RESEARCH ARTICLE

EVALUATION OF IN-VITRO ANTICOAGULANT ACTIVITY OF SRI LANKAN BLACK TEA (*Camellia sinensis*) AND THE EFFECT OF TEA POLYSACCHARIDES ON BLOOD COAGULATION PROCESS

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ABSTRACT

Warfarin and Heparin are extensively used clinically to prolong blood clotting to treat thrombotic diseases. However, they have side effects such as bleeding and thrombocytopenia. Because of these side effects, there has been a continuous search for alternative anticoagulant therapies that can offer efficacy without the same level of risk. The goal is to find anticoagulant therapies that strike a balance between effectively preventing blood clots while minimizing the risk of bleeding complications or other side effects commonly associated with traditional anticoagulants. Nature offers a wealth of compounds that have demonstrated various therapeutic benefits, and investigating natural sources for their anticoagulant potential could yield promising results. Hence, this study examined various Sri Lankan black tea (SBT) for anticoagulant potential by monitoring whole blood clotting, activated-partial-thromboplastin-time (aPTT), prothrombin-time (PT), and antiplatelet activity. Hot water extract, caffeine rich and poor, polysaccharide rich (PR) and poor extracts of tea were prepared following the literature procedures. Ca^{2+} induced whole blood clotting time of the extracts were evaluated using citrated arterial goat blood. Polysaccharide rich (PR) extract obtained from low-grown tea showed the highest anticoagulant activity (270 ± 7 s) when compared to the polysaccharide low extract (136 ± 2 s) with the negative control (100 ± 3 s). Polysaccharides have a close relationship with blood coagulation and heparin which is widely used as an anticoagulant is also a polysaccharide. The results showed that PR prolonged aPTT and PT significantly. Platelet aggregation assay indicated that PR has aspirin like antiplatelet behavior as well. Collectively, our results suggest that SBT, specifically PR, possesses antithrombotic activities and that the current study could provide information for the development of new anticoagulants based on tea polysaccharides.

Keywords: *Black tea, Tea polysaccharides, Anticoagulant, Antiplatelet*

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1. INTRODUCTION

Tea, which is made from tender shoots of *Camellia sinensis* is the most widely consumed beverage in the world [1, 2]. Recent studies have revealed that tea has various health benefits including, cholesterol reduction, hypertension lowering, anti-oxidant, anti-microbial, lowering the risk of cardiovascular diseases and anticancer properties. [3-8]. Tea polyphenols (catechins and other flavonoids), alkaloids (caffeine, theobromine, theophylline, etc), volatile oils, tannins and tea polysaccharides (PR) are the major classes of bioactive compounds responsible for these health benefits [2-8]. Based on the method of processing and the origin, there is a significant variation in their composition among different types of tea. Black, green and Oolong tea are the three major types of tea based on the method of processing [1-9]. Out of these, black tea is the most popular tea type representing about 80% of global tea consumption. Traditionally, Sri Lanka has been renowned for its role as a leading exporter of tea, known for producing some of the finest quality varieties. Apart from aforementioned tea types, there are several local-tea types based on the elevation at which the tea plants are grown and the processing grade. High grown, mid grown and low grown are the types based on elevation and OP (Orange Pekoe), BOPF (Broken Orange Pekoe Fannings) and dust etc. are categorized according to the processing grade. According to the bioactivity profiles, low grown black tea has high level of bioactive secondary metabolites [10-13].

Recent studies have also shown that water soluble tea polysaccharides have many bioactivities, such as reduction of blood sugar level, immunological benefits, anti-blood coagulation, anti-cancer, anti-HIV etc [14-16]. Tea polysaccharides are found to be mostly glycoconjugates in which a protein carries one or more carbohydrate chains covalently attached to a polypeptide backbone, usually via N- or O-linkages [15,17]. Initially, polysaccharides were considered as primary metabolites with the major role of energy storage [18-19]. However, now there is enough, evidence to consider special types of polysaccharides as secondary metabolites. The polysaccharides are among the less investigated classes of secondary metabolites with great hidden potential of bioactivity [20-22]. PR are a group of hetero polysaccharides extracted from tea leaves. Studies show that PR has many health benefits including antioxidant, anticancer, antiaging, antibacterial, antidiabetics and immune boosters. However, PR has not received enough attention [20-26].

Anticoagulant activity of PR has not been studied enough to elaborate its true potential [27,28]. Blood coagulation is a major defense mechanism against bleeding. Blood coagulation process is considered as a complex series of cascading-coordinated reactions involving plasma proteins and blood cells that eventually forms an insoluble clot. Most of the precursors (zymogens, procoagulants, proenzymes), which are essential for the coagulation cascade, are present in an inert form until the process is initiated by a trigger factor. Tissue factor, collagen and calcium ion are the major trigger factors. Most of the reactions involve in the coagulation process are enzymatically controlled (serine proteases) [29-33]. Coagulation process is composed of two pathways: intrinsic pathway – *contact activation* and extrinsic pathway-*tissue factor*. Activation of the extrinsic pathway initiate the hemostasis and thrombosis. The common mediator of both the intrinsic and extrinsic pathways is thrombin [34]. Thrombin initiates the platelet activation, as well as the production of factor V, VIII and IX, and mediates the proteolytic cleavage of fibrinogen to fibrin and then it binds to fibrin where it remains active [35]. Given the central role of thrombin in the development of a thrombus, many strategies for preventing and treating thromboembolic events have focused on inhibiting thrombin generation or blocking its activity [34,35].

The exploration of anticoagulant principles from natural sources, especially medicinal plants have gained much attention [36].

In this study, we examined the initial anticoagulant properties of diverse tea extracts, including tea polysaccharides. We also explored the influence of tea-elevation on the coagulation process and investigated the potential anticoagulation mechanism associated with PR.

2. MATERIAL AND METHODS

2.1 Materials

Anticoagulant analyzer - automated coagulometer, Coatron M2, TECO Corporation, Germany, 96 well plate reader-Molecular Devices, Spectra Max, USA, aPTT assay reagent and PT (Thromboplastin-D) reagents were purchased from Thermo Scientific (UK), Black tea was purchased from a Kotagala Plantation, Bulathsinghala.

2.2 Preparation of tea extracts

Four types of tea extracts were prepared according to published protocols.

2.2.1. Hot water extract

50.0 g of black-tea powder was suspended in 150 mL of distilled water and sonicated for 3 hours at room temperature followed by another 30 min at 60 °C. Filtered extract was then dried under vacuum to get the final dried product.

2.2.2. Caffeine rich and caffeine poor extracts

50.0 g of black-tea powder was extracted twice with 150 mL of ethyl acetate each time with sonication (3 hours at RT). Ethyl acetate extract was filtered and evaporated under reduced pressure to obtain caffeine rich extract [34, 35]. The residue was extracted as described in 2.2.1 to get caffeine poor hot water extract.

2.2.3. Polysaccharide rich and polysaccharide poor extract

50.0 g of black-tea powder was first extracted with 300.0 mL (150.0 mL x 2) of ethyl acetate with sonication to remove pigments and caffeine. The residue was next extracted with distilled waters as described in 2.2.1. The supernatant was concentrated under reduced pressure, then mixed with four volumes of 95% ethanol and kept at 4.0 °C in a refrigerator for 12 h. The precipitate was obtained by centrifugation and washed three times with absolute ethanol. The dried pellet was taken as the polysaccharide rich extract and the supernatant was dried and taken as the polysaccharide poor extract [27, 39].

2.3 Whole blood clotting time

Goat blood (arterial) was collected at the Colombo municipal slaughterhouse, Dematagoda, Sri Lanka, and immediately citrated using 3.1% sodium citrate solution. All prepared tea-crude extracts were resuspended in water, except, the caffeine rich extract, which was re-suspended in DMSO to obtain 10 mg/mL extracts. Citrated blood (4.0 mL) was combined with extracts (0.5 mL) in clean glass tubes. Distilled water and DMSO (0.5 mL) were used as the negative controls. Each test tube containing treated blood was added with 0.2 mL of 2 % calcium chloride and stoppered immediately. The content of each tube was thoroughly mixed, and the calcium induced clotting time was determined

by tilting each tube every 30 sec. until a firm clot was formed. If the blood did not clot by 10 min. it was considered as un-clotted.

2.4 Anticoagulation assay (PT and aPTT) for crude tea polysaccharides

The anticoagulant activity of the polysaccharides was determined using aPTT and PT as indicators according to the method provided by TECO Corporation, Germany. In the assay, normal human plasma prepared from healthy donors without a history of bleeding or thrombosis was used. Nine parts of human blood collected by venipuncture were drawn into 3.1% citrate solutions. Blood was centrifuged for 10 min at 1500 rpm, and the platelet rich plasma was obtained.

For aPTT clotting assay, citrated human plasma (20 µL) was mixed with the extract (5 µL), then 25 µL aPTT reagent was added to the mixture and incubated at 37 ℃ for 3 min. Preincubated (37 °C) 50 µL of 0.025 mol/L $CaCl_2$ solution was added and clotting time was recorded on an automated coagulometer (Coatron M2, Germany). In PT clotting measurement, citrated human plasma (20 µL) was mixed with the polysaccharide (5 µL) and incubated at 37 ℃ for 3 min, then 50 µL of PT reagent pre-incubated at 37 °C for 10 min was added and clotting time was recorded using the automated coagulometer. For both assay 5 µL of heparin was used as the positive control while 5 µL of DW (Distilled water) was used as the negative control.

2.5 Platelet aggregation assay

Preliminary Platelet aggregation assay was carried out at 37 °C with platelet rich plasma (PRP) having platelet counts between 2.5 and 3.0 x 10^9/L of plasma. All experiments were performed within 3 h of PRP preparation. The volume of 90 µL of PRP was preincubated with the 5 µL of tea polysaccharides dissolved in PBS (Phosphate Buffered Saline) (10 mg/mL). 5 µL of 1 mg/mL aspirin was used as the positive control while PBS was used as the negative control. The platelet activation was induced by adding 5 µL of 5 µM ADP (adenosine 5’ diphosphate). Measurement of platelet aggregation was monitored by the turbidity changes at 600 nm using 96 well plate reader for 30 min (Molecular Devices, Spectra Max, USA).

2.6 Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis involved a one-way analysis of variance (ANOVA). A value of P less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results and discussion

According to the literature, black tea has the highest number of bioactive metabolites. Among the black tea varieties, low grown black tea has the highest demand, owing to its unique color and aroma. When we compared the PT of tea polysaccharides obtained from tea grown at different elevations, low grown black tea turned out to be the best (**Table 01**) hence used throughout this study. Though there are number of publications on bioactive compounds form tea, there are limited records on the anticoagulant properties of tea. Still, most of these reports are qualitative and have focused on major metabolites such as caffeine, catechins, epigallocatechins, polyphenols, tannins.

Table 01: PT activity of PR depending on the elevation

Sample collection area	Tea type	PT (s)	Calculated INR*
Nuwara Eliya	High Grown	22 ± 2	1.5
Dimbula	High Grown	28 ± 1	1.9
Uva	High Grown	30 ± 1	2.0
Kandy	Mid Grown	39 ± 2	2.6
Ruhuna	Low Grown	45 ± 3	3.0
Bulathsinhala	Low Grown	46 ± 3	3.1
Negative control	-	15 ± 2	-

*INR = International Normalized Ratio

3.1 Whole blood clotting time

Goat blood obtained from the slaughterhouse; Colombo municipal council was used for the preliminary screening of the different tea extracts for anticoagulant activity. The blood was collected form five male animals and pooled in vials added with 3.1 % sodium citrate solution which is the recommended anticoagulant for the PT and aPTT analyses. All the analyses were done within four hours from the time of collection of blood. All tea extracts were dissolved in saline solution except caffeine extract since it is poorly soluble in aqueous solvent and 40 % DMSO was used as the solvent. Therefore, both saline and 40 % DMSO were used as the negative controls. However, there was no any significant difference between two negative controls.

Summary of the results of whole blood coagulation time for the prepared crude tea extracts are given in Table 02 with the percentage yield.

Table 02: Whole blood clotting time of different tea extracts

Fractions	**% yield**	**Concentration**	**Clotting time (seconds)**
Hot water	12.2	0.5 mL of 10 mg/mL	185 ± 6
Polysaccharide rich (PR)	4.5	0.5 mL of 10 mg/mL	270 ± 7
Polysaccharide poor (PP)	5.1	0.5 mL of 10 mg/mL	136 ± 2
Caffeine fraction (CF)	0.5	0.5 mL of 10 mg/mL	130 ± 7
Decaffeinated (DF)	9.2	0.5 mL of 10 mg/mL	167 ± 7
Control 1 (Saline)		0.5 mL of 0.9% NaCl	100 ± 3
Control 2 (DMSO)		0.5 mL of DMSO	100 ± 11

According to the whole blood clotting time, polysaccharide rich extract showed the highest activity followed by hot water extract. Low coagulation time with the PP (136 ± 2 s) compared to PR (270 ± 7) clearly indicates the anticoagulation activity can be

attributed to the tea polysaccharides (PR). The CF did not significantly alter the coagulation time. Lower whole blood coagulation time indicates that the responsible metabolites are either an extrinsic blocker, intrinsic blocker, platelet aggregation blocker or synergistic effect. Since PR showed the highest activity, it was selected for the further analyses.

3.2. Blood coagulation assays (PT and aPTT) for crude tea polysaccharides

Human blood was used for the PT and aPTT assay with a written consent of the donors. The blood was collected, 5 mL from each, from five healthy donors with no history of blood coagulation issues. The collected blood was pooled and fresh pooled plasma was used for the assays. In order to ensure the anticoagulant activity of isolated crude tea polysaccharide for extrinsic pathway (PT time) the extracts were subjected to a preliminary PT screening and the results are given in **Table 03**. The PR showed the highest activity, almost twice as the control. But when compared to the hot water extract of SBT, the PT time for PR is not significant.

Table 03: Preliminary PT of selected tea extracts

Extract type	Concentration	PT time/s	INR
Hot water	5 mg/mL	30 ± 2	1.80
Polysaccharide rich (PR)	5 mg/mL	39 ± 1	2.30
Polysaccharide poor	5 mg/mL	21 ± 2	1.25
Control (saline)	0.9% NaCl	14 ± 1	1.15

In order to get a better insight on PR's anticoagulation potential, concentration dependent effect of PR on PT and aPTT was evaluated and the results are given in **Table 04** with calculated INR values. The results for PT assay are same as before (**Table 03**) and it is concentration dependent. PR significantly increases the PT coagulation time at both 5 and 2 mg/mL concentrations compared to the control but not significant against the positive control. In the aPTT assay, PR at 5 and 2 mg/mL significantly increased the

coagulation time. Interestingly, at 5 mg/mL of PR, which is a crude extract, showed significant activity compared to the control at 0.5 mg/mL. Although the concentrations are different, this is significant as PR is a crude mixture being compared to pure Heparin.

Table 04: PT and aPTT profiles of the crude PR

	Concentration	**PT/s**	**INR**	**aPTT/s**
PR	20 mg/mL	*UC*	-	*UC*
	10 mg/mL	*UC*	-	*UC*
	5 mg/mL	**40 ± 3***	**3.25**	**270 ± 8*#**
	2 mg/mL	24 ± **3***	1.95	98 ± **5***
	1 mg/mL	14 ± **2**	1.14	59 ± **5**
Saline (09 % NaCl)		13.5 ± **1**	1.10	32 ± **3**
Heparin (Enoxaparin)	2 mg/mL	270 ± **12***	22.00	UC
	0.5 mg/mL	110 *	8.96	120 ± **4***

Each value represents the means ± SD (n=3). *P ＜ 0.05 as compared to negative control
#P ＜ 0.05 as compared to positive control, *UC*- unclotted

Considering both PT and aPTT data, it is conclusive that the PR is more effective in prolonging aPTT time than PT time. The aPTT assay represents the intrinsic pathway, which is mainly responsible for internal clotting. PR seems to have more potency to reduce the formation of internal clots. In addition to the auto coagulation-analyzer, both PT and aPTT assays were repeated manually. Manual assay revealed morphological differences of the clots formed with and without PR. A gel like clot formed without PR but with PR only an enhanced turbidity was observed and no gel like clot formation (**Figure 01**). The turbidity observed with added PR might be due to the fibrin activation which could have been perceived as clotting point by the auto- coagulation-analyzer when the same assay was done in the auto-mode. Blood clotting is a combination of two pathways, which are fibrin activation and platelet aggregation. A gel like clot is formed

when both pathways are active and activated fibrin is bound with activated platelets. In the presence of PR, it seems only the fibrin activation is viable while platelet activation is hindered.

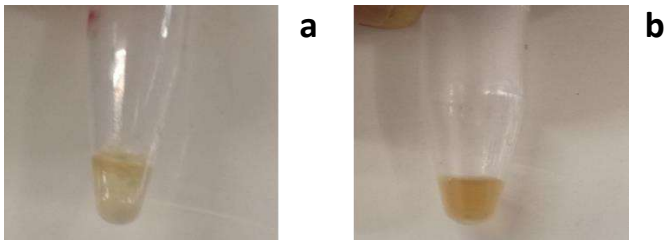

Figure 01: Morphological differences between the clots (**a:** Clot with DW, **b:** Fibrin formation with PR)

3.3. Platelet aggregation assay

Based on the morphological differences observed as described before (**Figure 01**), it was decided to assess the effect of PR on platelet activation. The best standard method for the *in vitro* test for platelet activation and aggregation level is to measure the light absorbance as depicted in **Figure 02** [40-42]. This assay is based on the principle that light passes more easily through a clear than a turbid solution, and will show low absorbance/scattering. Centrifugation of blood results in pelleting the red and white blood cells to produce a suspension of platelets in plasma, termed as platelet-rich plasma (PRP). In order to characterize platelet aggregation, the change in light transmission of PRP held in a cuvette can be observed. Here, as aggregates form, the turbidity of the solution is reduced and absorbance will be reduced and this is monitored by the detector which is sensitive to the level of light passing through the sample solution from the light source. The amount of light transmitted is indication of the extent of platelet aggregation. The results of the assay are shown in the **Figure 03**. The PBS (phosphate buffered saline), the negative control, added solution starts showing increased level of transmission just after few minutes, which is indicative of turbidity reduction due to platelet aggregation. With PR and Aspirin, the absorbance remains low/or transmission remains high for longer period indicating that the solution remains turbid as platelet aggregation is hindered by the additives. The platelet aggregation results of PR are more similar to the positive control Aspirin. This suggests that the PR has antiplatelet effect as

well in addition to the anticoagulation (block fibrin activation) effect. Therefore, crude PR has both aspirin like effect and heparin like effect as a potent anticoagulant.

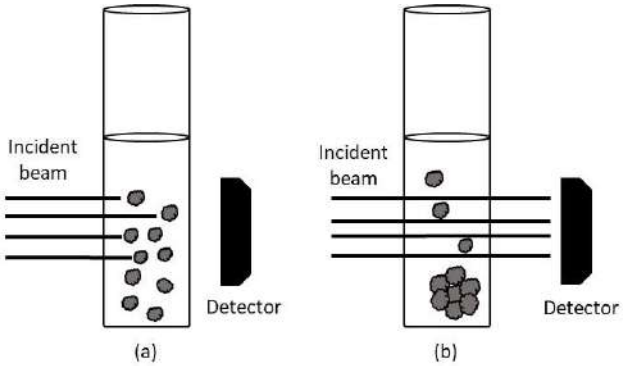

Figure 02. A schematic diagram of light transmission assay of platelet activation

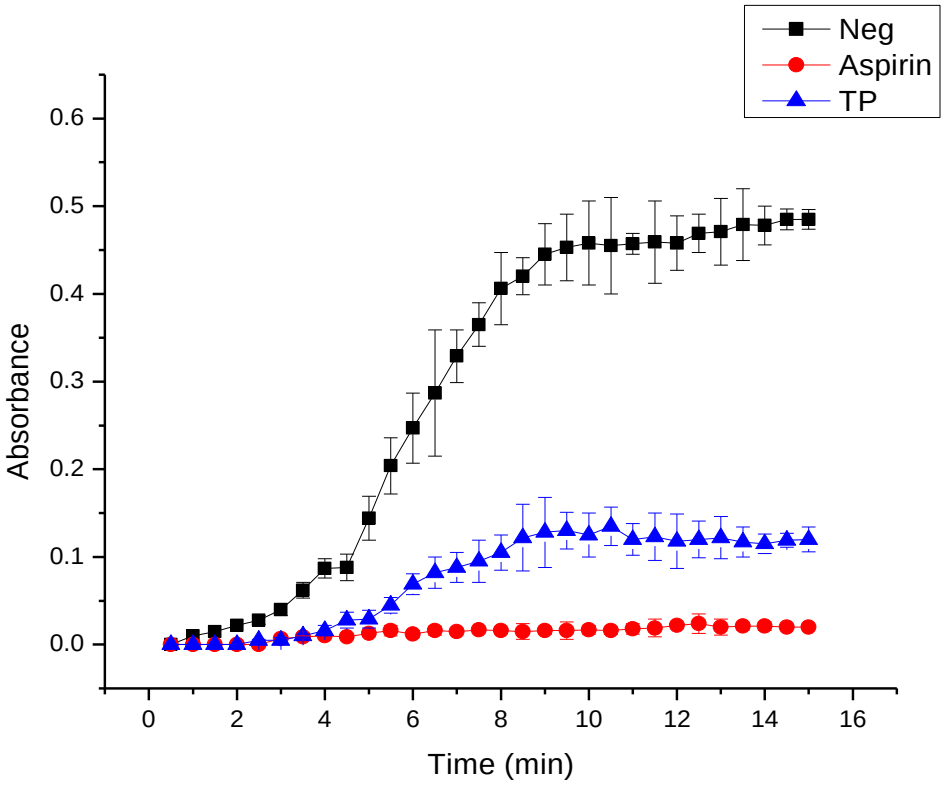

Figure 03: Antiplatelet activity of isolated PR and aspirin and PBS (Phosphate buffered saline) was used as the positive control and negative control respectively

4. Conclusion

In conclusion, this study shows that SBT (Sri Lankan Black Tea) possesses anticoagulation potential, especially the PR exhibited to affect the intrinsic pathway of the coagulation process and the platelet aggregation. PR is the least evaluated type of secondary metabolites of tea, this study might bring the due attention for PR. Crude PR seems to have a versatile mechanism to block both fibrin activation and platelet aggregation, which requires further studies to elucidate the mechanism. We hope this study will prove helpful to those involving in pharmacological strategies for the treatment or prevention of vascular diseases via the regulation of coagulation processes.

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