

Evaluation of Antithrombotic Activities of *Solanum xanthocarpum* and *Tinospora cordifolia*

Onila Lugun, Sobharani Bhoi, Priyanka Kujur, D. Vinay Kumar, William R. Surin

Department of Microbiology and Cell Biology, Indian Institute of Science, Bengaluru, Karnataka, India

ABSTRACT

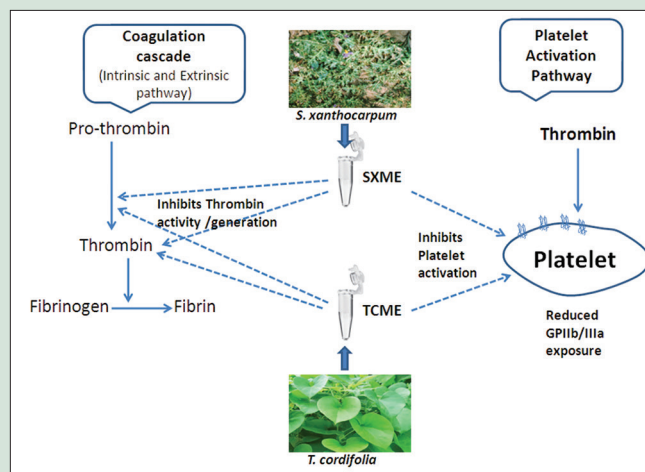
Background: *Solanum xanthocarpum* and *Tinospora cordifolia* have been reported to exhibit anti-inflammatory, antiarthritic, antioxidant, antiallergic, and hepatoprotective activities. The origins of many of the currently available antithrombotic treatments are from natural products and natural sources. **Objective:** To investigate the antithrombotic activities of methanolic leaf extracts of *S. xanthocarpum* (SXME) and *T. cordifolia* (TCME). **Materials and Methods:** Antithrombotic activities were assessed by thrombin inhibition assay, thrombin generation assay, platelet adhesion assay on collagen-coated surface, and platelet PAC1-FITC binding by flow cytometry. **Results:** SXME significantly inhibited thrombin activity at 5–20 mg/ml concentrations, whereas TCME inhibited thrombin activity at 500 µg/ml–5 mg/ml concentrations. Further, SXME inhibited thrombin generation at 2–20 mg/ml concentrations, whereas TCME exhibited significant inhibition at 200 µg/ml, suggesting that TCME has higher efficacy as compared to SXME. Moreover, SXME did not inhibit platelet adhesion on collagen-coated surface, whereas TCME inhibited platelet adhesion on collagen-coated surface at 5 mg/ml. Indomethacin showed significant inhibition in platelet adhesion at 300 µM. Further, SXME inhibited thrombin-induced platelet activation (PAC1-FITC binding) significantly at 1 mg/ml by about 80%, whereas TCME inhibited thrombin-induced platelet activation (PAC1-FITC binding) by about 40% at 1 mg/ml. **Conclusion:** These results strongly suggested that SXME and TCME possess antithrombotic activities. However, further studies are essential to find out the active constituent responsible for antithrombotic effect.

Key words: Collagen, platelet, *Solanum xanthocarpum*, thrombin, thrombosis, *Tinospora cordifolia*

SUMMARY

The methanolic extracts obtained from the leaves of *Tinospora cordifolia* and *Solanum xanthocarpum* were evaluated for antithrombotic activity by thrombin inhibition assay, thrombin generation assay, platelet adhesion assay and platelet activation assay by flow cytometry. These extracts inhibited thrombin activity and thrombin generation in rat plasma. Also, these extracts inhibited

thrombin induced platelet activation in PAC1-FITC binding study in flow cytometry.



Abbreviation Used: DVT: Deep vein thrombosis, TCME: *Tinospora cordifolia* methanolic extract, SXME: *Solanum xanthocarpum* methanolic extract, IL-1 β : interleukin-1 β , DMSO: dimethyl sulfoxide, PRP: Platelet rich plasma

Correspondence:

Dr. William R. Surin,
Department of Microbiology and Cell Biology,
Indian Institute of Science, Bengaluru - 560 012,
Karnataka, India.
E-mail: wrsurin@mcbli.isc.ernet.in
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INTRODUCTION

Cardiovascular diseases associated with intravascular thrombosis are the major cause of mortality and morbidity worldwide.^[1] Intravascular thrombosis leads to venous thromboembolism, stroke and ischemic heart diseases, deep vein thrombosis (DVT), and disseminated intravascular coagulation.^[2] Intravascular thrombosis can be divided into arterial thrombosis and venous thrombosis.^[3] Increased activation of coagulation factors, aging, and hyperaggregability of platelets results in intravascular thrombosis.^[4] Furthermore, deficiency of coagulation factors such as Protein C and Protein S can lead to DVT.^[5] Further, pathologies such as diabetes and atherosclerosis leads to increased activation of platelets and coagulation factors.^[6,7] Coagulation cascade and platelets play a major role in thrombus formation.^[8] Platelets circulate in the bloodstream and on vascular damage; they adhere to the damaged endothelium and initiate formation of platelet plug to inhibit blood loss from the site of injury. However, hyperaggregability of platelets or hyperactivity of coagulation factors leads to intravascular thrombosis.^[9] There are many antithrombotic interventions available in the market. However,

bleeding complications, recurrent stenosis, thrombocytopenia, need of continuous dose monitoring, and various side effects associated with these therapies prompts us to search for newer therapies with higher safety and efficacy profile.^[8,10-12]

The origins of many of the currently available antiplatelet therapies such as aspirin and anticoagulants such as heparin and coumarins can be traced to natural products and natural sources.^[13] Therefore, we proposed to study the antithrombotic activities of *Solanum xanthocarpum* and *Tinospora cordifolia*. *S. xanthocarpum* (Solanaceae) is a medicinal herb

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which grows in various parts of India and is commonly known as kantakari. Phytoconstituents present in *S. xanthocarpum* have been shown to possess antifertility, anti-inflammatory, antiallergic, antioxidant, hepatoprotective, nephroprotective, antibacterial, and anticancer activities.^[14-19] *T. cordifolia* (Menispermaceae) is a large spreading, glabrous, perennial, deciduous, climbing shrub distributed throughout India and South Asia. It is also commonly known as amruthu (Malayalam), amrutha balli (Kannada), gurcha (Hindi), guduchi (Marathi, Sanskrit), etc. It has many medicinal properties such as antidiabetic, anti-inflammatory, antiarthritic, antioxidant, antiallergic, anti-stress, antileprotic, antimalarial, hepatoprotective, and immunomodulatory activities.^[20,21] Extracts from *T. cordifolia* have been found to inhibit autoimmune disease such as rheumatoid arthritis. Furthermore, it was found to reduce the production of pro-inflammatory cytokines such as interleukin-1 β (IL-1 β), tumor necrosis factor- α , IL-6, and IL-17 in the rat adjuvant-induced arthritis model of human rheumatoid arthritis.^[22] Further, the various extract fractions and pure molecules of *T. cordifolia* exhibits anticancer and immunomodulatory activities.^[21]

Therefore, we collected the leaves of *S. xanthocarpum* and *T. cordifolia* and evaluated respective methanolic extracts for antithrombotic activities.

MATERIALS AND METHODS

S. xanthocarpum was collected from Sambalpur district of Odisha during January 2014, and a voucher specimen was deposited in the herbarium JCB at Centre for Ecological Sciences, Indian Institute of Science, Bengaluru, for reference (Ref No. HJCB 1096). *T. cordifolia* leaves were collected locally from Bengaluru region during March 2014, and a voucher specimen was deposited in herbarium JCB at Centre for Ecological Sciences, Indian Institute of Science, Bengaluru, for reference (Ref No. HJCB 1097). HEPES buffer, Tris base, Collagen type I, thrombin, heparin, apyrase, and indomethacin were from Sigma Chemicals Inc. (USA). PAC1-FITC antibody was from BD Biosciences Inc. (USA). Thrombin substrate III was from Calbiochem (USA). All the other chemicals used were of analytical grade.

Animals

Six male Wistar rats (200–250 g) were maintained at Central Animal Facility, Indian Institute of Science, and acclimatized to laboratory condition at room temperature 22°C \pm 2°C with 12 h light/dark cycle and relative humidity (55% \pm 10%) and were provided chow pellets and water *ad libitum*. All the experiments were performed in accordance with the guideline for the care and use of laboratory animals with the prior approval of the Institute Animal Ethics Committee of Indian Institute of Science, Bengaluru (CAF/Ethics/533/2016).

Plant extract preparation

Plants containing fresh and healthy leaves of *S. xanthocarpum* were collected. The leaves were washed thoroughly in distilled water and were air-dried. The leaves were then subsequently dried in a hot air oven at 37°C for 48 h. Then, the dried leaves were separated and grinded in a grinding machine. Then, 50 g of the leaves powder was dissolved in 500 ml of methanol and was placed in incubator shaker for proper mixing for 48 h. Then, it was filtered using Whatman No. 1 filter paper, and the filtered methanolic extract was collected in vials. The methanolic fraction was evaporated with rotary evaporator and 24 mg of the residue obtained. Then, it was stored at –20°C till further use. Before use, the residue was dissolved in dimethyl sulfoxide (DMSO) and labeled as methanolic extract of *S. xanthocarpum* (SXME). Similar procedure was followed to obtain methanolic extract from the leaves of *T. cordifolia*, and it was labeled as methanolic extract of *T. cordifolia* (TCME). The residue obtained from the leaves of *T. cordifolia* was 15 mg.

Thrombin inhibition assay

The thrombin inhibition assay was conducted as described by Surin *et al.*^[23] Briefly, SXME extract (10 μ g/ml–5 mg/ml) or TCME extract (10 μ g/ml–20 mg/ml) or DMSO (5 μ L) was incubated with Tris-buffer, pH-7.5 in 96-well plate (black, flat, and bottom). Then, thrombin substrate III (0.2 mM) was added followed by the addition of thrombin (3 nM). Fluorescence was read at 450 nm of emission and 390 nm of excitation in a fluorimeter after an interval of 1 h (Spectra Max M5e, Molecular Devices, Sunnyvale, CA, USA).

Thrombin generation assay

The thrombin generation assay was conducted as described by Surin *et al.*^[23] Briefly, three male Wistar rats weighing 250–300 g were anesthetized under ether. Blood was collected into a syringe containing 3.8% trisodium citrate by cardiac puncture in the ratio of 1:9. The blood was centrifuged at 500 g for 20 min at 20°C to obtain plasma. SXME extract (10 μ g/ml–5 mg/ml) or TCME extract (10 μ g/ml–20 mg/ml) or DMSO (5 μ L) was incubated with plasma in 96-well plates (black, flat bottom). Then, thrombin substrate III (0.2 mM) was added followed by the addition of thrombin (1nM). Then, the thrombin generation was read in fluorimeter after an interval of 1 h (Spectra Max M5e, Molecular Devices, Sunnyvale, CA, USA).

Washed platelet preparation and platelet adhesion on collagen-coated 96-well plate

The collagen (Rat tail, type I) stock solution 0.1% (w/v) was prepared in 0.1M acetic acid. Working solution was prepared by diluting 10 fold to 0.01%. About 100 μ l of collagen solution was added to each well of black 96-well plate and incubated for overnight at 2°C–8°C. Platelets were isolated as described by Karim *et al.*, with slight modifications.^[24] Three male Wistar rats (250–300 g) were anesthetized under ether. Blood was collected into a syringe containing 3.8% trisodium citrate by cardiac puncture in the ratio of 1:9. It was centrifuged at 275 g for 20 min at 20°C, and the platelet-rich plasma (PRP) was separated. PRP was incubated with 1 mM acetylsalicylic acid and 0.15 ADPase units of apyrase/ml were added to PRP and incubated for 30 min at 37°C. After addition of ethylenediaminetetraacetic acid (5 mM), PRP was centrifuged at 1500 g for 15 min. The cells were resuspended in buffer A (20 mM HEPES, 138 mMNaCl, 2.9 mMKCl, 1 mM MgCl₂, 0.36 mM NaH₂PO₄, 1 mM EGTA, supplemented with 5 mM glucose, and 0.6 ADPase units/ml of apyrase, pH 6.2). Platelets were washed and finally resuspended in buffer B (pH 7.4), which was same as buffer A, but without EGTA and apyrase. The final cell count was adjusted to 0.8–1.2 \times 10⁹/ml.

Washed platelets were incubated with mepacrine (200 μ M) at 37°C for 30 min. The washed platelets (200 μ l) were aliquoted into different vials. It was grouped into control, DMSO, and SXME extract group and TCME extract group. SXME or TCME extract were incubated for 30 min before being added to the collagen-coated surface. It was incubated for 1 h in collagen-coated plates at 37°C under dark conditions. Thereafter, it was washed thrice with phosphate buffer saline (pH: 7.4). The reading was taken in fluorimeter at excitation of 435 nm and emission at 525 nm (Spectra Max M5e, Molecular Devices, Sunnyvale, CA, USA).

PAC1-FITC binding assay by flow cytometry

Platelets were isolated from fresh human blood donated by healthy volunteers who have not taken any platelet inhibiting medicines 15 days before donating the blood. Washed platelets were prepared as per the protocol already described in the earlier paragraph. Platelets were incubated with increasing concentrations (300 μ g/ml–1 mg/ml) of SXME extract or TCME extract for 30 min at 37°C. Further, platelets

were incubated with PAC1-FITC (BD Biosciences, Inc. USA). Platelet activation was assessed by addition of thrombin (1U/ml). It was analyzed by flow cytometry at excitation of 488 nm and emission of 515–545 nm (BD FACSCanto™ II, BD Biosciences, Inc).

Statistical analysis

Comparisons between different groups were made using two-way ANOVA with Newman–Keuls multiple comparison tests. Microcal origin software version 6.0 was used for data analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of methanolic extract of *Solanum xanthocarpum* and TCME on thrombin activity *in vitro*

We evaluated the SXME and TCME for their effect on thrombin activity *in vitro*. SXME inhibited thrombin activity starting from 2 mg/ml in a concentration-dependent manner at 5 mg/ml, 10 mg/ml, and 20 mg/ml. However, SXME did not show thrombin inhibitory activities at lower concentrations 10 µg/ml–1 mg/ml [Figure 1]. TCME exhibited significant thrombin inhibition at 500 µg/ml, 1 mg/ml, 2 mg/ml, and

5 mg/ml. Further, marginal inhibition was observed at 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml concentrations. All these observations suggest that SXME and TCME possess thrombin inhibitory effect and TCME has higher efficacy as compared to SXME in inhibiting thrombin activity [Figure 1].

Effect of methanolic extract of *Solanum xanthocarpum* and *Tinospora cordifolia* on thrombin generation assay

Further studies were carried out to assess the effect of SXME (500 µg/ml–20 mg/ml) and TCME (5–200 µg/ml) on thrombin generation in rat plasma *in vitro*. It was observed that SXME significantly inhibited thrombin generation at higher concentration (2–20 mg/ml). The arbitrary fluorescence unit obtained was 882 ± 21 , 742 ± 16 , 654 ± 19 , and 542 ± 14 at 2 mg/ml, 5 mg/ml, 10 mg/ml, and 20 mg/ml, respectively, following the treatment with SXME. Further, arbitrary fluorescence unit obtained was 794 ± 20 at 200 µg/ml in the presence of TCME [Table 1]. This shows that TCME has higher efficacy as compared to SXME in inhibiting thrombin generation in plasma.

Effect of methanolic extract of *Solanum xanthocarpum* and *Tinospora cordifolia* on platelet adhesion on collagen-coated plate

The mepacrine-labeled washed platelets were incubated with different concentrations of SXME (1–20 mg/ml) followed by incubation on collagen in collagen-coated 96-well plates. No significant inhibition was observed in platelet adhesion in the presence of SXME [Figure 2]. TCME was evaluated at concentration 25 µg/ml–5 mg/ml. TCME exhibited significant reduction in platelet adhesion at 5 mg/ml, suggesting that TCME can inhibit platelet adhesion at higher concentration. Indomethacin, a potential COX inhibitor, showed significant inhibition on platelet adhesion at 300 µM [Figure 2].

Effect of methanolic extract of *Solanum xanthocarpum* and *Tinospora cordifolia* on PAC1-FITC binding by flow cytometry

Further, we evaluated various concentrations of SXME (100 µg/ml–1 mg/ml) and TCME (100 µg/ml–1 mg/ml) for their effect on platelet activation by flow cytometry induced by thrombin. It was observed that SXME inhibited the platelet activation significantly at 1 mg/ml by about 80% [Figure 3]. Furthermore,

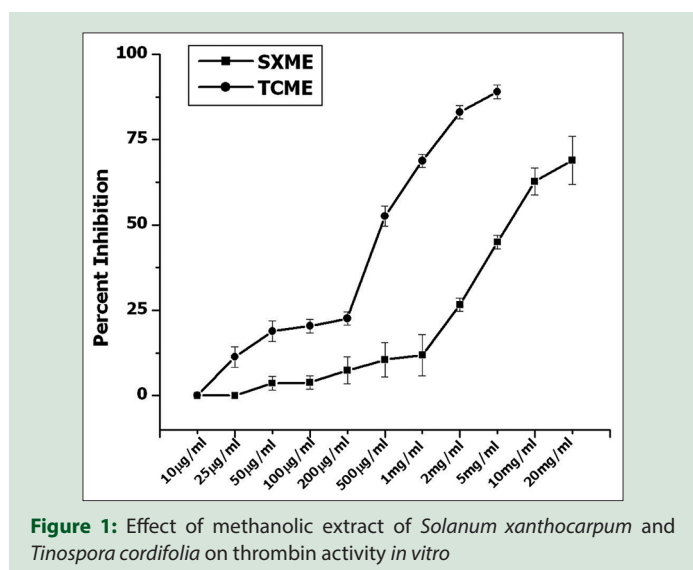


Figure 1: Effect of methanolic extract of *Solanum xanthocarpum* and *Tinospora cordifolia* on thrombin activity *in vitro*

Table 1: Effect of methanolic extract of *Solanum xanthocarpum* and methanolic extract of *Tinospora cordifolia* on thrombin generation assay on rat plasma *in vitro*

Sample concentrations	Control	Vehicle control/(methanol) (5 µl)	SXME (AFU)	TCME (AFU)	Heparin concentrations	Heparin (AFU)
	1130±11	1057±44				
5 µg/ml			-	964±39	1 U/ml	1177±104
10 µg/ml			1152±17	1129±8	3 U/ml	757±22
25 µg/ml			1256±20	1036±14	10 U/ml	622±6
50 µg/ml			1162±18	1007±14		
100 µg/ml			1182±30	905±27		
200 µg/ml			1032±25	794±20*		
500 µg/ml			1045±30			
1 mg/ml			915±28			
2 mg/ml			882±21*			
5 mg/ml			742±16*			
10 mg/ml			654±19**			
20 mg/ml			542±14**			

Thrombin generation in AFU. Data represented as mean±SEM (n≥4). For * $P < 0.05$ and for ** $P < 0.01$. SXME: Methanolic extract of *Solanum xanthocarpum*; TCME: Methanolic extract of *Tinospora cordifolia*; AFU: Arbitrary fluorescence unit; SEM: Standard error of mean

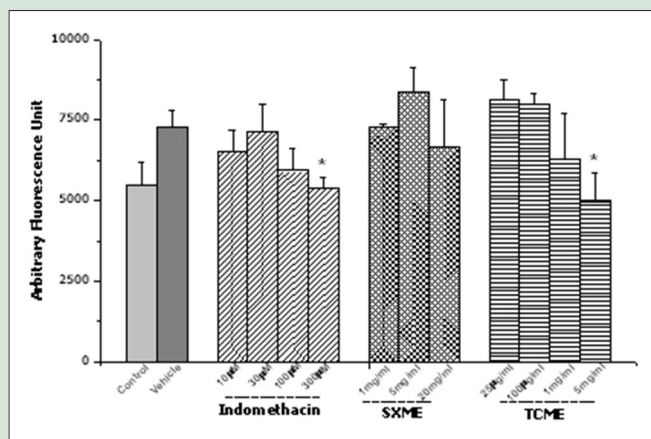


Figure 2: Effect of methanolic extract of *Solanum xanthocarpum*, methanolic extract of *Tinospora cordifolia*, and Indomethacin on platelet adhesion on collagen-coated surface *in vitro*. Data represented as mean \pm standard error of the mean ($n \geq 4$). For *: $P < 0.05$

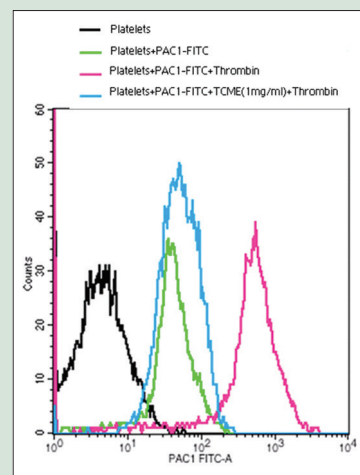


Figure 3: Effect of methanolic extract of *Solanum xanthocarpum* and *Tinospora cordifolia* on platelet PAC1-FITC binding in thrombin-induced platelet activation by flow cytometry. Data represented as mean \pm standard error of the mean ($n \geq 4$)

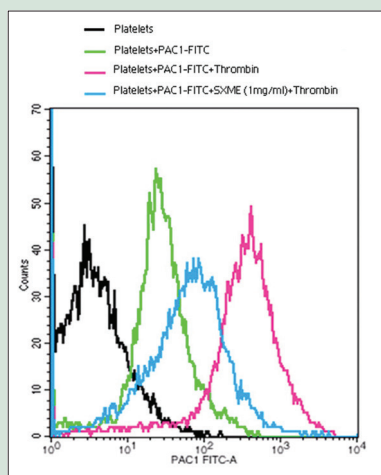


Figure 4: Effect of methanolic extract of *Tinospora cordifolia* on platelet PAC1-FITC binding in thrombin-induced platelet activation by flow cytometry

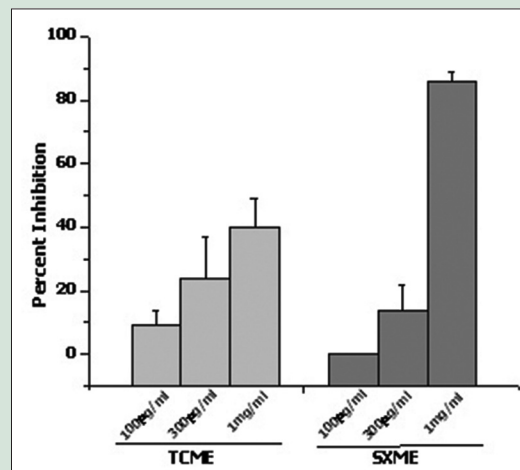


Figure 5: Effect of methanolic extract of *Solanum xanthocarpum* on platelet PAC1-FITC binding in thrombin-induced platelet activation by flow cytometry

TCME inhibited thrombin-induced platelet activation significantly in a concentration-dependent manner [Figures 4 and 5]. TCME exhibited about 40% inhibition in platelet activation at 1 mg/ml [Figure 3].

DISCUSSION

Thrombin is part of coagulation cascade, and therefore, we evaluated SXME for thrombin inhibitory activities. We found that SXME inhibited thrombin activity and thrombin generation. Furthermore, we assessed SXME for its effect on platelet-dependent thrombosis. Collagen is one of the agonist of platelet activation. Platelets get exposed to subendothelial collagen during vascular denudation. Collagen brings about platelet activation through GPIa/IIa and GPVI receptors present on platelets.^[25] However, SXME did not inhibit platelet adhesion on collagen-coated surface. All these observations suggest that SXME possess antithrombotic activity and exhibits both thrombin inhibitory and platelet inhibitory effect.

We assessed the effect of *T. cordifolia* methanolic extract on thrombin, the end product of coagulation cascade. There is increased thrombin activity in patients having thrombotic disorders.^[26] Increased thrombin activity leads to increased incidence of thromboembolic events.^[27] TCME exhibited significant thrombin inhibitory effect. Furthermore, TCME inhibited thrombin generation in rat plasma. Platelets play equally important role in thrombus formation. Therefore, we evaluated TCME for its effect on platelets. TCME inhibited platelet adhesion on collagen-coated surface suggesting that TCME possess both antiplatelet and anticoagulant effect.

GPIIb/IIIa is the final receptor which gets activated in the process of platelet activation. During platelet shape change and activation, there is increased expression of GPIIb/IIIa receptors on platelet surface. GPIIb/IIIa mediates platelet-platelet interaction called platelet aggregation through fibrinogen. Externalization of GPIIb/IIIa or activation of GPIIb/IIIa receptor is the final event in the process of platelet activation.^[28] PAC1 binds to fibrinogen receptor

GPIIb/IIIa on platelet activation. GPIIb/IIIa transforms from low-affinity state to higher affinity state during platelet activation.^[29] Therefore, we evaluated SXME and TCME against thrombin-induced platelet activation by flow cytometry. Human washed platelets were incubated with SXME or TCME and PAC1-FITC binding was assessed by adding thrombin. It was observed that SXME and TCME inhibited thrombin-induced platelet activation significantly in a concentration-dependent manner. All these observations suggest that SXME and TCME possess antiplatelet activity which seems to be mediated by thrombin pathway.

Further, these plants contain various flavonoids, alkaloids, sterols, and various glycosides. Therefore, various constituents of the SXME need to be isolated and studied separately for its individual and specific effect. It should be noted that *T. cordifolia* (stem) is one of the component of Abana, an herbal preparation approved by Department of Ayush, Ministry of Health and Family Welfare, Government of India. Abana has been demonstrated to inhibit ADP induced platelet aggregation in rat platelets. Further, Abana along with aspirin inhibited, inferior vena cava ligation induced DVT in rats.^[30]

On the other hand, GC-MS study on the bioactive components of *S. xanthocarpum* has shown the presence of 9,12,15-Octadecatrienoic acid, (Z, Z, Z), which possess anti-inflammatory, hypocholesterolemic, antihistaminic, antiarthritic, anti-coronary activities; Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester which has antiarrhythmic, antiinfarctal, anti-inflammatory, hypotensive, inotropic activities; 2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E) which has antioxidant, immune stimulant, and lipoxygenase-inhibitor activities; Vitamin E has protein kinase C inhibitor and antithrombotic, thromboxane-B2 inhibitory activities.^[31] Furthermore, there are many signaling pathways in platelet activation and adhesion. Therefore, further studies are needed to find out specific pathways being modulated by TCME and SXME.

CONCLUSION

These studies demonstrate that the methanolic extracts obtained from the leaves of *Tinospora cordifolia* and *Solanum xanthocarpum* possess antithrombotic properties. Further the studies show that the SXME and TCME mediated antithrombotic effect is due to the inhibition of thrombin induced platelet activation. Also, the results suggest that SXME and TCME inhibit thrombin activity and thrombin generation in coagulation cascade. Various constituents are present in these extracts. Further studies will reveal the constituent exhibiting antithrombotic activities.

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Conflicts of interest

There are no conflicts of interest.

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