



ANTICLOTTING PROPERTIES OF SRI LANKAN LOW GROWN ORTHODOX ORANGE PEKOE GRADE BLACK TEA (*CAMELLIA SINENSIS* LINN)

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ABSTRACT

A previous investigation has shown that, Sri Lankan high grown orthodox broken leaf grade black tea (Dust No.1, Broken Orange Pekoe Fannings and Broken Orange Pekoe) possesses *in vitro* blood anticlotting activity. However, anticlotting activity of whole leaf grade black teas is, as yet, unknown although, bioactivity of tea is known to vary with several factors including grade of tea and agroclimatic elevation. The present study evaluates anticlotting activity properties of Sri Lankan low grown orthodox Orange Pekoe (O.P.) grade black tea (whole leaf grade type) both *in vitro* (using goat blood) and *in vivo* (using rats). In *in vitro* study, different concentrations (1.25, 2.5, 5.0, 7.5 and 10.0 mg/ml) of black tea brew (BTB) was made using freeze dried sample and calcium induced clotting time was determined (N=42-64/group). In *in vivo* studies, different doses of (223, 446 and 1339 mg/kg) doses of BTB was orally administered daily to separate groups of rats (N=6/group) and their clotting time was assessed on days 1(1,2 and 3h post treatment), 8 (1h post treatment) and 16 (1h post treatment) of the treatment. BTB showed strong and long lasting anticlotting activity, up to 24 h with an all or none type of dose relationship in *in vitro* study. In contrast, a mild anticlotting activity was evident in *in vivo* study with a curvilinear dose response in the acute study and linear relationship in the subchronic study. It is concluded that, regular consumption of moderately strong Sri Lankan O.P. grade black tea has a potential as dietary therapeutic for the betterment of cardiovascular health.

Key words: Black tea, *Camellia sinensis*, Orange Pekoe grade, clotting, anticlotting, anticoagulant

INTRODUCTION

Anticoagulants are used for prevention and treatment of thromboembolic disorders [1] and in medical equipment such as test tubes, transfusion bags, renal dialysis equipment and as a rodenticide [2]. However, the undesirable side effects and the high prices of the conventional anticoagulant drugs used today have prompted research into development of novel anticoagulant therapeutics which are orally active, target specific (particularly on factor Xa and thrombin) efficacious, safe and affordable. In this regard, we launched a programme of research to explore the possibility of developing a potential anticoagulant using Sri Lankan orthodox black tea which is manufactured from dried tender terminal leaves and buds of the plant *Camellia sinensis* L. O. Kruntz, Family: Theaceae. Black tea is consumed in many countries across the world [3] and shown to possess several

health benefits [3]. Black tea is the most consumed beverage in the world second to water [3]. There are two main categories of orthodox black tea: broken leaf grades and whole leaf grades.

We have already shown that, three grades of broken leaf categories namely, high grown (above 1200m, average mean sea level) Dust No.1, Broken Orange Pekoe (B.O.P.) and Broken Orange Pekoe Fannings (B.O.P.F.) (particle size: 250 – 500, 850 – 1400 and 500 – 850 μ m respectively) teas possessed marked anticoagulant activity in *in vitro* [4]. However, the anticoagulant potential of teas belonging to whole leaf category is not assessed, but is worth examining: since it is known that, bioactivity of black tea depends on particle size and agroclimatic elevation among other things [5,6]. Hence, this study was initiated to bridge this gap by evaluating the anticoagulant potential of Sri Lankan low grown (below 600 m, average mean

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sea level) orthodox whole leaf grade black tea both in *in vitro* and *in vivo* using Orange Pekoe (O.P.) grade (particle size: 1400 – 2000 µm) tea.

MATERIALS AND METHODS

Experimental Animals: Investigation of *in vivo* anticoagulant activity was performed using healthy adult Wistar rats (225-250 g) purchased from the Medical Research Institute, Colombo, Sri Lanka. They were kept under standardized animal house conditions (temperature: 28-31 °C, photoperiod: approximately 12 hours natural light per day, relative humidity: 50-55%) at the animal house of the Department of Zoology, University of Colombo. All the animals were acclimatized for 14 days prior to the experiment. All rats had free access to pelleted food (CIC Feed Pvt. Ltd., Ekala, Sri Lanka) and domestic tap water. All the experiments were conducted in accordance with the internationally accepted laboratory animal use and care guidelines [7] and rules of the Faculty of Science, University of Colombo, for animal experimentation.

Source of tea: Topmost immature leaves and buds of *C. sinensis* plucked from the plantation of St. Jochims tea estate of the Tea Research Institute, Hedallana, Ratnapura, Sri Lanka (29 m above mean sea level: low grown) during November – December 2011 were used to process O.P. grade black tea by orthodox-rotovane technique at the estate factory. The composition of true to size particles defined for the O.P. grade black tea was determined using sieve shaker (Retsch AS 200, Retsch GmbH, Haan, Germany) with standard set of sieves (shaking time: 10 minutes and shaking speed: 50 vibrations/minute). Typical characters belonging to elevations were assessed organoleptically by professional tea tasters of the Tea testing unit, Sri Lanka Tea Board. Tea samples were packed in triple laminated aluminium foil bags (1 kg each) and stored at -20 °C until use.

Preparation of black tea brew (BTB): BTB was made according to the ISO standards (ISO 3103) adding 2g of O.P. grade black tea to 100 ml of boiling water and brewed for 5 min [8]. This contained 36.1% (w/v) tea solids in water. For *in vivo* studies, 1339 mg/kg (equivalent to 9 cups, 1 cup = 150 ml) of BTB in 3 ml of water was prepared by adding 10 g of O.P. grade black tea to 30 ml boiling water and brewed for 5 min. Then 446 mg/kg (equivalent to 3 cups) and 223 mg/kg (equivalent to 1.5 cups) doses of BTB were prepared by diluting appropriately with boiling water. For *in vitro* studies, tea brew that was prepared according to the ISO standard was freeze dried and different concentrations (1.25, 2.5, 5, 7.5

and 10 mg/ml) were prepared by dissolving appropriate weights of freeze dried tea solids in isotonic saline (0.9% NaCl w/v).

Evaluation of *in vitro* anticlotting activity of O.P. grade black tea: Anticlotting activity in *in vitro* of O.P. grade of black tea was assessed using 5 concentrations (1.25, 2.5, 5, 7.5 and 10 mg/ml) of freeze-dried tea samples in isotonic saline and citrated (citrated using 3.2 % Sodium Citrate solution) arterial goat blood which was collected from the Colombo Municipal slaughter house, Dematagoda, Sri Lanka in calcium induced clotting model as described by Ratnasooriya *et al.*, 2007 [4]. Briefly, 4 ml of citrated blood was mixed with 1ml of different concentrations of tea brew or isotonic saline (used as the control) (N = 42 -64/group) in clean, dry glass tubes (10 mm diameter, 5 cm height).

Then, 0.2 ml of 2 % Calcium Chloride solution was added to the tea – blood or tea – saline mixtures. Immediately, they were mixed thoroughly, tightly closed using a stopper and started a stop watch. The calcium induced clotting time was determined by tilting each tube every 30 sec. until a firm clot is formed. If the blood did not clot by 10 min., it was considered as unclotted. The samples which failed to clot at 10 minutes were incubated at 37 °C and observed at 24 hour for the appearance of a firm clot by tilting.

Investigation of acute *in vivo* anticlotting activity of O.P. grade black tea: In *in vivo* acute anticlotting activity was investigated in different groups of rats (N = 6/group) following oral administration of three doses of BTB [1.5 cups (223 mg/kg), 3 cups (446 mg/kg) and 9 cups (1339 mg/kg)]. The control animals (N=18) received 3 ml distilled water orally. Blood (0.25 ml) was collected to clean and dry glass tubes at 1, 2 and 3 h post treatment from the tails of rats in different groups using aseptic precautions under mild ether anaesthesia, closed using a stopper and the clotting time was determined immediately by tilting the each tube at every 30 sec until it forms a firm clot at the bottom of the tube.

Investigation of subchronic *in vivo* anticlotting activity of O.P. grade black tea: Sub chronic anticlotting activity was ascertained in another group of rats (N=24, 6/group) on days 8 and 16 following daily oral administration of different doses [1.5 cups (223 mg/kg), 3 cups (446 mg/kg) and 9 cups (1339 mg/kg)] of BTB or 3ml of water (control). At 8 and 16 days of the treatment, blood (0.25ml) was collected from the tails of rats using aseptic precautions under mild ether anaesthesia to clean and dry glass tubes at 1h after the treatment

and clotting time was determined as described above in acute studies.

Investigation of the effect of BTB of O.P. on one stage prothrombin time in rats: Twelve rats were divided into two equal groups (N = 6/group) and orally treated with 446 mg/kg dose of BTB or 3ml of water (control). After 1h of the treatment, 0.45 ml of blood was collected to a clean plastic appendorf tube with 0.05ml of 3.2 %sodium citrate.

Then, the one stage prothrombin time was determined using Hemostat Thromboplastin – SI kit (Human Gesellschaft fur Biochemia und Diagnostica mbH, Germany) as per instructions given by the manufacturer. Briefly, citrated blood was centrifuged for 15 minutes at 1500 g in a centrifuge (Sanyo MSE Micro Centaur Centrifuge, Sanyo, Japan) and plasma was separated. Plasma was incubated for 5min at 37 °C. Then 0.2 ml of the pre warmed (incubated at 37 °C) thromboplastin reagent (containing 2.6% of rabbit brain extract, 0.13% of CaCl₂) was added to 0.1 ml of plasma at 37 °C and started the stop watch. The mixture was stirred gently using a wire loop until a clot was formed. The time was recorded for the clot formation.

Statistical analysis: Data is represented as Mean ± standard error of mean (SEM). Statistical comparisons were made using Mann-Whitney U-test [9] using Minitab 14.0 statistical package. Significant level was set at P<0.05.

RESULTS

The result of sieve analysis showed that, 83.5% of tea particles were true size (1400 – 2000 µm) for O.P. grade black tea. This indicates the tea sample used in the study was typical to O.P. grade black teas. Further, organoleptic profile of the professional and experienced tea tasters' confirmed that the sample used can be accepted as well made high quality low grown O.P. grade Sri Lankan black tea.

In vitro anticlotting activity of O.P. grade black tea: The results of *in vitro* anticlotting activity of BTB of O.P. grade black tea are summarized in Table 1. As shown, 1.25, 2.5 and 5 mg/ml doses did not significantly (P > 0.05) alter the calcium induced clotting time. On the other hand, no clotting was observed with 7.5 and 10.0 mg/ml doses and therefore, clotting time was considered to 10 min. Interestingly, no clotting was observed even after 24 h in all the unclotted blood samples at 10 min.

Acute in vivo anticlotting activity of O.P. grade black tea: The results obtained with acute *in vivo* anticlotting activity are summarized in Figure 1. As shown, the mid dose (446 mg/kg) significantly (P < 0.05) prolonged the clotting time by 72%, 39% and 28% respectively at 1, 2, and 3h post treatment, while the low dose (223 mg/kg) significantly (P < 0.05) increased the clotting time in 1 and 2 hour post treatment by 55.53 and 27.73% respectively. In complete contrast, no significant (P > 0.05) anticlotting activity was evident with the highest dose (1339 mg/kg) at any time point tested. The acute *in vivo* anticlotting activity was curvilinearly dose dependent (r²= 1)

Subchronic in vivo anticlotting activity of O.P. grade black tea: The results of subchronic *in vivo* anticlotting activity of BTB of O.P. tea are depicted in Figure 2. As shown, all the three doses of BTB significantly (P < 0.05) increased the clotting time on days 8 and 16 of the treatment (223 mg/kg dose: by 33 and 55% respectively; 446 mg/kg dose: by 39 % on both days and 1339 mg/kg dose: by 61 and 50 % respectively).

Effect of O.P. grade black tea on prothrombin time : As shown in the Table 2, oral administration BTB of O.P. did not alter the prothrombin time in rats.

DISCUSSION

This study examined the blood anticlotting properties of Sri Lankan low grown, garden fresh, unblended (in terms of sieve analysis and organoleptic profile) orthodox O.P. grade black tea both in *in vitro* and *in vivo*. For *in vitro* studies shed goat blood samples were used and *in vivo* studies were conducted on rats mainly due to its easy availability in large quantities. Since blood coagulation mechanism is highly conserved in mammals [10], the results are valid and meaningful irrespective of the source of mammalian blood sample used. O.P. grade black tea was selected as it is the main whole leaf category manufactured in Sri Lanka [11] and because it is the most widely used tea in blending teas [12]. Since, O.P. grade orthodox black teas are produced only in factories of low grown agroclimatic elevation, it became obligatory to select this variety.

The results show, for the first time, that Sri Lankan low grown orthodox O.P. grade black tea possesses anticlotting activity both *in vitro* and *in vivo* (acute and subchronic). However, *in vitro* anticoagulant activity was strong and *in vivo* anticoagulant activity was mild. Interestingly, heparin a commonly clinically used anticoagulant also acts both in *in vitro* and in *in vivo* [13]. We have

previously shown that, Sri Lankan high grown orthodox black tea belonging to Dust grade No.1, B.O.P. and B.O.P.F., which are broken grade teas possess anticlotting activity in *in vitro* (4). However, anticlotting activity of these three varieties of tea were more potent compared to O.P. grade tea, a whole leaf grade tea used in this study [4]. Nevertheless, their anticlotting profile was similar to what is evident in this study [4]. This difference in potency may be attributed to the variation in extraction of water soluble phytoconstituents such as flavanoids (catechins), polyphenols (theaflavins and thearubigins) and caffeine [3,14] from the tea sample to tea brew possibly due to their dissimilarity in particle size: extractable solids to the tea brew from Dust No.1, B.O.P.F. and B.O.P. were respectively 43.7% [15], 44% and 41% [4] while from O.P. tea this is 36.7%. Alternatively, this difference in potency may be attributed to difference in altitude and climate from where the tea samples originated: Dust No.1, B.O.P. and B.O.P.F. were high grown teas (4) whilst O.P. was low grown tea. It is of interest to note that, a potency difference between different grade of black tea was reported previously for tea induced diuresis: O.P. > Dust No.1 > B.O.P.F. [15,16,17].

The *in vitro* anticlotting activity observed in this study exhibited an all or none type of dose response relationship with a threshold concentration as reported earlier for Dust No.1, B.O.P., B.O.P.F. teas [4]. Moreover, as with Dust No.1, B.O.P., B.O.P.F. [4], in this study also the blood did not clot even up to 24 h following the addition of tea brew. This suggests an operation of a similar mechanism among the different tea samples in eliciting *in vitro* anticoagulatory activity. All or none type of dose relationships are rare, but not confined to haematological studies. For instance, such relationships were reported with impairment of human sperm motility with melatonin [18] and in induction of analgesic activity with aqueous leaf and stem extracts of *Psychotria sarmentosa* [19]. On the other hand, *in vivo* anticlotting activity of O.P. grade tea was mild but appears to exhibit a curvilinear type of dose response relationship in acute studies and a linear relationship in subchronic studies. Further, the *in vivo* anticlotting activity seen in this study cannot be compared with the previous studies with Dust No.1, B.O.P., B.O.P.F. [4] as *in vivo* studies has not been conducted.

The precise mechanisms precipitating anticlotting activity in the present study is not known, as yet. Nevertheless, some meaningful putative mechanisms can be proposed. Classically, blood clotting (thrombogenesis) has long being viewed as a 'cascade' of proteolytic reactions inducing

several clotting factors (sixteen) which is initiated via two different and independent mechanisms: the extrinsic (tissue factor) and intrinsic (contact) pathways that ultimately converge in to a common pathway of coagulation [13,20]. Factor Xa is known to act as the clotting factor at this convergent point [13,20]. It is widely accepted that *in vitro* clotting is predominantly initiated by intrinsic pathway [10, 13] although some recent data challenges this assumption [20]. However, if this classical pathway mechanism of blood clotting is correct, then the O.P. triggered *in vitro* anticlotting activity observed in this study can be attributed to blocking or interference of one factor and/or several factors of clotting operating in the intrinsic pathway (such as kininogen, kallikrein, factor VIII, IX, XI and XII), and/or common pathway (such as prothrombin, fibrinogen and Ca^{2+}) especially the Ca^{2+} (factor IV). Black tea is reported to possess metal ion chelating activity due to its flavanoids [3] and quercetin [3]. On the contrary, O.P. induced *in vitro* anticlotting activity could be mediated by direct inhibition of thrombin (which converts fibrinogen into fibrin clot) as reported for heparin [13].

In this study, one-stage prothrombin time remained unaltered in rats treated with O.P. grade tea although their clotting time was prolonged. This indicates that, *in vivo* anticoagulant activity, evident in this study, is not mediated via blocking or interference of clotting factors in the extrinsic (such as factor VII and platelet phospholipids) and common pathways of clotting (such as prothrombin, fibrinogen). Further, this indicates that, O.P. tea does not act as the anticoagulant, warfarin, which acts as a vitamin K antagonist [2,13]. Thus, it is very likely that *in vivo* anticlotting activity in this study is mediated via binding and direct inhibition of thrombin (both free and clot bound) with no involvement of natural clotting factors as reported with the most potent natural inhibitor of thrombin, hirudin [21] and new generation of oral anticoagulants (such as dabigatran) [22] and/or directly inhibiting the factor Xa as reported with rivaroxaban, apixaban [22]. In complete contrast, O.P. tea may mediate its *in vivo* anticoagulant activity by promoting the activity of antithrombin circulating in the blood and/ or thrombomodulin secreted by the endothelial cells (which dissolves blood clots) [13].

Based on the results of our previous study on thrombolytic activity [4] and anticlotting activity in this study, it can be proposed that Sri Lankan low grown O.P. grade orthodox black tea may act as a potential dietary therapeutic for the betterment of cardiovascular health. Further, the results hint the

potential of developing pharmaceuticals from Sri Lankan black tea for cardiovascular disorders.

therapeutic for the betterment of cardiovascular health.

CONCLUSION

This study shows, for the first time that, Sri Lankan low grown orthodox O.P. grade black tea of whole leaf grade type possesses both *in vitro* and *in vivo* blood anticlotting activities and regular consumption of moderately strong Sri Lankan O.P. grade black tea has a potential as dietary

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Conflict of interest: None

Table 1: Effect of O.P. grade black tea on *in vitro* anticlotting activity

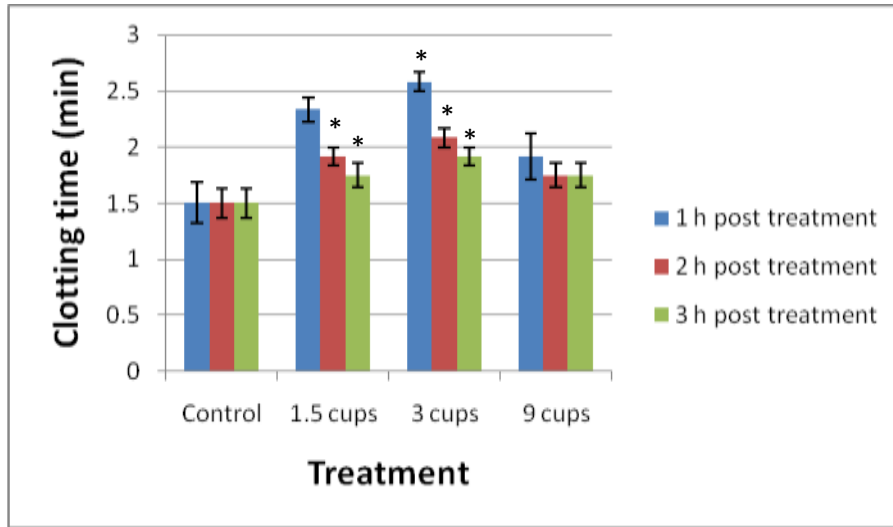
Concentration of tea brew	Clotting time (min)
Control (N = 64)	1.0 ± 0.0
1.25 mg/ml (N = 47)	1.0 ± 0.0
2.5 mg/ml (N = 45)	1.0 ± 0.0
5 mg/ml (N = 49)	1.0 ± 0.0
7.5 mg/ml (N = 42)	10.0 ± 0.0 *
10 mg/ml (N = 42)	10.0 ± 0.0 *

Results are expressed as Mean ± SEM, min - minutes
 *- P < 0.05; compared to control (Mann-Whitney U test)

Table 2: Effect of BTB of O.P. tea on prothrombin time

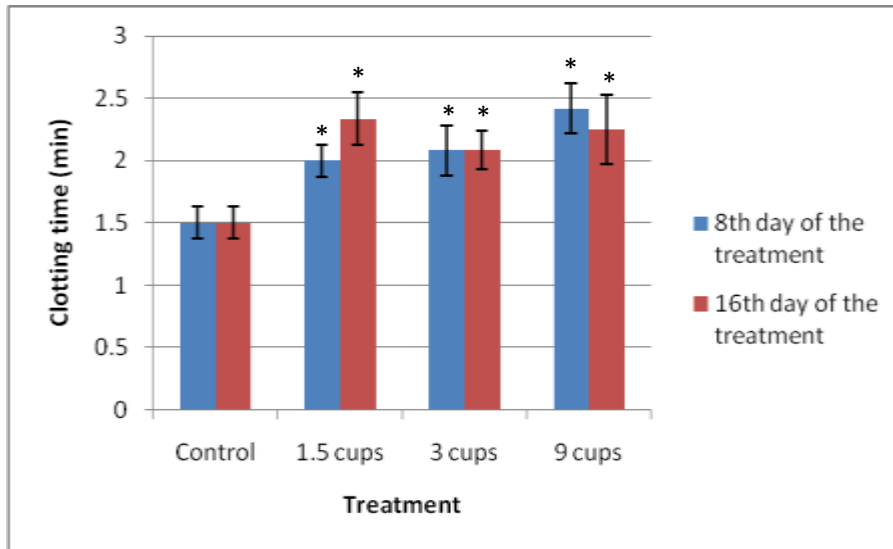
Treatment	Prothrombin time (sec)
Control (water)	21.44 ± 0.42
BTB (446 mg/kg)	21.38 ± 0.39

Results are expressed as Mean ± SEM, sec - seconds



*- P < 0.05; compared to control (Mann-Whitney U test)

Figure 1: Effect of O.P. grade black tea on acute *in vivo* anticlotting activity at 1, 2 and 3h following oral administration of BTB of O.P. tea or water (control) (Mean ± SEM)



*- P < 0.05; compared to control (Mann-Whitney U test)

Figure 2: Effect of O.P. grade black tea on subchronic *in vivo* anticlotting activity on days 8 and 16 of treatment of BTB of O.P. tea or water (control) (Mean ± SEM)

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