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Anti-thrombotic activity of isolated β-sitosterol from roots of *Hemidesmus indicus* Linn in rat model

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Abstract
Thrombosis is the basic pathophysiological procedure, brings about majority strokes and heart attacks which are serious causes of death all over the world. The present work was aimed to study the anti-thrombotic activity of isolated compound β-sitosterol (BS) from roots of herb Anantamul (*Hemidesmus indicus* Linn). A number of engrossing biomedical properties have been attributed to BS. Anti-thrombotic activity was evaluated by *in-vitro* analysis of different experimental paradigm i.e. Free radical scavenging activity, Clot lysis assay, Malondialdehyde (MDA) production in blood platelets and *in-vivo* models such as Rat arterio-venous shunt silk thread model, Rat venous thrombosis model, Carrageenan induced thrombosis. The statistical results obtained from the experimental models inferred that isolated BS imputed to its anti-thrombotic activity due to the anti-oxidant property, clot lysis property, inhibition of lipid peroxidation in platelet, inhibition of platelet lysis and effect on coagulation cascade.

Keywords: Anantmool, *Hemidesmus indicus*, β-sitosterol, thrombosis, clot lysis, platelets

Introduction
The formation or presence of a blood clot in a blood vessel both venous and arterial called as thrombosis. It is a major cause of morbidity and mortality worldwide with high impact on health and socioeconomic issues [1]. Data from the World Health Organization (WHO) indicate that the most important current reasons of death are ischemic heart disease and cerebrovascular disease (cerebral ischemia) [2]. With respect to the arteries, majority strokes and heart attacks are triggered by thrombosis and secondary to interrupted atherosclerotic plaques. Atrial fibrillation patients have a fivefold greater risk of having a stroke because of their propensity for left atrial thrombosis and subsequent cerebral embolism [3]. Venous thromboembolism (VTE) includes deep vein thrombosis (DVT) and pulmonary embolism (PE) and is the third most common vascular disorder after heart attack and stroke. In contrast to arterial thrombosis, venous thrombus is formed in the deep venous system, usually in the legs under low shear condition and is predominantly composed of fibrin and red blood cells. Such a thrombus can break off and enters into the pulmonary venous circulation, leading to PE, which if large enough can cause circulatory embarrassment and leading to death [4, 5].
In the developing countries about 80% of the world population primarily used herbal medicines for primary health care. They have proven the safety, efficacy, cultural acceptability and lesser side effects. Herbal medicine accommodates the phytoconstituents which are a part of the physiological functions of living flora and hence believed to have better compatibility with the human body [6]. Many medicinal plants like *Ocimum basilicum, Careya arborea, Allium sativum, Vernonia amygdalina, Jatropha curcas are* been recommended and used for the treatment. On the other hand, herbal remedies that have biologic activities are also reported to have side effects and interactions [7, 8]. Thus due to lack of systematic scientific evaluation and safety information, herbal remedies are still not considered as first line of treatment for thrombosis.
Currently several classes of drugs like oral anticoagulants, anti-platelet drugs are available in market but, an overarching efficacy and safety. In the arterial thrombosis treatment

particularly, push toward combining antithrombotic drugs to prevent recurrence of ischemia in patients with acute coronary syndromes (ACSs).

Although this approach showed outcome in a decreasing rate of recurrent ischemia, the incidence of bleeding has increased, to the extent that contemporary registry data indicate that approximately 4% of such patients experience a major hemorrhage, and up to 14% require a blood transfusion [9]. These findings are alarming because there is mounting evidence that bleeding is related with unfavorable cardiovascular outcomes and carries the same risk of death [10, 11]. In spite of advancement in this field and better understanding the pathology of thrombosis, current therapies are either inefficient or associated with adverse effects. This necessitates the development/exploration of safe and potential drug to treat thrombosis which can offer an effective therapy with lesser side effects [12, 13].

Materials and Method

- Isolation and standardization of secondary metabolite from selected medicinal plant.
- Evaluation and Safety assessment of isolated secondary metabolite potential against experimental thrombosis by *in vitro* and *in vivo* method.
- Predict the mechanism of action of isolated secondary metabolite

A. Selection of Indigenous Plant: The roots of *Hemidesmus indicus* Linn. R. Br. (Family: Asclepiadaceae) were procured, authenticated and identified.

B. Crude Drug Extraction from the Plant: The dried roots were grinded to get coarse powder and subjected for extraction. 100 g of powdered roots were extracted in methanol (70%) by continuous stirring. The filtrate was collected and vacuum evaporated to dryness. B-sitosterol was isolated from root extract of *Hemidesmus indicus* Linn. R. Br. (Family: Asclepiadaceae). (Reference 14: SK Roy, M Ali; MP Sharma; R Ramachandran, Indian J Chem, 2002, 41B (11), 2390-2394.: β-sitosterol was isolated by column chromatography using mobile phase i.e. chloroform: ethyl acetate: methanol by gradient elution and further characterized by HPTLC and TLC)

Experimental Design

A. *In vitro* Screening Method

- **Free Radical Scavenging Activity [15]:** The free radical scavenging activity of β-sitosterol, based on the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined. B-sitosterol (0.1 ml) was added to 3ml of a 0.004% MeOH solution of DPPH. Absorbance at 517nm was measured after 30 min and the percentage inhibition activity was calculated. IC_{50} value was calculated from the equation of line obtained by plotting a graph of concentration (µg/ml) versus % inhibition.
- **Clot Lysis Assay [15]:** Blood collected from fresh rats was further distributed in different pre weighed sterile micro centrifuge tube (0.5 ml/tube) and incubated at 37 °C for 45 minutes. Without disturbing clot formation, serum was completely removed. Each tube having clot was again weighed to determine the clot. To each micro centrifuge tube containing pre-weighed clot, different concentration of β-sitosterol solution, streptokinase and distilled water was added. All tubes were incubated at 37 °C for 90 minutes and clot lysis was observed.
- **Malondialdehyde (MDA) production in blood platelets [16]:** Citrated blood (from healthy rat) was centrifuged at 800 rpm for 8 minute at room temperature to obtain platelet rich plasma (PRP). Further the blood was centrifuged at 3000 rpm for 10 minutes to obtain platelet poor plasma (PPP). To 0.3 ml of PRP different concentrations of isolated β-sitosterol solution was added. The final concentrations obtained were 0.1, 0.25, 0.5 and 1 mg/ml and incubated for 15 min at 37 ºC. ADP (0.03 ml of 3 µM) and trichloroacetic acid in 0.6 M HCl was added and further centrifuged for 10 min at 3000 rpm. To the obtained clear supernatant 1% thiobarbituric acid was added in a ratio of 1:0.2 and immersed in boiling water bath for 30 min. The absorbance was measured at 532 nm.

A. *In vivo* Screening Method

All experiments were carried out at (Therapeutic Drug Monitoring) TDM laboratory, Matunga, Mumbai- 400 019, following the guidelines set by the institutional Animal Ethics Committee (IAEC). The protocol number issued was AT12012501.

- **Rat Arterio-Venous Shunt Silk Thread Model [17, 18]:** Rats were randomly divided into five groups (n=6) I- untreated control, II, III and IV- are plant extract/fraction treated groups, V- standard treated group. The right carotid artery and the jugular vein cannulation were done using polyethylene tubes (two 8 cm) on anesthetized rats. These tubes were linked to a central part containing 5cm silk thread (known weight) and filled with a heparin saline solution. The extracorporeal circulation was maintained for 15 min, during which time a thrombus formation will take place via adhesion to silk thread. The shunt was removed and thread with its associated thrombus was removed and weighed immediately.
- **Rat Venous Thrombosis Model [19]:** Rats were randomly divided into five groups (n=6) I- untreated control, II, III and IV-are plant extract treated groups, V- standard treated group. The anesthetized rat abdomen was opened by a midline incision, and the inferior vena cava was exposed. The blunt needle was inserted between renal veins for stenosis and ligation of vena cava was done with the silk suture. The needle was retracted after the ligation. The filter paper disk (3 mm diameter) presoaked with 10% $FeCl_3$ solution was placed on the external surface of the vena cava for 5 min. The saline soaked gauze was placed to cover the viscera for 1 hr. The animals were sacrificed and thrombus was removed and weighed.
- **Carrageenan induced thrombosis [20]:** Rats were randomly divided into six groups (n=6) I- untreated control, II- normal control, III, IV and V-are plant extract/fraction treated groups, VI- standard treated group. K-Carrageenan was injected in dorsal tail vein in all groups except in Group I in who saline was injected. The length of the infracted region at the tail tip was recorded at 6, 24 and 48 hr. after the κ-Carrageenan injection.

Results

The Physicochemical Properties of isolated β-sitosterol:

Color	light yellowish
Odor	characteristic odor
Melting point	131-133˚C

- Acute Oral Toxicity was performed as per revised OECD guideline 423 [21]. The dose of isolated β-sitosterol selected was 2000 mg/kg (as per the guidelines). Food consumption of all extract treated animals was found to be comparable to control group. There were no changes in behavior pattern and body weight. On the 14th day animals were sacrificed and subjected to necropsy but gross morphological examination not showed any abnormality in treatment group. There were no significant changes in the organ to body weight ratio in the treated as well as control group.

- **DPPH Radical Scavenging Activity**

Concentration (microgram/ml)	Percentage inhibition	
	Ascorbic acid (Standard)	Isolated β-sitosterol
10	22.62 ± 0.61*	2.33 ± 0.66
50	51.70 ± 0.96*	12.74 ± 1.03*
100	76.44 ± 1.73*	28.38 ± 1.43*
500	84.23 ± 1.03*	36.03 ± 1.22*
1000	89.99 ± 0.17 *	48.43 ± 1.16*
3000	94.67 ± 0.58*	85.61 ± 0.76*

All values are expressed in mean ± SD (n=6). Statistical analysis was done using one-way ANOVA followed by post Dunnett's test. Statistical significance was considered *p<0.05 when compared with Control.

- **Clot Lysis Assay**

Treatment	% Clot Lysis
Control	0.02 ± 0.02
IBRHI (0.05mg/ml)	67.01 ± 1.73*
IBRHI (0.1mg/ml)	68.75 ± 0.92*
IBRHI (0.2mg/ml)	69.96 ± 0.41*
IBRHI (0.4mg/ml)	71.21 ± 0.30*
IBRHI (0.8mg/ml)	72.70 ± 1.47*
IBRHI (1.0mg/ml)	74.47 ± 0.88*
Streptokinase (15,000 IU)	74.94 ± 0.99*

All values are expressed in mean ± SD (n=6). Statistical analysis was done using ONE WAY ANOVA further followed by post Dunnett's test. Statistical significance was considered *p<0.05 when compared with Control. {IBRHI: Isolated β-sitosterol from roots of *Hemidesmus indicus*}

- **Malondialdehyde (MDA) Production in Blood Platelets**

Treatment	NMOL of MDA formed/ 4*108 Platelet	% Inhibition
Control	11.15 ± 0.04	---
IBRHI (0.1mg/ml)	5.09 ± 0.05*	54.39
IBRHI (0.2mg/ml)	3.93 ± 0.06*	64.79
IBRHI (0.4mg/ml)	3.01 ± 0.07*	73.04
IBRHI (0.8mg/ml)	1.67 ± 0.06*	85.01
IBRHI (1.0mg/ml)	1.37 ± 0.06*	87.70

All values are expressed in mean ± SD (n=6). Statistical analysis was done using ONE WAY ANOVA further followed by post Dunnett's test. Statistical significance was considered *p<0.05 when compared with Control. {IBRHI: Isolated β-sitosterol from roots of *Hemidesmus Indicus*}

- **Rat Arterio-Venous Shunt Silk Thread Model**

Treatment	Wt. of thrombus in mg	% Inhibition
Normal Control	46.69 ± 0.71	0.00
Dose I (250 µg/kg)	36.15 ± 0.59*	23.18
Dose II (500 µg/kg)	31.24 ± 0.35*	33.61
Dose III (1000 µg/kg)	27.82 ± 0.57*	40.89
Standard (Aspirin)	26.45 ± 0.24*	43.79

All values are expressed in mean ± SD (n=6). Statistical analysis was done using ONE WAY ANOVA further followed by post Dunnett's test. Statistical significance was considered *p<0.05 when compared with Control.

- **Rat Venous Thrombosis Model**

Treatment	Wt. of thrombus in mg	% Inhibition
Normal Control	10.92 ± 0.53	0.00
Dose I (250 µg/kg)	8.69 ± 0.32*	14.32
Dose II (500 µg/kg)	8.10 ± 0.40*	20.17
Dose III (1000 µg/kg)	6.56 ± 0.55*	35.29
Standard (Aspirin)	5.94 ± 0.12*	41.39

All values are expressed in mean ± SD (n=6). Statistical analysis was done using ONE WAY ANOVA further followed by post Dunnett's test. Statistical significance was considered *p<0.05 when compared with Control.

- **Carrageenan Induced Thrombosis Model**

Treatment	6.00 hrs.	24.00 hrs.	48.00 hrs.
Normal Control	---	---	---
Carrageenan Control	42.13±1.89	43.18±1.72	41.43±1.59
Dose I (250 µg/kg)	42.69±1.77	31.16±1.33*	22.28±0.80*
Dose II (500 µg/kg)	44.10±.48	25.79±1.00*	22.35±0.84*
Dose III (1000 µg/kg)	42.28±1.77	26.74±1.31*	22.46±0.85*
Standard (Aspirin)	42.90±1.42	25.40±0.86*	22.78±0.80*

All values are expressed in mean ± SD (n=6). Statistical analysis was done using ONE WAY ANOVA further followed by post Dunnett's test. Statistical significance was considered *p<0.05 when compared with Control

Discussion

The β-sitosterol was isolated from the roots of *Hemidesmus indicus* Linn. R. Br. (Family: Asclepiadaceae). The compound was isolated and characterized by solvent extraction, Column chromatography, TLC, HPTLC techniques. The free radical scavenging activity results shows that the isolated β-sitosterol has got the anti-oxidant property in dose dependent manner which can be responsible for its antithrombotic effect. To study to fibrinolytic (activity like tissue plasminogen activator) potential of β-sitosterol, an *In vitro* model was selected in which clot formed was kept in contact with isolated β-sitosterol or streptokinase and its clot dissolution property was studied. There clot lyses observes found to be significant at all the doses of isolated β-sitosterol indicating its thrombolytic activity which was comparable to the streptokinase antithrombotic effect [15]. The isolated compound i.e. β-sitosterol when incubated with PRP for 15

min it inhibited ADP induced MDA production in a dose dependent manner. It indicates that isolated β-sitosterol inhibited the lipid peroxidation in platelet induced by ADP [16]. Hence, it can be considered that isolated β-sitosterol may have some effect on COX pathway. Oral administration of isolated β-sitosterol to rats for 3days significantly and dose dependently inhibited the A-V shunt induced thrombosis on cotton thread and thrombus formation in stasis combined with ferric chloride induced thrombosis in inferior vena cava of rats as there was decrease in the weight of thrombus formed compared to control group. In A-V shunt induced thrombosis model platelets adhere to the thrombogenic surface (cotton thread), followed by aggregation of platelets and finally leading to thrombus formation. The thrombus formed here mainly contains platelets along with it, it also contains erythrocytes meshed with fibrin [17, 18, 19]. Thus, this model concludes that isolated β-sitosterol probably give anti thrombotic activity by inhibiting platelet aggregation. In contrast to arterial thrombosis which occurs in high shear condition and is rich in platelet, the venous thrombosis is produced by stagnant flow of blood where the pooling of the blood in particular site leads to activation of coagulation cascade and hypercoagulability leading to formation of thrombus rich in fibrin [20, 21, 22]. The venous stasis model indicates that isolated β-sitosterol can have some effect on the coagulation cascade or pathway which is responsible for clotting of blood. In carrageenan induced thrombosis model Thromboxane/Prostaglandin I2 balance is responsible for thrombosis induction in the tails of rats [23, 24, 25]. Isolated β-sitosterol showed a dose dependent inhibition of infarction. Acute toxicity study of isolated β-sitosterol when performed in rats showed that it is safe when taken at the dose of 2000 mg/kg.

Conclusion

Present study indicates that isolated β-sitosterol from the roots of *Hemidesmus indicus* Linn. R. Br. (Family: Asclepiadaceae) can act as anti-thrombotic activity due to the anti-oxidant property, clot lysis property, inhibition of lipid peroxidation in platelet, inhibition of platelet lysis and effect on coagulation cascade. Further there is needed to study in detail different mechanism/(s) which are responsible for the anti-thrombotic activity of β-sitosterol obtained from roots of *Hemidesmus indicus*.

List of Abbreviations

Word or Phrase	Abbreviations
World Health Organization	WHO
Venous thromboembolism	VTE
Deep vein thrombosis	DVT
Pulmonary embolism	PE
Acute coronary syndromes	ACSs
Thin layer chromatography	TLC
High pressure thin layer chromatography	HPTLC
2, 2-diphenyl-1-picrylhydrazyl	DPPH
Malondialdehyde	MDA
Platelet rich plasma	PRP
Platelet poor plasma	PPP
Therapeutic drug monitoring	TDM
Institutional Animal Ethics Committee	IAEC
Isolated β-sitosterol from roots of *Hemidesmus Indicus*	IBRHI

Conflict of Interest

Authors declared that there is no 'Conflict of Interest'.

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