Screening of selected medicinal plants of Assam for assessment of anticoagulant property and preparation of active anticoagulant fraction from selected plants

4.1 Brief Introduction:

Cardiovascular diseases are responsible for millions of deaths every year worldwide. Thrombosis is one of the leading causes of thromboembolic disorders. For more than five decades, anticoagulant drugs such as low molecular mass heparins, vitamin K antagonists, and their derivatives have been the major players in the clinical setting. The efficacy of these drugs remains disputed and the deleterious life-threatening side effects of these drugs have also been documented [1-3]. Plants may serve as the alternative sources for the development of new anticoagulant agents due to their biological activities. Several of the medicinal plants used for the treatment of thromboembolic diseases in different systems of traditional medicine have shown anticoagulant/antithrombotic activity; however, most of these plants remain to be scientifically investigated [4-6]. Further, the consumption of dietary anticoagulants or phytochemicals possessing anticoagulant properties can ultimately reduce or eliminate the risks of thromboembolic diseases [7,8].

The present chapter describes the screening of different plant extracts for anticoagulant activity under different extraction conditions. The two plants namely *Leucas indica* and *Momordica charantia* showing best anticoagulant activity were selected for further studies. Furthermore, a novel, cost effective and simple method for preparing an active anticoagulant preparation (AAP) from the aqueous leaves extract of *L. indica* was also presented in this chapter. The preparation and characterization of anticoagulant active fraction (AAF) from *M. charantia* is described in chapter 7 of this thesis.

4.2 Results

4.2.1 The aqueous extract of the leaves of *Leucas indica* and *Momordica charantia* showed superior anticoagulant activity

The results of the anticoagulant activity of different solvent extract of plants selected under the study are shown in Table 4.1. It was observed that highest anticoagulant activity was displayed by aqueous shade dried leaves extract of *L. indica* and whole fruits extract of *M. charantia.* It is noteworthy to mention that extraction with water having the highest dielectric constant (DEC) yields best anticoagulant activity. Therefore, these extracts were considered for further study to avoid inclusion of salts for purification purpose.

4.2.2 Optimization of extraction conditions of *L. indica* and *M. charantia*

The results of the anticoagulant activity of aqueous leaves extract of *L. indica* and fruit extract *M. charantia* under different extraction conditions is shown in Table 4.2. It was observed that optimum anticoagulant activity for *L. indica* was achieved post 4 h aqueous extraction at pH 7.4. However,*M. charantia*, showed optimum anticoagulant activity at 1 h of stirring, pH 7.4 followed by 10 min of sonication. Nevertheless, the extraction carried out at 4 °C or at room temperature did not influence the anticoagulant activity of the extracts suggesting the phytochemicals are stable at room temperature (Table 4.2).

Table 4.1. Anticoagulant activity shown by different extracts. Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to different extraction condition each other, $*p < 0.05$. For anticoagulant activity assay procedure, see section 3.2.8.1. NA: No activity; ND: Not determined.

Sl No	Botanical name	Parts Used	Conditions	Anticoagulant activity (Units) (1.0 µg/ml)					
				Ethanol (100%)	Methanol (100%)	Ethanol (50%)	Ethanol (25%)	Ethanol (12.5%)	Water
			Dielectric constant (DEC) →	25.0	32.5	51.5	67.0	71.3	78.0
1	*Leucas indica*	Leaves	Fresh	13.2 ± 1.2	11.2 ± 1.2	13.8 ± 0.8	14.2 ± 1.8	15.6 ± 2.1	18.2 ± 2.5
			Shade dried	14.0± 1.8	16.7 ± 2.1	15.8 ± 1.2	15.6 ± 1.2	18.4 ± 2.2	30.5 ± 3.2*
2	*Momordica charantia*	Leaves	Fresh	6.2 ± 1.5	5.0 ± 0.8	6.8 ± 0.6	7.2 ± 0.8	9.3 ± 1.4	11.0 ± 1.8
			Shade dried	8.8 ± 1.2	7.5 ± 1.2	8.9 ± 0.8	11.2 ± 1.4	12.2 ± 0.8	14.2 ± 1.6
		Fruits	Whole fruit	10.2 ± 2.2	12.4 ± 2.2	12.2 ± 1.1	13.6 ± 0.6	17.5 ± 2.5	21.7 ± 2.5*
			Seedless fruit	9.5 ± 1.8	11.3 ± 1.8	11.2 ± 1.0	13.2 ± 1.2	15.8 ± 1.4	17.2 ± 1.6
3	*Solanum indicum*	Fruits	Ripe	1.6 ± 1.6	3.2 ± 0.2	ND	ND	ND	5.6 ± 1.1
			Unripe	5.5 ± 2.1	4.2 ± 0.6	ND	ND	ND	7.2 ± 2.2
4	*Centella asiatica*	Leaves	Fresh	4.0 ± 2.2	1.2 ± 0.02	ND	ND	ND	4.6 ± 1.2
			Dried	2.5 ±1.3	6.2 ± 1.0	ND	ND	ND	8.5 ± 2.5
5	*Allivum sativum*	Clove	Ripe	5.5 ± 2.1	6.8 ± 1.2	ND	ND	ND	16.5 ± 1.9
			Unripe	3.5 ± 3.2	4.2 ± 0.8	ND	ND	ND	11.2 ± 2.2
6	*Hibiscus rosasinensis*	Flowers	Fresh	NA	NA	ND	ND	ND	NA
			Dried	NA	NA	ND	ND	ND	ND

Table 4.2. Anticoagulant activity of aqueous leaves extract *L. indica* and fruit extract *M. charantia* under different extraction conditions. Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to different extraction parameters, * $p < 0.05$.

Parameters	**Anticoagulant activity (units) (1.0 µg/ml)**	
Time of stirring (h)	***L. indica***	***M. charantia***
1	21.2 ± 2.8	21.7 ± 2.5
2	22.4 ± 1.2	20.3 ± 3.2
4	30.5 ± 2.2*	21.2 ± 1.8
6	30.0 ± 1.8*	20.2 ± 3.2
8	28.3 ± 6.3*	22.1 ± 1.5
12	27.5 ± 2.7*	21.2 ± 2.5
pH (4 h & 1 h stirring is kept constant for *L. indica* & *M. Charantia*, respectively)		
6.5	25.6 ± 1.8	14.7 ± 2.2
7.0	29.2 ± 1.6	21.4 ± 1.6
7.4	36.5 ± 2.8*	27.7 ± 1.4*
8.0	32.2 ± 2.8	23.8 ± 3.1
Temperature (4 h & 1 h stirring is kept constant for *L. indica* & *M. Charantia*, respectively, at pH 7.4)		
4 ºC	36.5 ± 2.8	27.7 ± 1.4
Room temperature, RT (23 ± 2 ºC)	36.5 ± 2.8	27.7 ± 1.4
Sonication (min) (4 h & 1 h stirring is kept constant for *L. indica* & *M. Charantia*, respectively, at pH 7.4 & RT)		
5	36.5 ± 2.8	28.3 ± 3.2
10	36.5 ± 2.8	33.7 ± 1.4*
15	36.5 ± 2.8	30.2 ± 2.0

4.2.3 Preparation of active anticoagulant fraction (AFLI) from aqueous leaves extract of *L. indica*

Fractionation of crude aqueous leaves extract of *L. indica* through anion-exchange matrix resulted in separation of its component into nine peaks (Fig. 4.1a). Peak1 (AEX_1) eluted with the equilibration buffer (unbound fractions) at pH 7.4 showed significant fibrinogenolytic and anticoagulant activities (Table 4.3). Cation-exchange chromatography of the AEX_1 peak separated it into eight fractions (CEX_1 to CEX_8) (Fig. 4.1b). The

cation-exchange unbound peak CEX_1 eluted with the equilibration buffer demonstrated further superior fibrinogenolytic as well as anticoagulant activities (Table 4.3) and this fraction was labeled as active fraction of *L. indica* (AFLI). A summary of purification of AFLI is shown in Table 4.3. The AFLI represented 2.85 % of the total protein loaded to the column.

The HPLC gel-filtration of CEX_1 fraction resolved it into three peaks (AF_GF1 to AF_GF3) (Fig. 4.1c); however, none of the showed anticoagulant activity better than AFLI (Table 4.3). Moreover, passing the AFLI through 3 kDa cut off membrane did not improve its anticoagulant and fibrinogenolytic activities (Table 4.3).

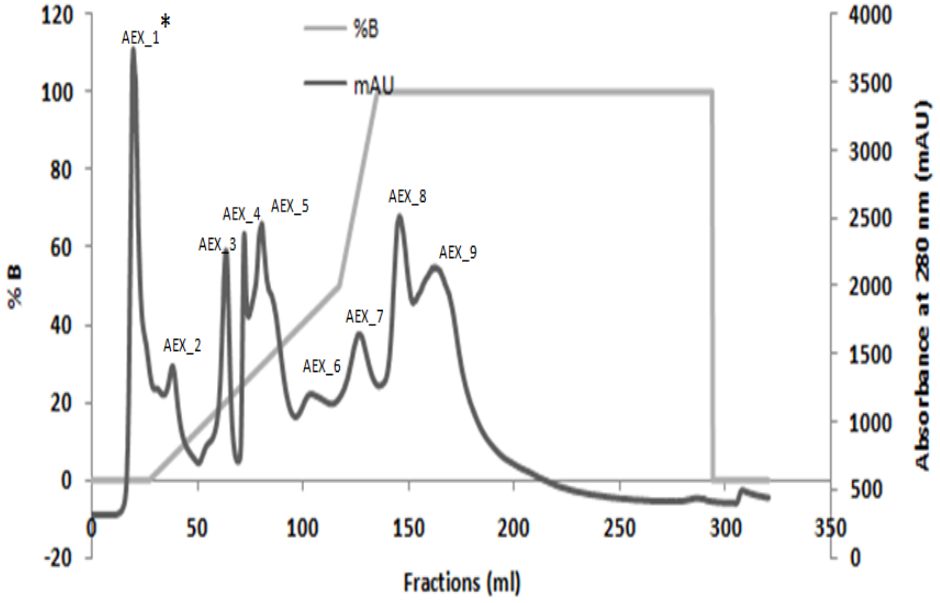


Fig. 4.1a. Fractionation of crude aqueous shade leaves extract of *L. indica* cation exchange on Prep^{TM} anion exchange DEAE-cellulose FF 16/10 column. The first peak (AEX_1) corresponds to the elution of fraction showing highest anticoagulant and fibrinogenolytic activities.

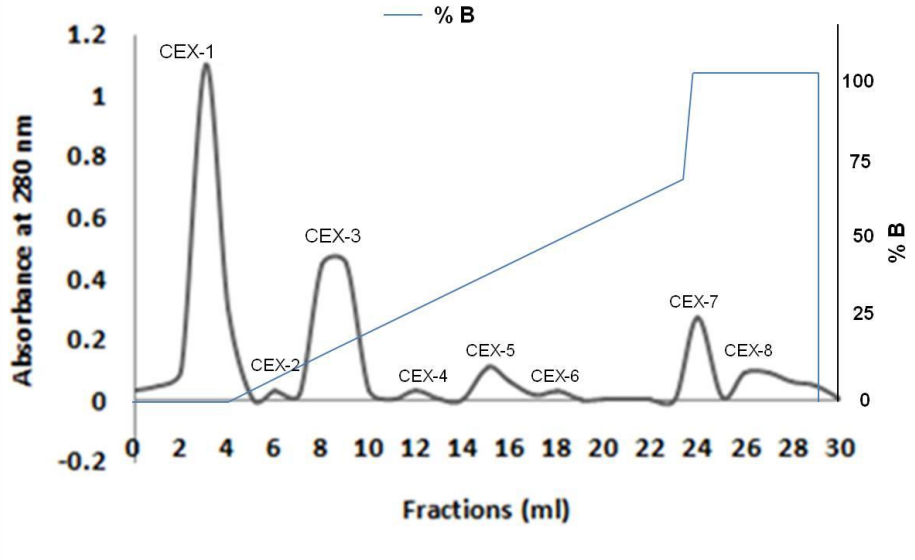


Fig. 4.1b. Fractionation of the anion-exchange unbound fraction (AEX_1 peak) on cation exchange CM-cellulose (20 mm X 60 mm) column. The first peak (CEX_1) corresponds to the elution of fraction showing highest anticoagulant and fibrinogenolytic activities.

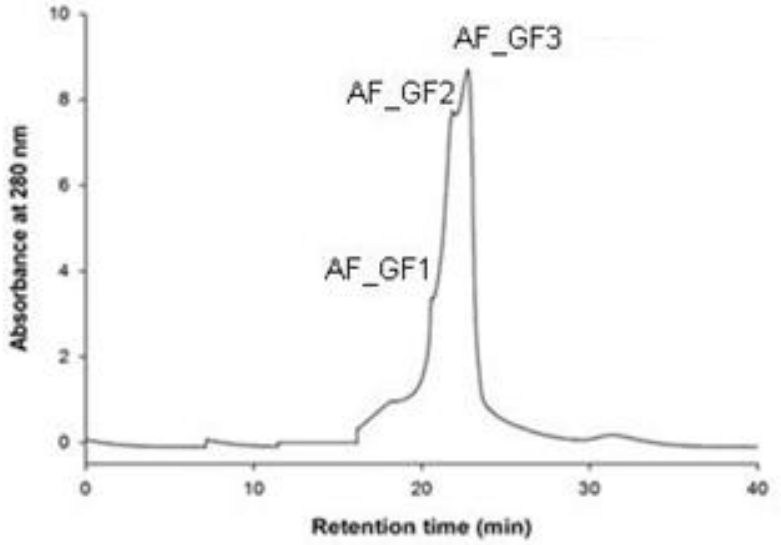


Fig. 4.1c. Gel filtration chromatography of the AFLI on Shodex KW-803 column (5 µm, 8 x 300 mm).

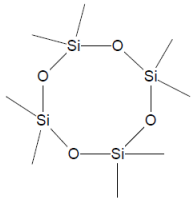
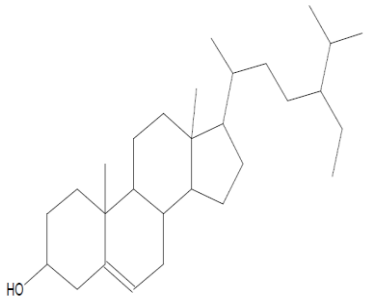
Table 4.3. A summary of purification of AFLI from aqueous leaves extract of *L. indica*. Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to crude aqueous extract, * $p< 0.01$. For the assay of anticoagulant and fibrinogenolytic activity, see procedure in sections 3.2.7.4 and 3.2.8.1, respectively.

Fractions	**Specific activity (Units/mg)**	
	Anticoagulant	**Fibrinogenolytic**
1. Crude aqueous leaves extract	$30.5 \times 10^3 \pm 1.00$	8.10 ± 0.64
2. Anion exchange chromatography		
Unbound fraction		
AEX_1	$34.06 \times 10^3 \pm 1.00$ *	7.90 ± 0.30
Bound Fractions		
AEX_2	$1.53 \times 10^3 \pm 0.30$	0.75 ± 0.05
AEX_3	$0.60 \times 10^3 \pm 0.36$	0.58 ± 0.27
AEX_4	$1.06x\ 10^3 \pm 0.15$	0.62 ± 0.12
AEX_5	$0.43 \times 10^3 \pm 0.05$	0.15 ± 0.05
AEX_6	$0.83 \times 10^3 \pm 0.32$	0.19 ± 0.01
AEX_7	$1.20 \times 10^3 \pm 0.70$	0.88 ± 0.12
AEX_8	$1.83 \times 10^3 \pm 0.25$	0.41 ± 0.11
AEX_9	$0.93 \times 10^3 \pm 0.15$	0.22 ± 0.11
3. Cation exchange chromatography		
Unbound fraction		
CEX_1 (AFLI)	$46.4 \times 10^4 \pm 2.8$ *	12.13 ± 1.02 *
Bound fractions		
CEX_2	$4.10 \times 10^3 \pm 1.15$	0.73 ± 0.43
CEX_3	$1.33 \times 10^3 \pm 0.66$	0.98 ± 0.19
CEX_4	$0.70 \times 10^3 \pm 0.36$	0.43 ± 0.12
CEX_5	$2.80 \times 10^3 \pm 0.80$	1.08 ± 0.22
CEX_6	$3.10 \times 10^3 \pm 0.26$	1.30 ± 0.20
CEX_7	$1.10 \times 10^3 \pm 0.26$	1.06 ± 0.15
CEX_8	$4.03 \times 10^3 \pm 0.20$	1.28 ± 0.45
4. Gel filtration chromatography		
GF_AF1	$14.2 \times 10^3 \pm 2.4$	2.30 ± 0.8
GF_AF2	$21.2 \times 10^3 \pm 2.4$	3.25 ± 1.0
GF_AF3	$32.5 \times 10^3 \pm 3.5$	5.50 ± 1.4
5. AFLI 3- KDa cut off membrane		
i) AFLI- 3KDa membrane retained fraction	$32.5 \times 10^3 \pm 3.5$	5.50 ± 1.4
ii). AFLI- 3KDa membrane flow through fraction	$42.0 \times 10^3 \pm 4.24$	10.2 ± 2.1

4.2.4 Gas chromatography–mass spectrometry analysis of AFLI

GC-MS analysis revealed the presence of β-sitosterol (16 - 20%), methyl palmitate (7 - 10%) and octamethyltetracyclosiloxane (9 - 12%) as the major phytoconstituents or active ingredients of AFLI (Table 4.4).

Table 4.4. Analysis of AFLI by GC–MS to identify its major constituents.

S No	Phytochemicals	Compound nature	Structure	Molecular mass (g/mol)
1	Cyclotetrasiloxane, octamethyl-	Organosilicone		296.61
2	Hexadecanoic acid, methyl ester (Methyl palmitate)	Unsaturated fatty acid		270.45
3	β-Sitosterol	Phytosterol		414.70

4.2.5 SDS-PAGE analysis of AFLI

By 12.5% SDS-PAGE analysis, the molecular mass of major protein constituent of AFLI was found to be 35 kDa (Fig. 4.2)

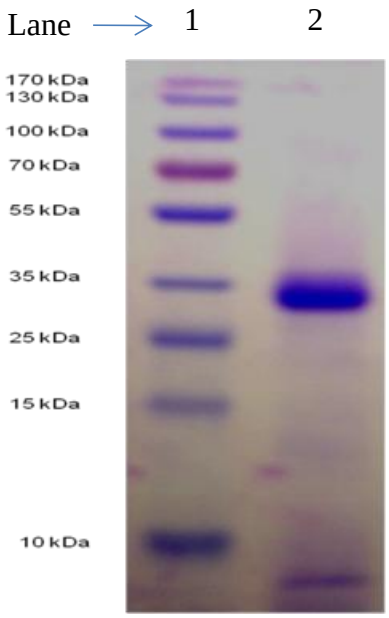


Fig. 4.2. 12.5% SDS-PAGE analysis of AFLI. Lane 1, protein molecular markers; Lane 2, reduced AFLI (30 µg).

4.2.6 LC-MS/MS analysis of major protein constituent of AFLI

By LC-MS/MS analysis of ~35 kDa protein, a major constituent of AFLI, did not show significant similarity with any plant protein deposited in NCBI protein databases suggesting it is an uncharacterized protein isolated from a plant source. However, one of the MS-MS derived tryptic fragment of this ~35 kDa protein viz. IITHPNFNGNTLDNDIMLIK (2283.6 Da) demonstrated conserved domain of trypsin-like superfamily suggesting this protein may be a previously uncharacterized protease from plant source. Biochemical characterization revealed protease activity contained in this active fraction (see chapter 5) which may be correlated to this ~35 kDa protein. The other tryptic sequences of this ~35 kDa protein viz. LGEHNIDVLEGNEQFINAAK (2219.39 Da), VATVSLPR (842 Da), LSSPATLNSR (1316.46 Da), APVLSDSSCK (1006.13) did not show similarity to Lamiaceae family of proteins deposited in databases. The detailed characterization of this~35 kDa protein is presented in Chapter 5 of this thesis.

4.2.7 Formulation of active anticoagulant preparation (AAP)

The AFLI and ß- sitosterol were mixed in different ratios [1:1, 5:1 and 10:1 (w/w)]. The anticoagulant activity of AFLI was found to be significantly enhanced when it was mixed with ß- sitosterol at 5:1 (AFLI: ß- sitosterol) ratio (Table 4.5). This formulation is termed Active Anticoagulant Preparation (AAP) of the present invention.

Table 4.5. Anticoagulant and antithrombin (in terms of fibrinogen clotting time of thrombin) activities of the AFLI and AAP. Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to AFLI, * $p < 0.05$;** $p < 0.01$.

Samples (1.0 µg/ml)	**Anticoagulant activity (Units/mg)[a]**	**Antithrombin actvity (Units/mg)[b]**
AFLI	$46.4 \times 10^3 \pm 0.8$	$69.25 \times 10^3 \pm 3.75$
ß- sitosterol	$35.5 \times 10^3 \pm 1.2$	$48.14 \times 10^3 \pm 3.1$
AFLI : ß- sitosterol (1:1)	$66.0 \times 10^3 \pm 1.7$*	$90.25 \times 10^3 \pm 6.6$*
AFLI : ß- sitosterol (5:1)	$82.6 \times 10^3 \pm 7.07$**	$104.5 \times 10^3 \pm 2.61$**
AFLI : ß- sitosterol (10:1)	$83.0 \times 10^3 \pm 5.05$**	$111.5 \times 10^3 \pm 3.5$**

[a]One unit of anticoagulant activity is defined as 1 s increase in clotting time of the control PPP in presence of sample

[b]One unit of inhibition of fibrinogen clotting time of thrombin is defined as 1 s increase in clotting time of fibrinogen by thrombin in presence of sample. Only thrombin showed fibrinogen clotting time of 39.9 ± 1.3 s (mean ± S.D., n=3)

4.2.8 Dose- and time-dependent anticoagulant activity of AAP and AFLI

Both AAP and AFLI dose- dependently prolonged the Ca^{2+} clotting time of platelet poor plasma (PPP); however, after a dose of 5.0 µg/ml of AAP a saturation in anticoagulant activity was observed (Fig. 4.3a).

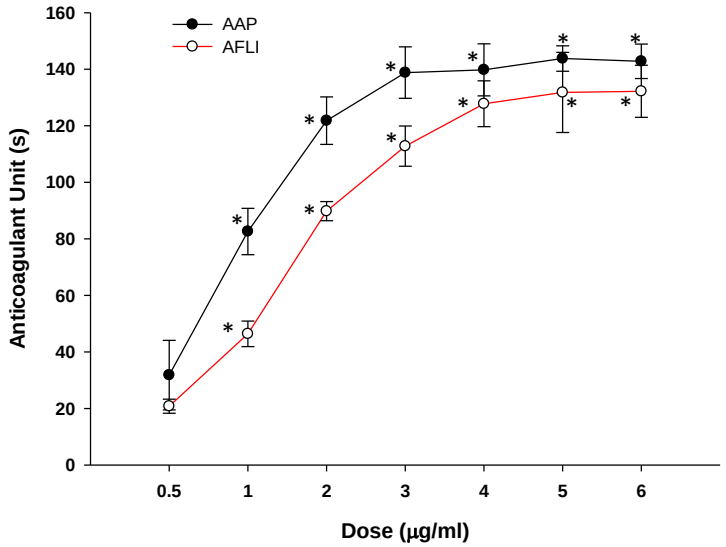


Fig. 4.3a. Dose- dependent *in vitro* anticoagulant activity of AAP/AFLI against human PPP. All values are means ± S.D. of twelve independent experiments. The Ca-clotting time of PPP under identical experimental conditions (control) was found to be 91.2 s. Significance of difference with respect to control (without AAP/AFLI), * $p< 0.01$.

AAP demonstrated optimum anticoagulant activity at 10 min of pre-incubation with PPP (Fig. 4.3b). The anticoagulant potential of AAP was found to be superior to heparin, warfarin and nattokinase; however, less ($p<0.01$) than argatroban (Fig. 4.3c). At a concentration of 1- 10 µg/ml, AAP had no effects on APTT of human plasma; however, it significantly ($p<0.05$) enhanced the PT of PPP (Fig. 4.3d).

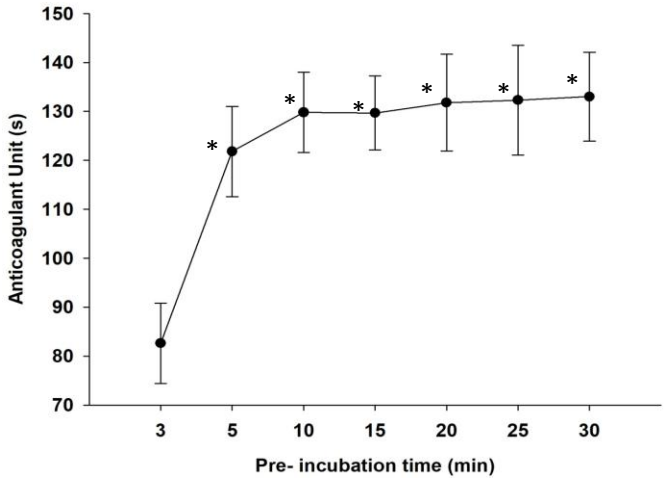


Fig. 4.3b. Time- dependent anticoagulant activity by AAP against human PPP. The AAP (5.0 µg/ml) was pre-incubated with 300 µl of PPP from 3 to 15 min at 37 °C, pH 7.4 before the addition of 250 mM $CaCl_2$ to initiate the clot formation. The Ca- Clotting time of PPP under identical experimental conditions (control) was found to be 91.2 s. Values are mean ± S.D. of twelve determinations. Significance of difference with respect to control (without AAP), * $p< 0.01$

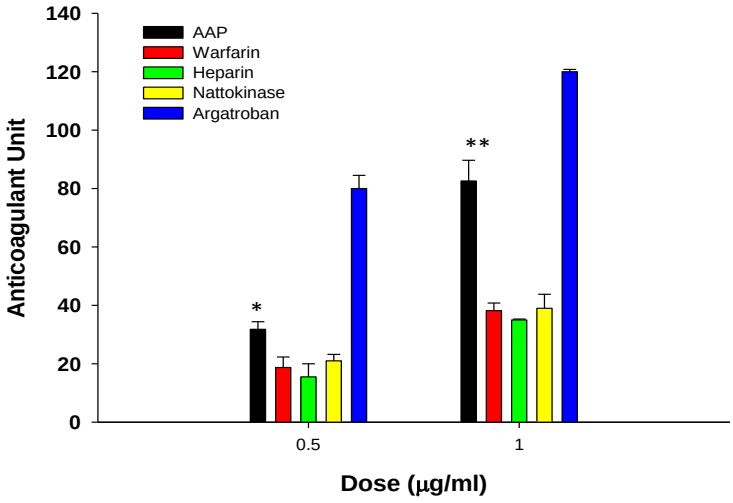


Fig. 4.3c. Comparison of *in vitro* anticoagulant activity of AAP, warfarin, heparin, nattokinase, and argatroban against goat PPP. Values are mean ± S.D. of three determinations. Significance of difference with respect to control, * p< 0.05; ** p< 0.01.

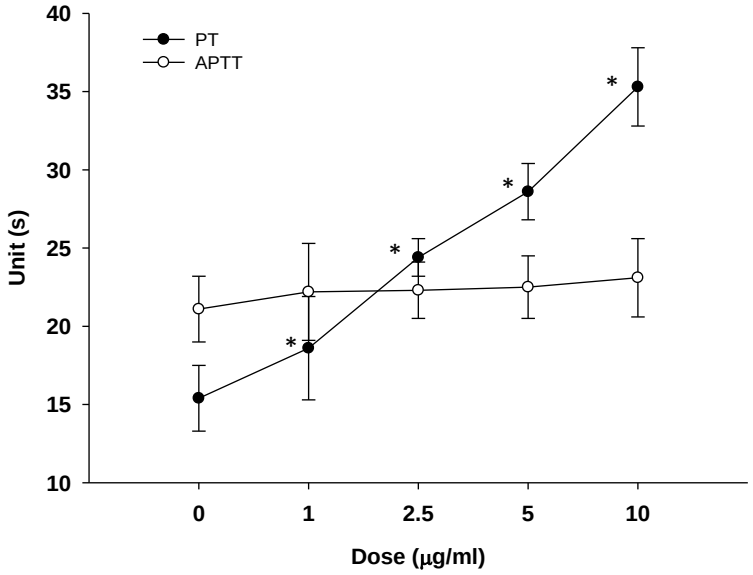


Fig. 4.3d. The *in vitro* effect of AAP on APTT and PT time of PPP from human blood. Values are mean ± S.D. of twelve determinations, Significance difference with respect to control, * $p < 0.01$

4.2.9 Inhibition of catalytic activity of thrombin and FXa by AAP

AAP significantly inhibited the amidolytic activity of thrombin (Fig. 4.4a) and FXa (Fig. 4.4b). In a dose-dependent manner, AAP prolonged the fibrinogen clotting time of thrombin (Fig. 4.4c). The optimum inhibition was observed at 15 min of pre-incubation of thrombin with AAP (Fig. 4.4d). Further, AAP at a dose of 1.0 µg/ml showed 100% inhibition of prothrombin activation by FXa (Fig. 4.4e).

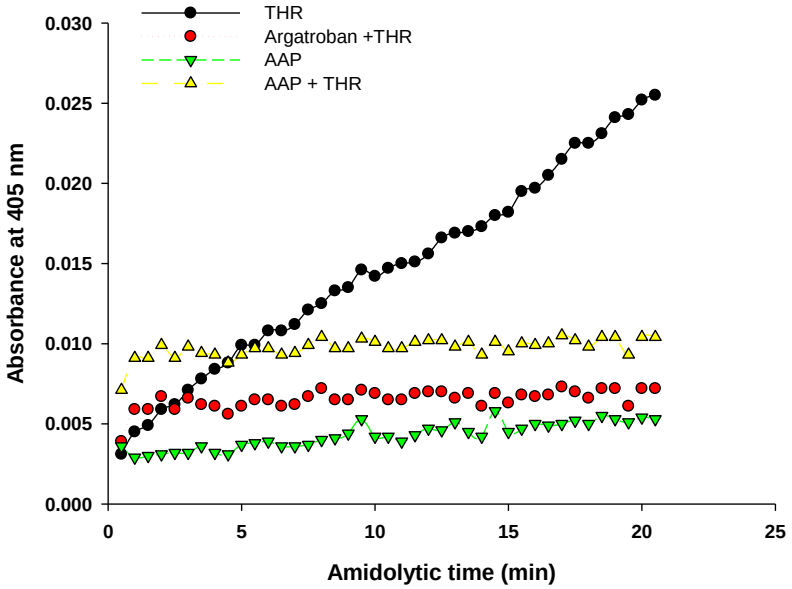


Fig. 4.4a. Effect of AAP (1.0 µg/ml) on amidolytic activity of thrombin (36.6 nM) against its chromogenic substrate T1637 (0.2 mM). The values are mean of triplicate determinations.

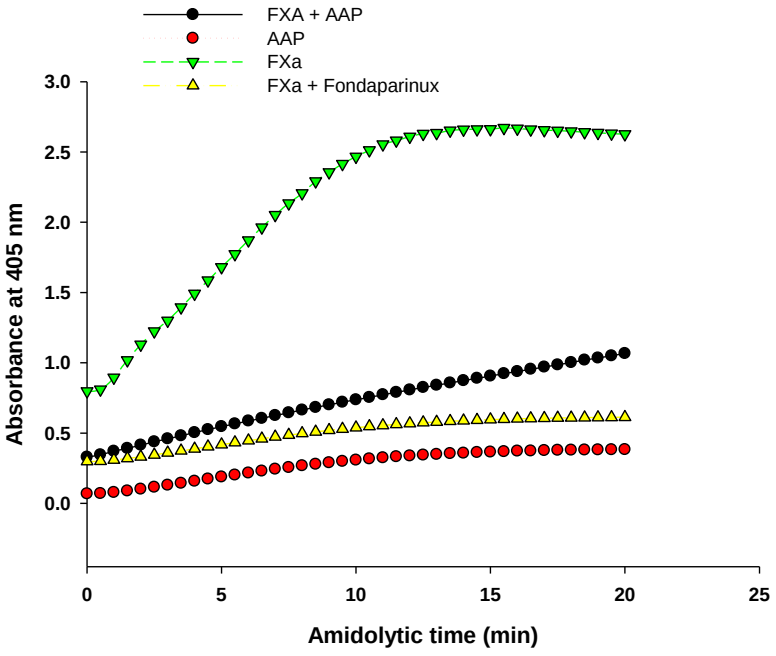


Fig. 4.4b. Effect of AAP (1.0 µg/ml) on amidolytic activity of FXa (0.13 mM) against its chromogenic substrate F3301 (0.2 mM). The values are mean of triplicate determinations.

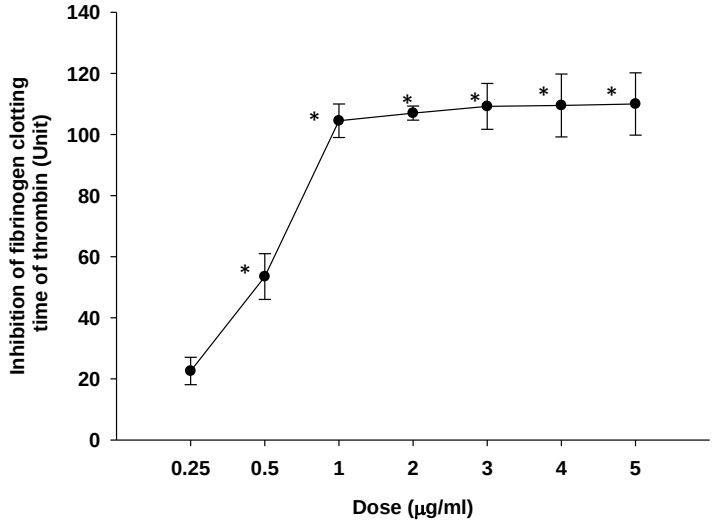


Fig. 4.4c. Dose-dependent (0.25-5 µg/ml) inhibition of fibrinogen clotting time of thrombin by AAP at 37 °C, pH 7.4. The values are mean ± S.D. of triplicate determinations. Significance of difference with respect to control (without AAP), * $p < 0.05$

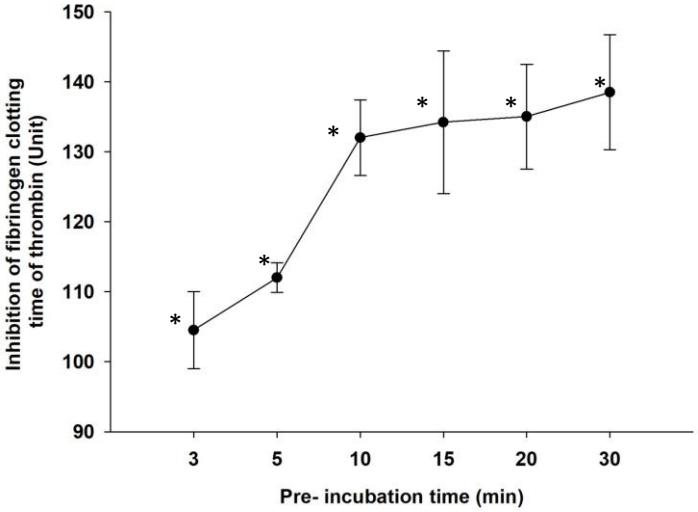


Fig. 4.4d. Time-dependent inhibition of fibrinogen clotting time of thrombin by AAP (1.0 µg/ml) at 37 °C, pH 7.4. The fibrinogen clotting time of thrombin under identical experimental conditions (control) was found to be 39.17 ± 1.54 s. The values are mean ± S.D. of triplicate determinations. Significance of difference with respect to control (without AAP), * $p < 0.05$.

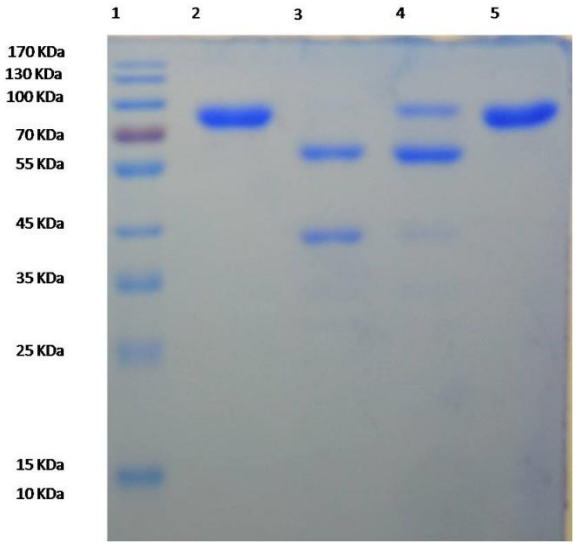


Fig. 4.4e. Inhibition of prothrombin activation property of FXa by AAP. After reduction with β-mercaptoethanol, degradation products were separated by 12.5% SDS-PAGE. Lane 1, protein molecular markers; lane 2, 12 µg PTH; lane 3, PTH (12 µg) incubated with FXa (0.1 µg) for 30 min at 37 ^{0}C, pH 7.4; lane 4, [FXa (0.1µg) pre-incubated with AAP (1.0 µg) for 15 min] + PTH (12 µg); lane 5, PTH + AAP.

4.2.10 Fibrin(ogeno)lytic activity of AAP

To elucidate the mode of action of AAP, digested fragments of fibrin/fibrinogen were separated by 12.5% SDS-PAGE. The fibrin/fibrinogen degradation pattern demonstrated that AAP preferentially degraded the Aα- chain of fibrin and fibrinogen and slow degradation of Bβ-chain of fibrinogen (Figs. 4.5a, b); however, the γ-chain of fibrinogen/fibrin was not degraded within 2 h of incubation (Figs. 4.5a, b). This result is indicative of the fact that the AAP contains a fibrin(ogen)olytic protease.

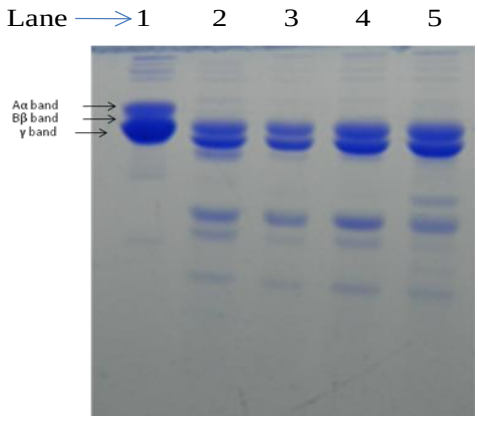


Fig. 4.5a. Kinetics of fibrinogenolytic activity of AAP. After reduction with β-mercaptoethanol, degradation products were separated by 12.5% SDS-PAGE. Lane 1, control human fibrinogen (0.25% w/v in 20 mM K-phosphate buffer, 150 mM NaCl, pH 7.4); lanes 2-5, human fibrinogen degradation by AAP (100 µg/ml) at 30, 60, 90 and 120 min of incubation, respectively at 37 ^{0}C, pH 7.4.

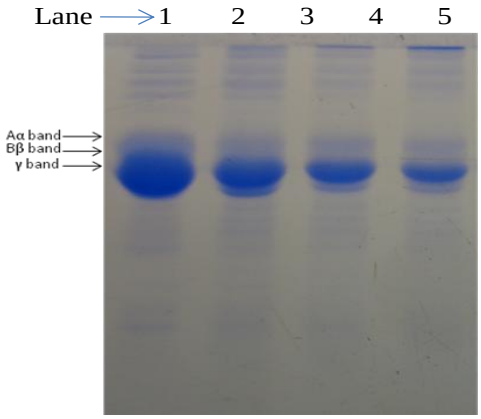


Fig. 4.5b. Kinetics of fibrinolytic activity of AAP. After reduction with β-mercaptoethanol, degradation products were separated by 12.5% SDS-PAGE. Lane 1, control human fibrin; lanes 2 - 4, human fibrin degradation by 100 µg/ml AAP at 60, 90 and 120 min of incubation, respectively at 37 ^{0}C, pH 7.4

4.2.11 Spectrofluorometric analysis to determine the interaction of AAP with thrombin/ fibrinogen/ FXa

A steady decrease in the fluorescence intensity of thrombin was observed in presence of AAP (Fig. 4.6a). However, the interaction between AAP and FXa (Fig. 4.6b) as well as

between AAP and fibrinogen (Fig. 4.6c) resulted in an increase in the fluorescence intensity of interacting proteins as compared to the fluorescence intensity of individual protein.

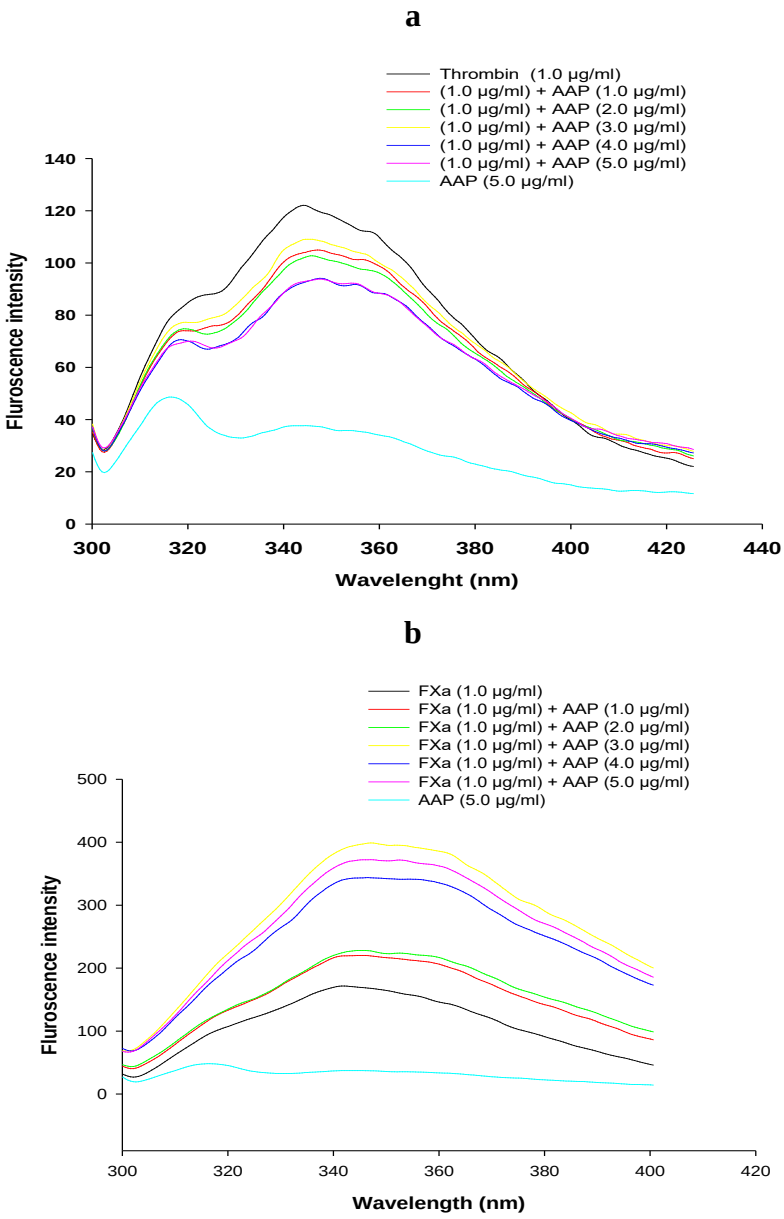


Fig. 4.6. Fluorescence spectra showing **a)** interaction of thrombin (1.0 µg/ml) and **b)** interaction of FXa (1.0 µg/ml) with different concentrations of AAP (1.0-5.0 µg/ml). Data represent the average of triplicate determinations.

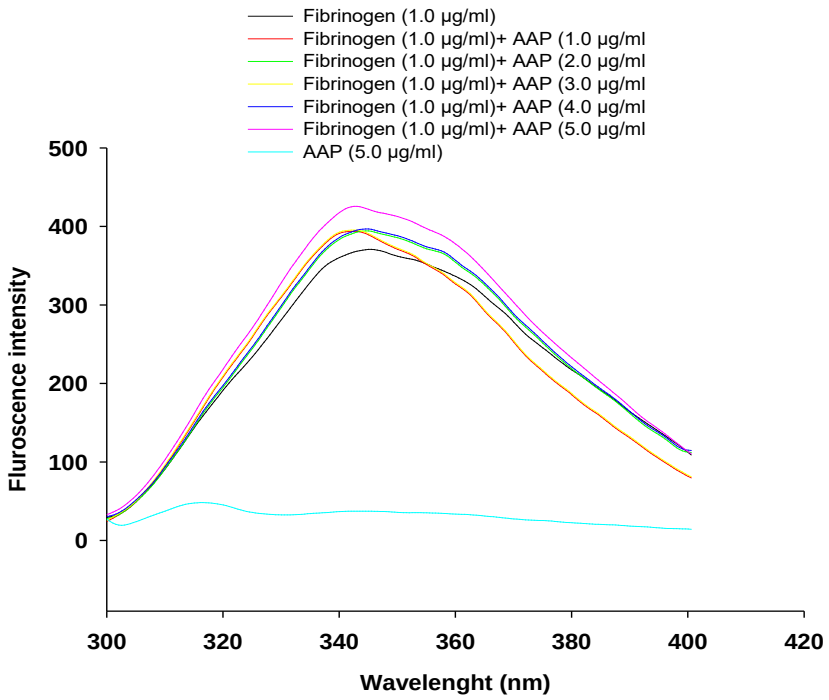


Fig. 4.6c. Fluorescence spectra showing interaction of fibrinogen (1.0 µg/ml) with different concentrations of AAP (1.0-5.0 µg/ml). Data represent the average of triplicate determinations.

4.2.12 AAP demonstrated *in vitro* thrombolytic activity

The *in vitro* thrombolytic potency of AAP and commercial thrombolytic agents (streptokinase, plasmin, nattokinase) is shown in Table 4.6. AAP was found to be superior to streptokinase, plasmin, and nattokinase in dissolving the blood clot. Nevertheless, the thrombolytic potency of AAP, nattokinase, and plasmin towards a heat-treated blood clot was reduced to 90%, 75%, and 60%, respectively, of their original activity to dissolve an unheated blood clot (Table 4.6). Streptokinase showed negligible activity (<1%) in dissolving a heated blood clot (Table 4.6).

Table 4.6. A comparison of the *in vitro* thrombolytic activity among AAP, nattokinase, plasmin, and streptokinase under identical experimental conditions. Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to control, * $p < 0.01$.

Agent	**Clot bursting activity (mg of blood clot lysed/µg of test sample)**
A. None-heated blood clot	
Control (1X PBS, pH 7.4)	0.3 ± 0.02
AAP	15.8 ± 0.5*
Streptokinase	13.8 ± 0.4
Nattokinase	12.7 ± 1.2
Plasmin	13.2 ± 0.8
B. Heated blood clot	
Control (1X PBS, pH 7.4)	0.28 ± 0.02
AAP	14.2 ± 2.5*
Streptokinase	0.6 ± 0.1
Nattokinase	9.2 ± 1.8*
Plasmin	7.8 ± 0.6

4.2.13 AAP is devoid of hemolytic activity and cytotoxicity against mammalian cells and did not affect the cytochrome P450 enzymes

AAP did not show *in vitro* hemolysis against washed erythrocytes and cytotoxicity against HEK 293 cells (Figs. 4.7a, b). AAP at the tested doses did not inhibit the activity of CYP isoforms enzymes (Fig. 4.7c)

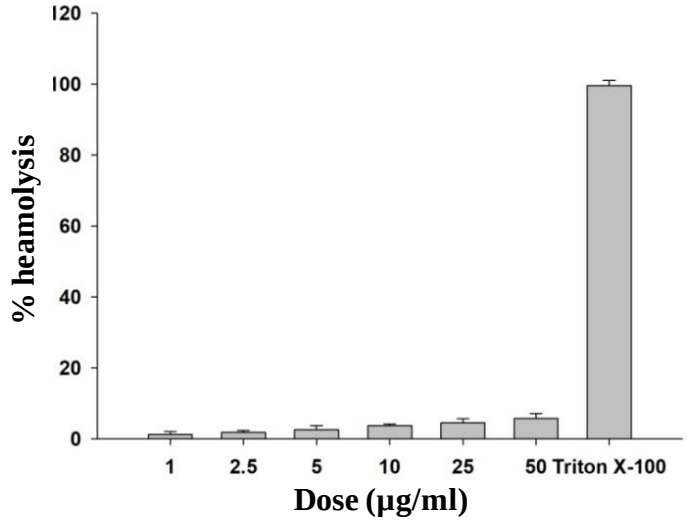


Fig. 4.7a. *In vitro* hemolysis assay of AAP. Erythrocytes suspension (5%, v/v) prepared from goat blood were treated with AAP (1.0-100 µg/mL) or 0.1% Triton x-100 (100% hemolysis) for 90 min at 37˚C. The values are mean ± SD of triplicate determinations.

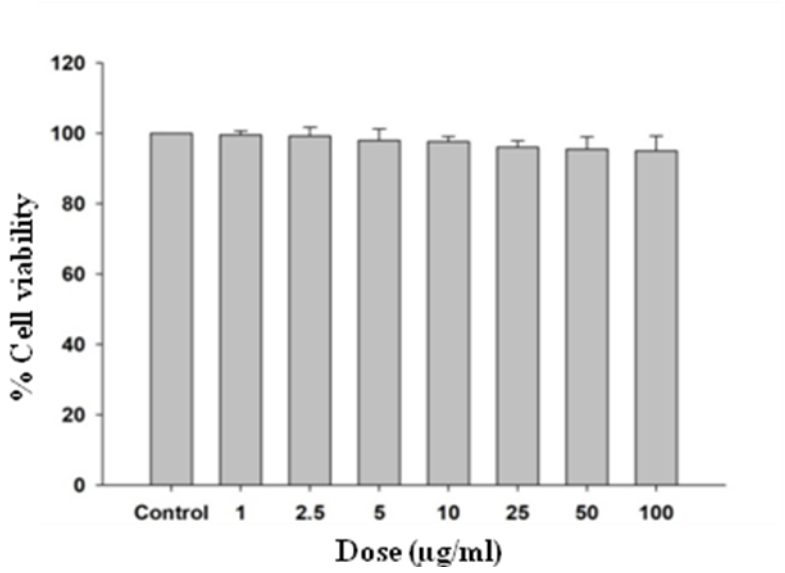


Fig. 4.7b. *In vitro* cell viability by MTT assay. HEK 293 cells (1.0 x 10^5 cells /ml) were treated with AAP (0- 100 µg/ml) for 24 h at 37 ˚C. All values are mean ± SD of triplicate determinations.

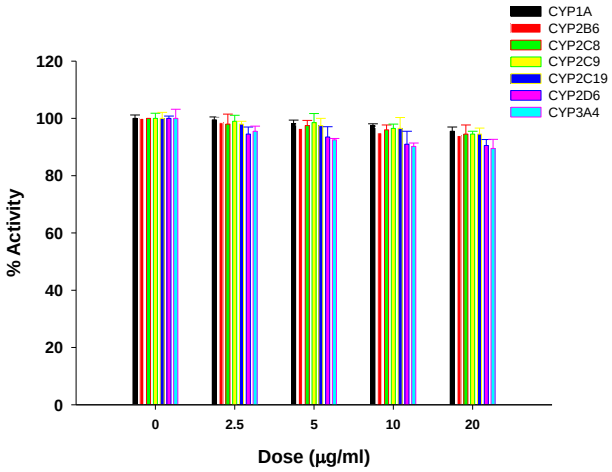


Fig. 4.7c. Effect of AAP (100 µg/ml) on different Cytochrome P450 isoforms. Values are mean ± S.D. of three determinations.

4.2.14 AAP showed antiplatelet activity and also inhibited the collagen and ADP-induced platelet aggregation

The dose-dependent platelet de-aggregation (antiplatelet) activity displayed by same concentrations of AAP and aspirin is shown in Fig. 4.8. AAP showed significantly higher platelet deaggregation property as compared to aspirin (Fig. 4.8). AAP also exhibited dose-dependent inhibition of the collagen and ADP-induced aggregation of PRP (Fig. 4.9).

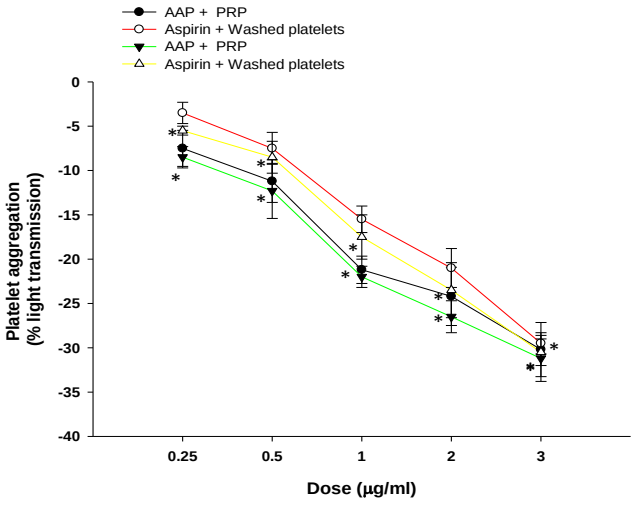


Fig. 4.8. Dose-dependent platelet deaggregation by AAP/aspirin. Different concentrations (0.25–3 µg/ml) of AAP or aspirin were incubated with platelet rich plasma at 37°C and the absorbance was recorded at 540 nm. Significance of difference with respect to aspirin, * p< 0.01.

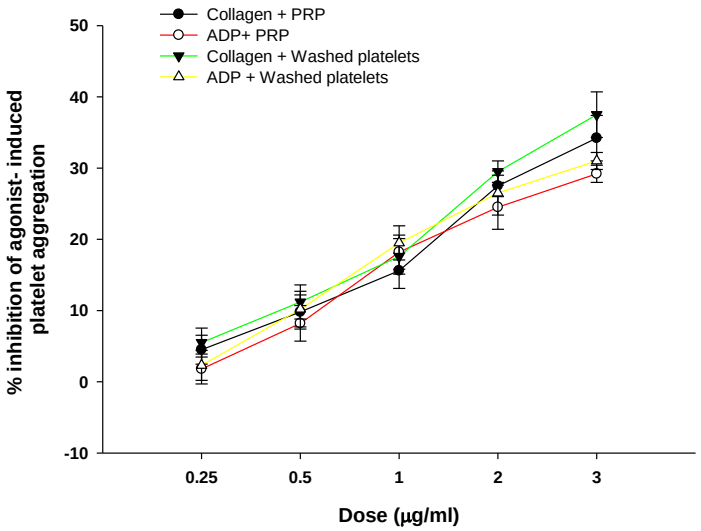


Fig. 4.9. Dose-dependent inhibition of collagen/ADP-induced platelet aggregation by AAP. Different concentrations of AAP (0.25-3.0 µg/ml) were incubated with PRP at 37°C for 10 min followed by addition of collagen (6.2 nM)/ADP (30 µM) to the reaction mixture. Data represents the mean ± SD of triplicate experiments.

4.2.15 Determination of *in vivo* toxicity of AAP in Swiss albino mice

AAP at a dose of 2000 mg/kg was found to be non-toxic to mice and showed no adverse effects and/or behavioral changes in treated-mice up to 7 days of oral administration (Table 4.7).

Table 4.7. A comparison of behavioral and physical parameters in AAP-treated mice. The AAPat a dose of 2000 mg/kg was orally administered to mice for 7 days.

Parameters	**Control**	**AAP-treated (2000 mg/kg)**
Survival of mice	No death	No death
Grip Strength (s)	22.5 ± 2.1	21.0 ± 1.8
Rectal temperature	35.3 ºC	35.7 ºC
Drooping of eyelids	Not observed	Not observed
Movement inability	Not observed	Not observed
Changes in body weight	Not observed	Not observed
Food and water intake	Normal	Normal

4.2.16 Effect on hematological and serum biochemical parameters of AAP-treated mice

The hematological parameters of blood from AAP-treated mice (7 days post-treatment) did not show any significant deviation as compared to the control group of mice (Table 4.8). In addition, none of the serum parameters of the treated mice were found to be altered ($p>0.05$), as compared to the control group of mice (Table 4.9). Plasma IgG, IgA, and IgE contents of AAP-treated mice did not differ significantly ($p>0.05$) from those of the control group of mice (Table 4.10).

Table 4.8. Hematological parameters of AAP-treated (2000 mg/kg) and control group of mice after 7 days of oral administration of AAP. The values are mean ± SD of 6 mice. There was no significant difference of values ($p>0.05$) between control and AAP-treated groups of mice.

	Values	
Parameters (Unit)	**Control mice**	**AAP-treated mice (2000 mg/kg)**
WBC(m/mm^3)	5.06 ± 0.36	4.85 ± 0.98
Lymphocytes(%)	36.17 ± 3.00	30.60 ± 0.61
Monocytes(%)	5.67 ± 0.20	5.13 ± 0.06
Neutrophils(%)	50.63 ± 6.62	61.33 ± 0.31
Eosinophils(%)	10.63 ± 0.26	12.20 ± 0.10
Basophils(%)	0.10 ± 0.00	0.17 ± 0.12
Total RBC(m/mm^3)	8.77 ± 0.12	7.65 ± 0.53
MCV(fl)	44.23 ± 2.60	48.77 ± 1.99
HCt(%)	36.20 ± 0.40	35.17 ± 1.77
MCH(pg)	15.77 ± 0.63	18.60 ± 2.12
MCHC(g/dl)	37.13 ± 0.66	31.17 ± 4.80
RDW	15.73 ± 0.16	12.70 ± 2.00
Hb(g/dl)	12.73 ± 0.25	12.67 ± 0.64
MPV(fl)	6.93 ± 0.10	6.80 ± 0.17
PCt(%)	0.31 ± 0.11	0.29 ± 0.03
PDW	8.37 ± 0.16	8.07 ± 0.25

Table 4.9. Some biochemical properties of serum of control and AAP-treated (2000 mg/kg) mice after 7 days of oral administration of AAP. Values are mean ± SD of 6 mice. There was no difference of values ($p>0.05$) between control and AAP-treated groups of mice.

	Values	
Parameters (Unit)	**Control**	**AAP-treated**
LDL(mg/dl)	13.30 ± 0.60	13.20 ± 0.50
HDL(mg/dl)	18.80 ± 0.40	16.60 ± 1.10
Glucose(mg/dl)	70.00 ± 5.20	67.00 ± 5.50
Urea(mg/dl)	53.80 ± 3.20	53.00 ± 3.30
Total protein(g/dl)	6.30 ± 0.40	6.10 ± 0.30
Uric acid(mg/dl)	1.30 ± 0.40	1.30 ± 0.20
Triglycerides	52.10 ± 2.60	50.40 ± 3.20
Cholesterol (mg/dl)	46.80 ± 3.40	48.20 ± 2.10
Creatinine(mg/dl)	1.00 ± 0.20	1.40 ± 0.20
SGPT(U/L)	91.30 ± 6.60	95.40 ± 1.70
SGOT(U/L)	162.30 ± 4.90	166.50 ± 3.10

Table 4.10. Serum immunoglobulin parameters of the AAP-treated (2000 mg/kg) mice after 7 days of oral administration. Values are mean ± SD of 6 mice. There was no significant difference of values ($p>0.05$) between control and AAP-treated groups of mice.

Parameters (mg/ml)	**Vehicle (control)**	**AAP-treated (2000 mg/kg) mice**
IgG	1.8 ± 0.34	1.7 ± 0.6
IgA	0.9 ± 0.03	1.2 ± 0.08
IgM	1.3 ± 0.2	1.1± 0.35

4.2.17 Histopathological study of tissues obtained from AAP-treated and control group of mice

Light microscopic examination of the liver, kidney, and cardiac tissues of the AAP-treated mice did not show any gross morphological alterations or pathophysiological symptoms as compared to the same tissues from control group of mice (Fig. 4.10).

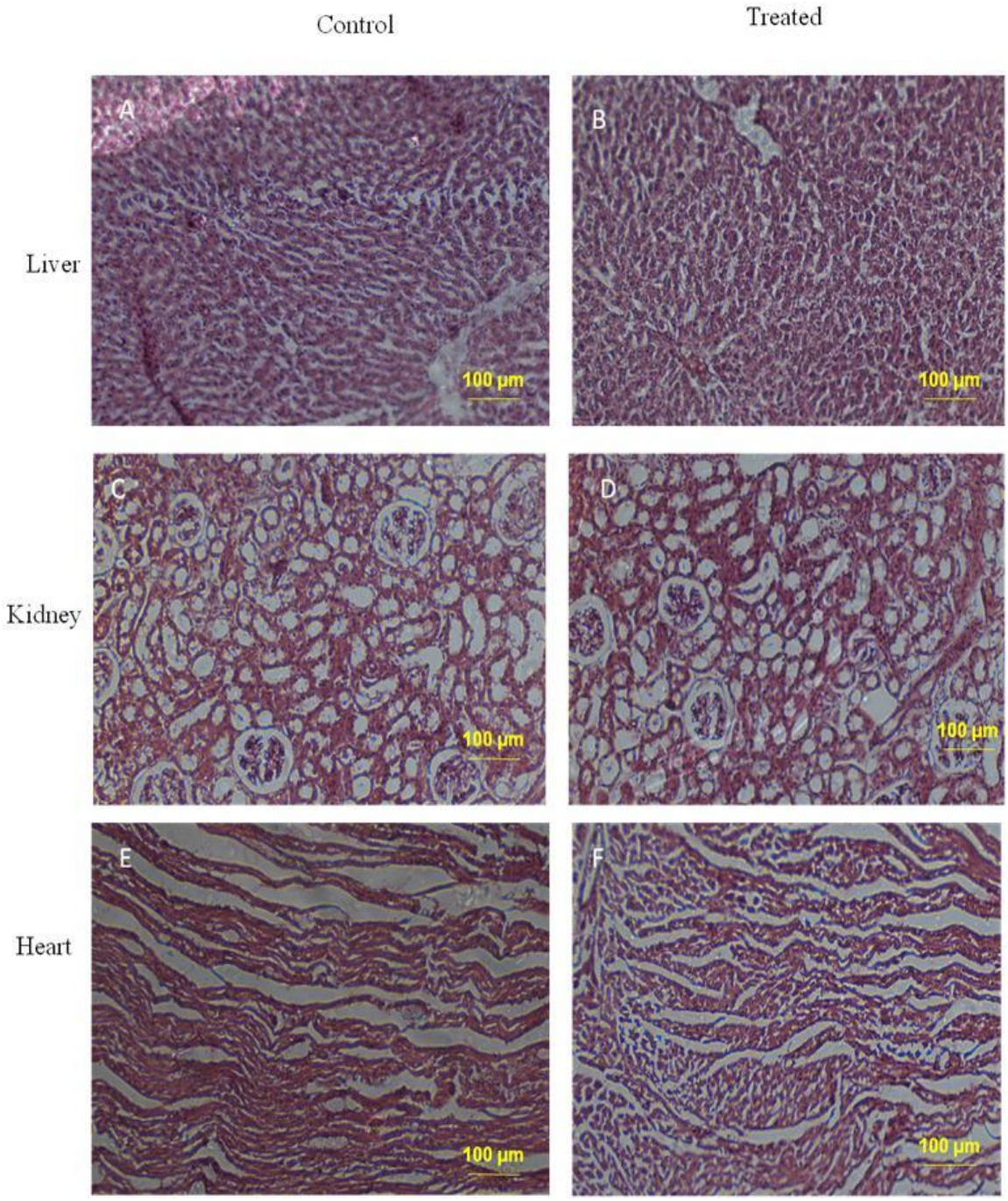


Fig. 4.10. Light microscopic observation of the effect of AAP on liver, kidney, and heart tissues of mice. The tissues were processed for microscopic observation by hematoxylene-eosin (H & E) staining as described in section 3.2.13.2. Tissues obtained from mice treated with 1X PBS served as control. (A) Control liver tissue, (B) AAP-treated liver tissue, (C) Control kidney tissue, (D) AAP-treated kidney tissue, (E) Control heart tissue, and (F) AAP-treated heart tissue. Magnification l0X: Bar = 100µm.

4.2.18 AAP demonstrated *in vivo* anticoagulant and defibrinogenating activity

AAP demonstrated dose-dependent *in vivo* defibrinogenation of mice plasma (Fig. 4.11) with a corresponding dose-dependent increase in the *in vitro* tail bleeding time, Ca-clotting time, and PT of PPP in the treated group of mice as compared to the control group of mice (Table 4.11).

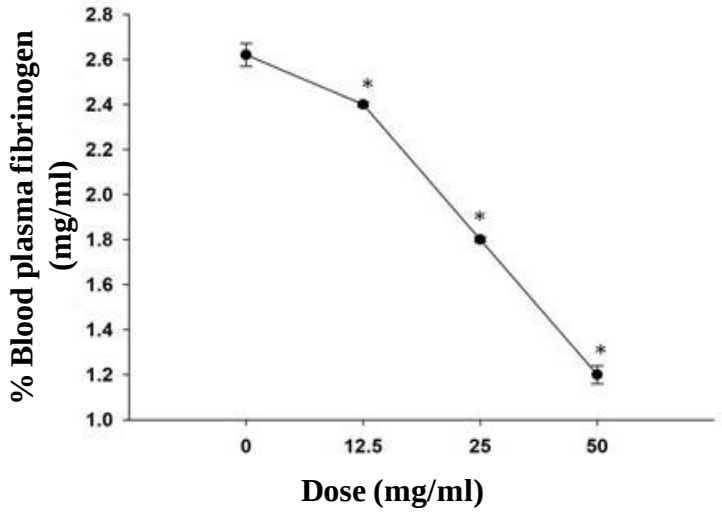


Fig. 4.11. Dose-dependent *in vivo* defibrinogenating activity of AAP after 5 h of oral administration in mice (n=6). Values are means ± S.D. of triplicate determinations. Significance of difference with respect to control, *p< 0.05

Table 4.11. A comparison of *in vivo* anticoagulant activity of AAP and nattokinase treated Swiss albino mice (n=6). The blood was withdrawn 5 h after oral administration of AAP (12.5- 50 mg/kg) or nattokinase (50 mg/kg).Values represent mean ± SD of six determinations. Significance of difference with respect to control, * $p < 0.01$. INR = ($prothrombin_{test}$ / $prothrombin_{control}$).

	PT (s)	**PT (INR)**	**APTT (s)**	**APTT (INR)**	**Tail bleeding time (s)**	**Plasma clotting time (s)**
Control mice	14.8 ± 0.77	1.0	28.3 ± 1.4	1.0	45.3 ± 2.5	175.5 ± 9.1
AAP- treated mice (12.5 mg/kg)	18.9 ± 1.4*	1.2	30.2 ± 2.1	1.06	66.4 ± 1.4*	216.3 ± 8.8*
AAP- treated mice (25.0 mg/kg)	26.9 ± 1.4*	1.8	31.0± 1.2	1.09	96.4 ± 7.2*	231.5 ± 8.7*
AAP- treated mice (50.0 mg/kg)	41.9 ± 2.1*	2.8	32.0 ± 3.1	1.1	128.5 ± 4.5**	253.5 ± 13.7*
Nattokinase- treated mice (50 mg/kg)	38.6 ± 2.8*	2.5	37.3 ± 3.6	1.3	120.0 ± 4.1**	211.3 ± 13.1*

4.2.19 Antithrombotic effect of AAP against k-carrageenan-induced tail thrombus in mice

AAP dose-dependently (12.5 to 50 mg/kg) inhibited the thrombus formation in the tail of k-carrageenan-treated mice (Table 4.12). The percent inhibition of thrombus formation induced by k-carrageenan in mice tail by AAP and nattokinase is shown in Table 4.12. At a dose of 50 mg/kg, both AAP and nattokinase showed identical antithrombotic effect (Table 4.12).

Table 4.12. The effect of AAP and nattokinase on k-carrageenan induced mice 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	**Doses**	**% inhibition of thrombus formation in mice tail after ƙ-carrageenan treatment**		
		24 h	**48 h**	**72 h**
AAP	12.5 mg/kg	12.0 ± 2.1*	15.8 ± 2.5*	22.2 ± 1.2*
	25.0 mg/kg	17.2 ± 1.8*	35.5 ± 1.2*	37.2 ± 2.5**
	50.0 mg/kg	27.4 ± 2.4*	48.00 ± 3.2**	58.3 ± 2.2**
Nattokinase	50.0 mg/kg	26.5 ± 1.4*	46.18 ± 2.5**	52.3 ± 3.8**

4.3 Discussion

In the present study, we developed a simple, novel, and cost effective process for preparing an anticoagulant active fraction (AAP) possessing fibrinogenolytic, thrombin and FXa inhibitory activities from aqueous leaves extract of *L. indica*. To the best of our knowledge, this is the first report showing preparation of AAP which has a great clinical significance for the development of low cost herbal drug to treat cardiovascular disorder. The proteomics analyses suggest that AAP contains previously uncharacterized novel plant protease.

The fibrinolytic system removes fibrin from the vascular system, thus preventing hemostatic clots from becoming too large and occluding the vessel. Hereditary and acquired abnormalities of fibrinolysis can lead to an increased risk of bleeding or thrombosis [9,10]. Based on their specificity for the α-chain and β-chain of fibrin/fibrinogen, the fibrin(ogen)olytic enzymes are classified as α and/or β fibrinogenases [11-13]. The α-fibrinogenase from different medicinal plants, such as *Costaria costata* [14] and *Codium fragile* [15] were reported to hydrolyse only the Aα-subunit of fibrinogen.The AAP contains an αβ-fibrinogenase, because it can degrade both α- and β-chains of fibrinogen/fibrin.

Several mechanisms could explain the anticoagulant action of drug molecules, such as the inhibition of thrombin, FXa; inhibition of platelet aggregation; vitamin k antagonism and/or defibrinogenation of blood plasma [12,15-20]. AAP significantly prolonged the *in vitro* Ca^{2+} clotting time of PPP indicating it is a potent anticoagulant preparation.

Our study suggests that the anticoagulant action of AAP is contributed by its fibrinogenolytic property, its potency in dual inhibition of thrombin and FXa, and its antiplatelet effect to prevent the blood clot formation. Because it delayed the APTT of PPP; therefore, it may be suggested that AAP affected both intrinsic and extrinsic pathways of blood coagulation [11,13]. In traditional medicine whole plants or mixtures of plants are used rather than isolated compounds. There is evidence that crude plant extracts and/or fractions often have greater activity rather than the individual components [21]. This may be due to additive and/or synergistic activity of the components to enhance the activity [21,22]. It is interesting to note that the most abundant phytochemicals of AAP-β-sitosterol (16 - 20%), methyl palmitate (7 - 10%) and octamethyltetracyclosiloxane (9 - 12%) have

not shown potent anticoagulant or antiplatelet activity individually or in several combinations indicating that these components of AAP act synergistically only in the plant extract to exert their anticoagulant activity. Our results provide an example of complementation of biological activity of bioactive components present in AAP.

Due to the great sensitivity and selectivity of fluorescence spectroscopy, this powerful tool can be used to study protein-protein interactions [23,24]. Our previous studies have also shown that fluorescence spectroscopy is a reliable tool to study protein-protein interactions due to its great selectivity and sensitivity [19,24,25]. Furthermore, this technique has been proven to be useful in determining the stoichiometry of binding [23]. The three aromatic amino acids viz., tyrosine, tryptophan and phenylalanine, which have distinct absorption and emission wavelengths, contribute to intrinsic fluorescence of a protein. However, the tryptophan residue is excited at 280 nm and shows emission in 340-345 nm range, and it has stronger fluorescence and higher quantum yield compared to tyrosine and phenylalanine residues [24]. Depending on the nature of the protein molecules, their interaction may result in either a decrease in yield owing to quenching of fluorescence of tyrosine and/or phenylalanine residues by a nearby tryptophan residue [23] or it can lead to an increase in fluorescence intensity of the protein complex compared to the individual components of the complex ([19,26]. Therefore, the nature of protein-protein interactions may lead to an increase or decrease in the fluorescence intensity.

In our spectrofluorometric analysis, a steady decrease in the fluorescence intensity of thrombin was observed in the presence of AAP suggesting the quenching of the tryptophan residues of thrombin when bound to AAP [16,19,23,24]. Likewise, the spectrofluorometric analysis demonstrated interactions between AAP and FXa/fibrinogen that led to an increase in fluorescence, compared to the fluorescence intensity of individual proteins. These results explain the mechanism of inhibition of thrombin and FXa by AAP by binding with their active site or at some other site.

As explained above, antiplatelet activity is another way to prevent the blood coagulation [27,28]. ADP is the oldest and one of the most important agonists of platelet activation. ADP induces platelet shape change, exposure of fibrinogen binding sites, aggregation, and influx and intracellular mobilization of Ca^{2+}. ADP-induced platelet aggregation is

important for maintaining normal hemostasis, but aberrant platelet aggregation manifests itself patho-physiologically in myocardial ischemia, stroke, and atherosclerosis [29,30]. Collagen is the most thrombogenic component of the subendothelium [31-33]. Following vascular damage, collagen is exposed to circulating platelets and both acts as a substrate for the adhesion of platelets [34-36] and induces platelet activation [36]. The prevailing evidence proposes that two receptors are involved in the platelet response to collagen; integrin $\alpha_2\beta_1$ acts to adhere platelets to collagen, allowing platelets to interact with the lower affinity receptor glycoprotein VI, which is mainly responsible for platelet activation [35-37]. AAP showed potent antiplatelet activity and was capable of inhibiting the collagen and ADP-induced platelet aggregation to prevent the clot formation, the precise mechanism of which needs to be explored.

A blood clot (thrombus) develops in the circulatory system which consolidates a mechanism in human body to repair the injured blood vessel [38,39]. If thrombus is formed when it is not needed, this can produce significant consequences like embolism, ischemia, heart attack, stroke, and so forth [39,40]. Embolism occurs when blood clot is formed inside a blood vessel or an artery and remains there which fully or partially block blood supply to a part of body resulting potentially severe consequences [39]. The clot bursting activity of AAP was not completely abolished in dissolving the heat-treated blood suggesting clot lysing action of AAP was partially dependent on endogenous factors of blood [12]. Usually, patients who develop atrial fibrillation require anticoagulants to prevent the risk of clot formation, which could otherwise initiate cardiovascular diseases [41]. AAP not only delays the progressive coagulation of blood but it also possesses thrombolytic potency, making it a suitable candidate for therapeutic applications as an antithrombotic and thrombolytic agent.

The thrombus forms via the effects of cruor, anticoagulation, the fibrinolytic system, haemorheology, vascular endothelial cells, platelets, and other factors [42], and animal thrombosis models are effective for evaluating the activity of thrombolytic agents. As an experimental model of peripheral obstructive disease, the k-carrageenan-induced thrombosis in mice is used, because of its advantages of being a simple method for inducing thrombus in small laboratory animals and observations are easy without having to

kill the animals [12,42]. Our results suggested that AAP can prevent tail thrombosis induced by k-carrageenan and it may be useful as a prophylactic antithrombotic drug.

It is an important prerequisite that before assessing the pre-clinical safety of a new drug molecule, its cytotoxicity against mammalian cells should be assessed in *in vitro* conditionsAAP at a high dose lacks cytotoxicity and haemolytic activity against mammalian cells suggesting its safety. Further, when AAP was given at a dose of 2000 mg/kg, which is approximately 160 times greater than its anticoagulant dose (12.5 mg/kg), it did not produce acute toxicity or adverse pharmacological effects in mice, indicating its preclinical safety and high therapeutic index.These results should encourage clinical trials of AAP as a possible anticoagulant antithrombotic drug to prevent cardiovascular diseases.

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