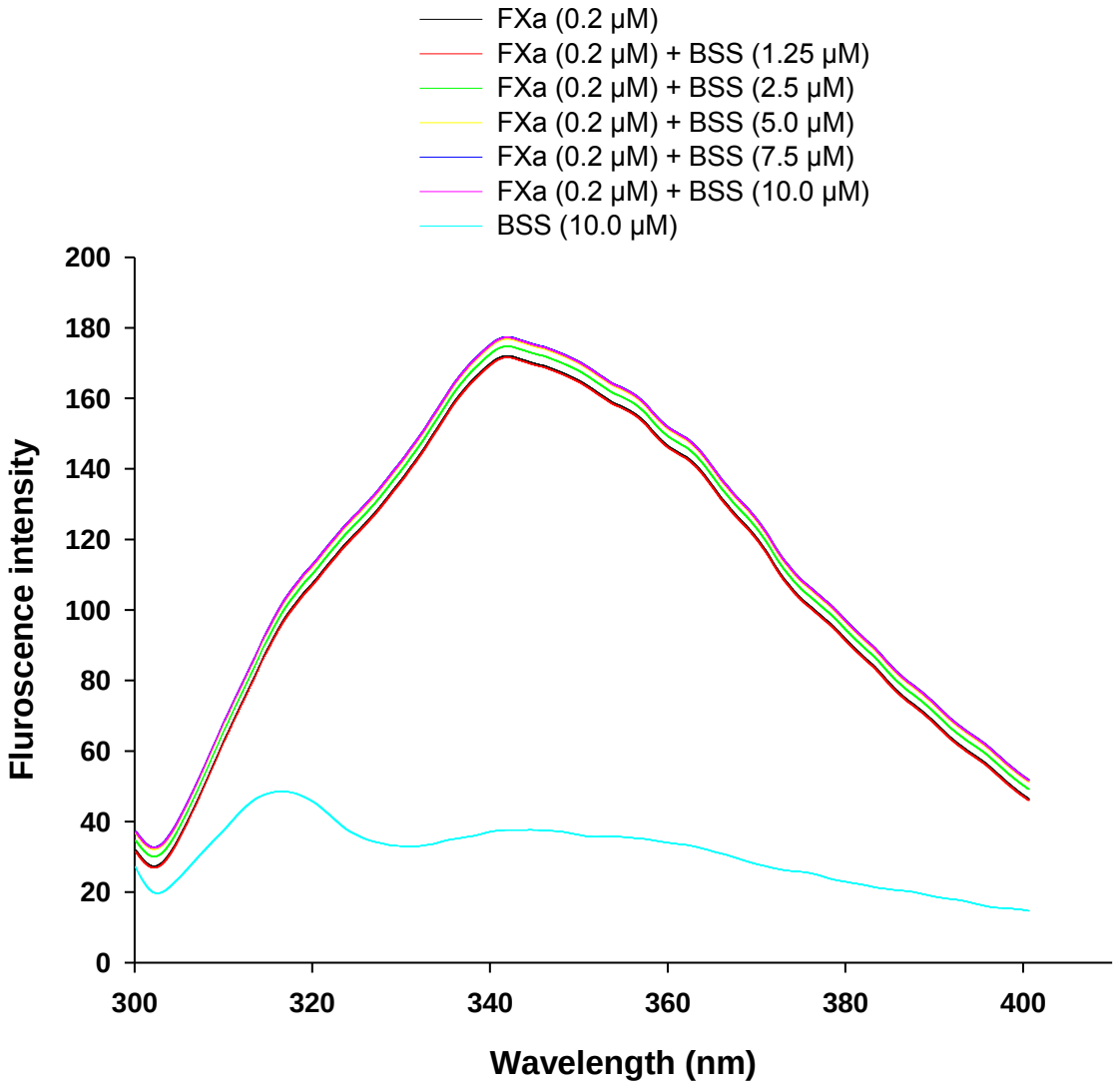

Fig. 6.7c. Fluorescence spectra showing interaction of FXa (0.2 µM) with different concentrations of BSS (1.25-10.0 µM).

Isothermal calorimetric titration was also performed to determine the interaction between BSS and thrombin. The titration of thrombin with BSS was a strong exothermic reaction (generation of heat) and it showed a sigmoidal saturation curve indicating a direct binding interaction between BSS and thrombin (Fig. 6.8). The best fit for the titration curve was obtained with a one binding site model with a binding constant (*Ka*) of 109 ± 20.5 M^{-1}, ΔH = -8371 ± 729.2 cal/mol, ΔS= -18.8 cal/mol/deg for the interaction between BSS and thrombin (Fig. 6.8).

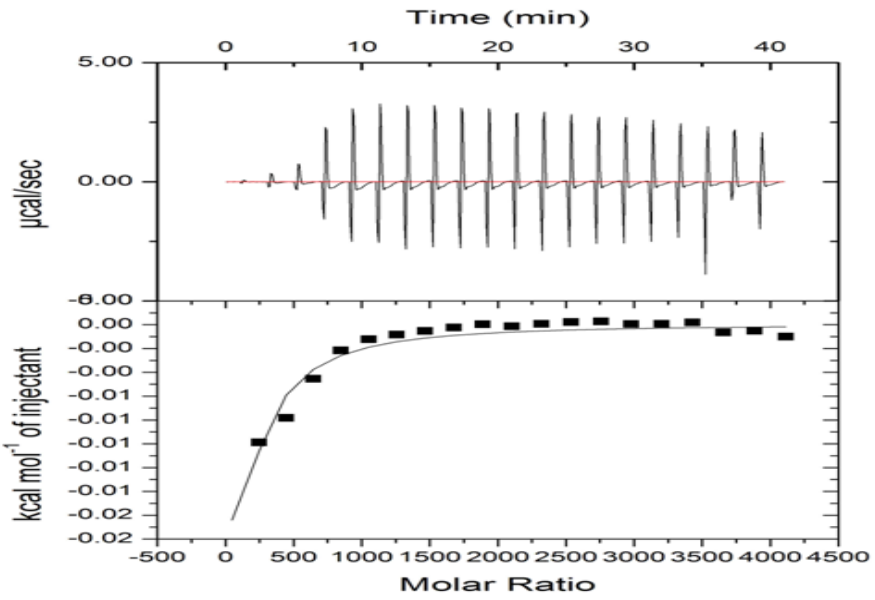

Fig. 6.8. ITC profile for BSS (10 µM) binding to thrombin (200 µM). Upper panel shows heat change upon ligand addition; lower panel shows an integrated ITC isotherm and its best fit to a one-site binding model.

6.2.5 BSS demonstrated antithrombotic activity

As shown in Table 6.1 the thrombolytic potential of BSS was compared to commercial thrombolytic agents (streptokinase, nattokinase, and plasmin). The *in vitro* thrombolytic activity of BSS was found to be lower than nattokinase and streptokinase. BSS and streptokinase demonstrated negligible activity in dissolving the heated blood clot (Table 6.1).Further, BSS did not possess plasmin-like activity (Fig. 6.9a), though it activated the plasminogen to form plasmin that subsequently hydrolyzed the substrate for plasmin (Fig. 6.9b).

Table 6.1. Comparison of *in vitro* thrombolytic activity shown by BSS, plasmin, streptokinase, and nattokinase under identical experimental conditions. The values are mean ± S.D. of triplicate determinations. [a] $p < 0.05$. Significance of difference with respect to negative control; [b]$p < 0.05$compared to positive controls

	Mg of clot lysed/µM of sample
Non-heated blood clot	
Negative control (1X PBS, pH 7.4)	2.1 ± 0.3
BSS	7.7 ± 0.3 [a,b]
Streptokinase	15.5 ± 0.4[a]
Nattokinase	14.8 ± 1.2[a]
Plasmin	15.8 ± 1.6[a]
Heated blood clot	
Negative control (1X PBS, pH 7.4)	0.7 ± 0.2
BSS	1.0± 0.1[b]
Streptokinase	1.3± 0.1[b]
Nattokinase	9.9 ± 1.8[a]
Plasmin	9.2 ± 0.3[a]

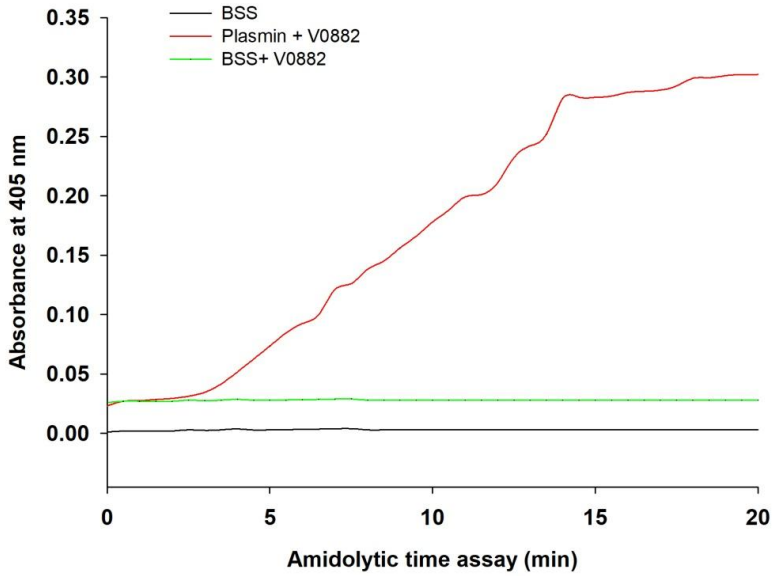

Fig. 6.9a. Effect of BSS (1.0 µM) on the chromogenic substrate of plasmin, V0882, D-Val-Leu-Lys-p-nitroanilidedihydrochloride (0.2 mM). The plots are means of three independent measurements.

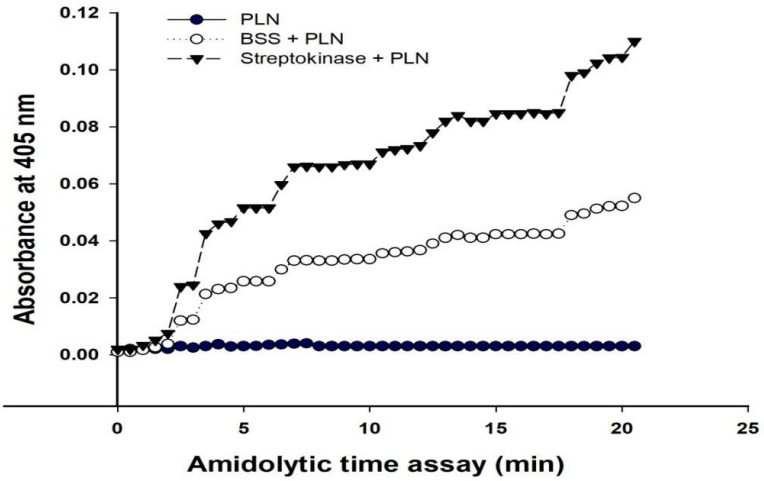

Fig. 6.9b. Effect of BSS (1.0 µM) on amidolytic activity of plasminogen (36.6 nM) against the chromogenic substrate of plasmin, V0882, D-Val-Leu-Lys-p-nitroanilidedihydrochloride (0.2 mM). The plots are means of three independent measurements.

6.2.6 BSS was devoid of hemolytic activity or cytotoxicity against mammalian cells

BSS at a concentration of 50 µM (which was 20 times greater than its *in vitro* minimum anticoagulant dose of 2.5 µM) did not show any adverse effects on the viability of

mammalian HEK 293 cells (Fig. 6.10a) indicating its lack of cytotoxicity against mammalian cells. BSS also did not cause *in vitro* hemolysis of mammalian erythrocytes (Fig. 6.10b).

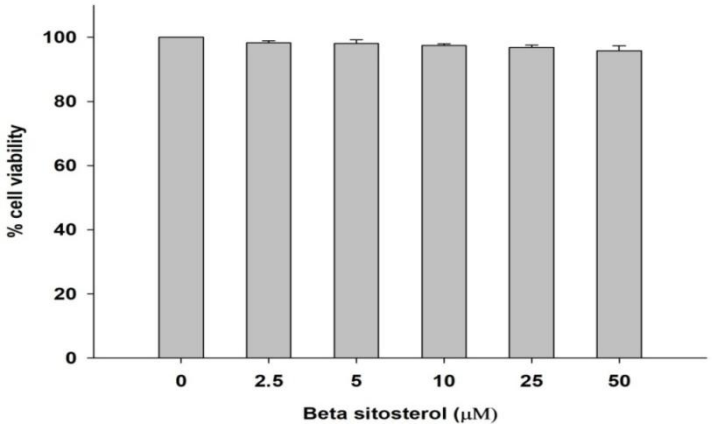

Fig. 6.10a. *In vitro* cell viability assay using MTT assay. The HEK 293 cells were treated with BSS (2.5-50 μM) for 24 h at 37˚C. All values are means ± SD of triplicate determinations.

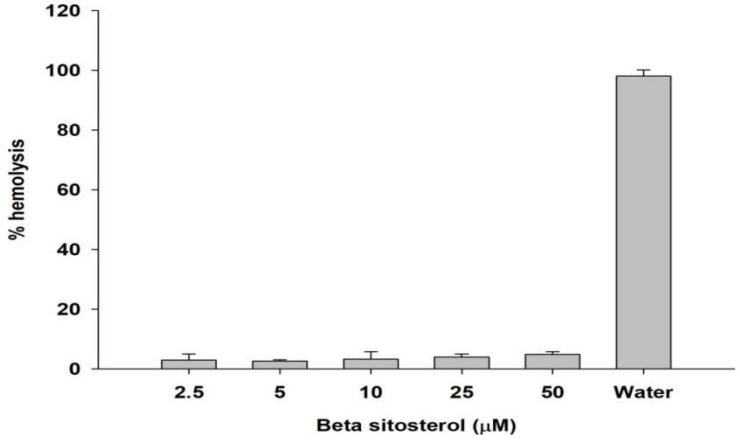

Fig. 6.10b. *In vitro* blood haemolysis assay. The human erythrocytes (5%, v/v) were treated with BSS (2.5-50 μM) for 90 min 37˚C. All values are mean ± SD of triplicate determinations.

6.2.7 BSS demonstrated *in vivo* anticoagulant activity but was devoid of fibrinogenolytic activity

As shown in Table 6.2, tail bleeding time, plasma clotting time, and APTT were significantly prolonged in BSS-treated mice, in comparison to the control group ($p<0.01$). Nevertheless, the *in vivo* anticoagulant potency of BSS was lower than the *in vivo* anticoagulant activity of heparin/nattokinase/argatroban (Table 6.2). BSS did not demonstrate *in vivo* defibrinogenation of mice plasma (Fig. 6.11).

Table 6.2. Comparison of *in vivo* anticoagulant activity of BSS, heparin, nattokinase, and argatroban-treated Swiss albino mice. Blood was withdrawn 5 h after *i.p*. injection of 50.0 mg/kg of BSS/ heparin/ nattokinase/ argatroban. Values represent mean ± SD of six determinations. Significance of difference with respect to control, * $p < 0.01$. INR = ($prothrombin_{test}$ / $prothrombin_{control}$).

Drugs (50 mg/kg)	**PT (s)**	**PT (INR)**	**APTT (s)**	**APTT (INR)**	**Tail bleeding time (s)**	**Plasma clotting time (s)**
1X PBS (control)	14.8 ± 0.7	1.0	28.3 ± 1.4	1.0	45.3 ± 2.5	175.5 ± 9.1
BSS	16.9 ± 0.6	1.13	46.8 ± 4.0*	1.63	61.0 ± 2.6*	193.5 ± 6.7*
Heparin	57.0 ± 4.3*	3.84	33.0 ± 2.0	1.16	153.3± 8.5*	215.6 ± 3.7*
Nattokinase	38.6 ± 2.8*	2.60	37.0 ± 3.6*	1.30	120.0 ± 4.1*	211.3± 12.8*
Argatroban	59.5± 2.5*	4.0	66.2 ± 4.2*	2.33	143.5 ± 5.6*	238.6 ± 9.25*

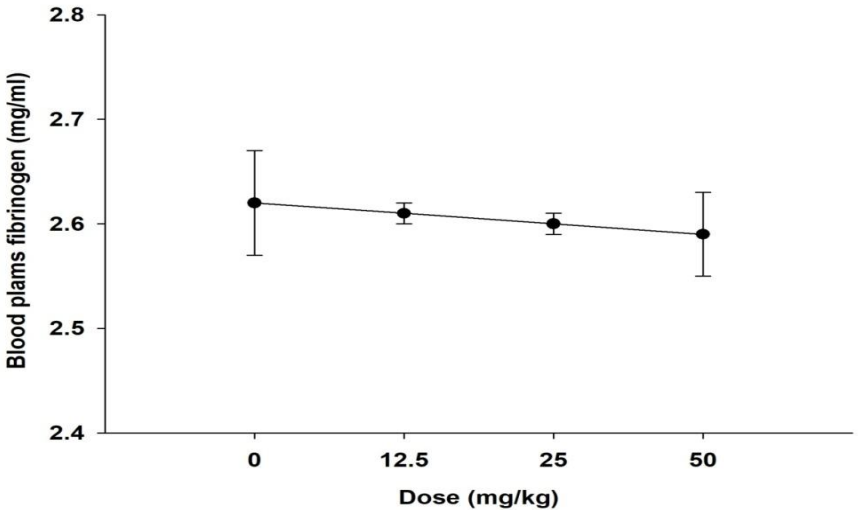

Fig. 6.11. Dose- dependent *in vivo* defibrinogenating activity of BSS 5 h after *i.p*. injection in mice. The values are means ± S.D. of triplicate determinations. $p > 0.05$.

6.2.8 Antithrombotic effect of BSS in the carrageenan-induced mouse tail thrombosis model

BSS dose-dependently inhibited thrombus formation in the tail of carrageenan-treated mice (Fig. 6.12, Table 6.3). The percent inhibition of thrombus formation induced by k-carrageenan in the mouse tail by BSS, nattokinase, and argatroban is shown in Table 6.3.

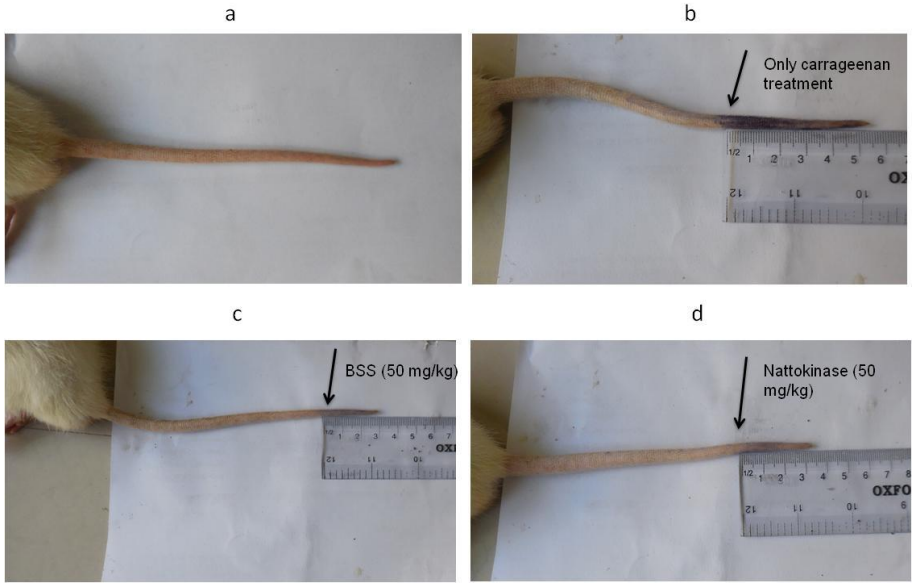

Fig. 6.12. The effect of BSS and nattokinase on κ-carrageenan-induced mouse tail thrombus length (48 h after κ-carrageenan injection). **a)** Tail without κ-carrageenan injection, **b)** Control group of mice treated with 0.9 mg/kg κ-carrageenan only, **c)** BSS

(50 mg/kg) pre-treated group of mice injected with 0.9 mg/kg κ-carrageenan**, d)** Nattokinase (50 mg/kg) pre-treated group of mice injected with 0.9 mg/kg κ-carrageenan. Arrows indicate thrombus formation region (wine and black-color) in tail of mice.

Table 6.3. The effect of BSS and nattokinase on k-carrageenan-induced mouse tail thrombus model at 24, 48, and 72 h post treatment. Values are mean ± SD of six mice.Significance of difference with respect to control group of mice;* $p < 0.05$, ** $p < 0.01$.

Drugs	**Dose**	**% Inhibition of thrombus formation in mice tail after ƙ-carrageenan treatment**		
		24 h	**48 h**	**72 h**
BSS	12.5 mg/kg	10.5 ± 3.1*	14.52 ± 3.5*	23.2 ± 1.8*
	25.0 mg/kg	15.2 ± 2.8*	31.26 ± 2.2*	36.5 ± 2.8**
	50.0 mg/kg	23.4 ± 2.1*	42.30 ± 2.8**	47.3 ± 3.1**
Nattokinase	50.0 mg/kg	26.5 ± 1.2*	46.18 ± 2.7**	52.3 ± 3.8**

6.3 Discussion

The present study is the first to report the *in vitro* and *in vivo* anticoagulant potency of BSS and explored its anticoagulant mechanism. Several key mechanisms are involved; for example, defibrinogenation of blood, inhibition of platelet aggregation, and/or interference with components of the blood coagulation cascade where anticoagulant drugs exert their anticoagulant effects [8-11]. Our findings suggest that the anticoagulant activity of BSS is correlated with its antithrombin property. Prolongation of the APTT and marginal effects of BSS on PT indicates that BSS inhibits the intrinsic pathway of coagulation [12,13]. The exact mechanism of prolongation of the APTT by BSS should be explored in near future since other direct and indirect thrombin inhibitors increase both PT and APTT.

Due to their greater sensitivity and selectivity, fluorescence spectroscopy and ITC were used to study the thrombin-BSS interaction [11,14-16]. Both of these studies showed interactions between BSS and thrombin. The +ve ΔH value in the ITC experiment suggests that the interactions are enthalpy-driven with the primary contributions to the complex stabilization likely resulting from electrostatic interactions and/or hydrogen bonds. Further, the binding energy that results from BSS and thrombin that was obtained in the *in silico* study agrees with reports for thrombin inhibitors such as pachydictyol A and isopachydictyol A from marine sources [17].

The catalytic activity of thrombin is also regulated by two recognition domains: exosite-I and exosite-II that bind to fibrinogen and heparin /AT-III, respectively [18,19]. Both sites are distant from the catalytic pocket but are involved in the specific binding of thrombin to several macromolecular substrates, inhibitors, and modulators [18,19]. Since BSS inhibited the catalytic activity of thrombin towards its small chromogenic substrate, the interaction with the active site of thrombin appears to be by an uncompetitive mechanism. Therefore, the mode of BSS-mediated thrombin inhibition differed from that of dabigatran etexilate or argatroban (commercial direct thrombin inhibitors) that follow a competitive model of thrombin inhibition [20-22].

Fibrinogen clotting and thrombin-induced aggregation of platelets are mediated by enzymatic cleavage of fibrinogen bound to exosite-I of thrombin and protease-activated receptors (PAR-1 and PAR-4, which are present on the surface of platelets) by thrombin, respectively [18,23]. Because BSS inhibited the amidolytic activity, fibrinogen clotting, and platelet aggregation properties of thrombin to an equal extent, it

would be reasonable to assume that the binding of BSS to the catalytic site of thrombin inhibited its enzymatic activity and subsequently diminished the fibrinogen clotting and platelet aggregation by thrombin. Further, pre-incubation of BSS/thrombin prior to the addition of heparin/AT-III did not jeopardize the catalytic activity of thrombin, which suggests that BSS does not bind to exosite-II (the heparin/AT-III binding site) of thrombin. In any case, further structural analyses would be needed to pinpoint the thrombin binding region of BSS.

Some reports have demonstrated the production of plasminogen activator in cultured endothelial cells from bovine carotid and in lung and kidney tissues [24,25]. In the present study; however, plasminogen is activated by BSS to form plasmin to dissolute the thrombus. Our results suggest that the thrombolytic potency of BSS depends on the activation of plasminogen to form plasmin since endogenous plasminogen is inactivated by heating the blood [26], which results in the loss of clot-bursting activity of BSS and streptokinase. Usually, patients who develop atrial fibrillation require anticoagulants to prevent the risk of clot formation, which could otherwise lead to cardiovascular diseases [27]. BSS delays the progressive coagulation of blood, and our results suggest that BSS plays a dual role in antithrombotic and thrombolytic activities.

Carrageenan-induced thrombosis in mice is a simple method for inducing thrombus in a small laboratory animal and observations are easy without having to kill the animals [13,28]. Our results suggest that BSS can prevent tail thrombosis induced by k-carrageenan, and therefore, could be a useful prophylactic antithrombotic drug.

Contradictory results have been presented to correlate the plasma levels of plant-sterol with CVDs [29]. Although the largest prospective trials and genome wide association studies suggest that high plasma levels of plant sterols are associated with increased CVD risk nevertheless some other studies have reported no such association and even an inverse relationship [29-31]. Thus, the available data cannot confirm an increased CVD risk with level of plant sterols, but cannot rule it out either. Only detailed interventional studies will provide deeper insight into the effects of plant-sterol-enriched food on the occurrence or prevention of CVDs.

Although the anticoagulant activity of BSS is lower than commercial drugs, potency may not be the sole reason for choosing an agent. Drug safety and the effectiveness of an anticoagulant when administered orally should also be considered. However, recent study shows that supplementation of BSS to the anticoagulant active fraction of plant

extract synergistically significantly enhanced anticoagulant activity as compared to the crude plant extract or the active fraction [32]. The literature suggests that BSS is generally considered as an effective nutritional supplement, safer to administer, non-toxic [33,34], inhibits mutagenecity [35], and has no effect on reproductive systems [36]. BSS is well tolerated in recommended doses for up to six months [6,7]. According to the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the acceptable daily intake (ADI) of BSS is 40 mg/kg human body weight/day and the No-Observed-Adverse-Effect-Level (NOAEL) is 4200 mg/kg_HBW/day. These doses are much higher than the *in vivo* anticoagulant dose of BSS suggesting its high therapeutic index. These results encourage clinical trials of BSS as an anticoagulant drug that may be used in combination with other drugs to prevent cardiovascular diseases.

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