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Evaluation of anticoagulant and thrombolytic activity of *Berberis orthobotrys* in animal model

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Abstract

Fundamental to this research was the evaluation of anticoagulant and thrombolytic potential of *Berberis orthobotrys* using clotting time, prothrombin time and activated partial thromboplastin time performed at 2.5, 5, 10% concentration. Aqueous-methanol extract significantly ($p < 0.05$) increased the coagulation parameters both in human blood as well as in rabbit blood and exhibited the more salient effect on prolongation of these coagulation parameters at 10% concentration. Aqueous methanol extract (25, 50 and 100 mg/kg) produced the preferential increase in bleeding time and clotting time nevertheless the effect was more perceptible after 90 min at 100 mg/kg. Moreover, the crude extract presented a substantial increase in coagulation parameters after 7 days of administration in rabbits with more pronounced effects at the highest dose. HPLC profile of aqueous methanol extract revealed the presence of quercetin, p-coumaric acid, gallic acid, vanillic acid, 4-hydroxy-3-methoxy benzoic acid. Thus, the present study provides the pharmacological basis for its medical use in cardiovascular and other thrombotic disorders.

Introduction

Blood coagulation is a defense mechanism to maintain the veracity of closed, high-pressure mammalian circulatory system after blood vessel injury. Blood coagulation abnormalities result in thrombosis, atherosclerosis and inflammation by the activation of enzymes in coagulation cascade and platelets (Gou et al., 2003). The stages of coagulation, therefore, include vessel constriction, platelet plug formation, coagulation as well as fibrin generation and healing and repair (Anderson and Spencer, 2003). However, three principal factors for thrombus formation are so-called Virchow's triad i.e., endothelial injury, blood flow stasis or turbulence and hyper-coagulability of blood (Adiba et al., 2013).

In the developed countries, thromboembolic disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks are the main causes of morbidity and mortality (Dickneite et al., 1995). The use of anti-

coagulant (heparin, warfarin) is linked to many limitations and adverse effects. The most incurable complication associated with this therapy is bleeding. Other adverse effects also limit the use of anticoagulants like, heparin-induced thrombocytopenia, drug-drug interactions, erratic dose-response relationship, and forceful monitoring need and in some cases reocclusion may occur. Conventional medicine has been defined as knowledge of collection, skills and practices which are based on the theories and views of different cultures that help in health maintenance as well as in prevention, diagnosis, improvement or treatment of physical and mental illness (Mahmood et al., 2011). Herbal medicine has been frequently used for historical, cultural and biological reasons due to their easy, continued availability (Bhattarai, 1989), better compatibility and also because of less testing time, higher safety, improved efficacy, compliance, least adverse effects and cost-effectiveness (Khanna et al., 1986).



As the medicinal plants like *Berberis calliobotrys* (Alamgeer et al., 2016), *Careya arborea*, *Melastoma malabathricum*, *Eichhornia crassipe*, *Jatropha curcas* and *Mentha longifolia* (Alamgeer et al., 2018), *Porana volubilis* etc are the pool of many cheap and safe therapeutic agents (Jain et al., 2014). A traditional medicinal plant namely, *Berberis orthobotrys* Bien ex Aitch was selected for the current research work and an attempt made for the confirmation of possible antithrombotic effects of the selected plant so that it could be helpful in the therapy of thrombotic disorders (Khan and Khatoon, 2007).

Materials and Methods

Chemicals and reagents used in the present research

Trisodium citrate, heparin (Leo Pharmaceuticals, Denmark), methanol (Merck, Germany), prothrombin reagent, activated partial thromboplastin reagent (Mac and Rains Pharmaceuticals, Pakistan) were purchased from the relevant companies.

Experimental animals

Healthy adult rabbits of either sex, weighing from 1.0-1.5 kg of 10-12 months were used. Rabbits were kept in a fine lighted air-conditioned room ($26 \pm 1^\circ\text{C}$) under standard environmental conditions (12 hours light-dark cycles). They were fed on standard green leafy, seasonal vegetables, green leaves of alfalfa and water *ad libitum*. *In vitro* studies were also performed on the blood samples obtained from the healthy human.

Collection and authentication of medicinal plant

B. orthobotrys roots were collected from the District Gilgit, Pakistan during the month of June 2014 and was identified and authenticated by Dr. Shair Wali, Department of Botany, Karakorum International University, Gilgit, Baltistan, Pakistan. A voucher (No. BO-15-12) has been deposited in the herbarium, Faculty of Pharmacy, University of Sargodha for future reference.

Preparation of aqueous methanolic extract

By using cold maceration process, the aqueous methanolic (70:30) extract of *B. orthobotrys* was prepared. The grounded plant material (2 kg) was soaked in 5 L of water-methanol mixture (70:30) for 72 hours at room temperature. The whole material was filtered after three days of intermittent shaking and the filtrate evaporated under reduced pressure using rotary evaporator. The crude extract was then air-dried to obtain a solid mass with a yield of 19% (Alamgeer et al., 2013).

In vitro experiments

Effect on clotting time

The ability of *B. orthobotrys* to inhibit the *in vitro* coagulation of blood collected either from human volunteer or rabbit was quantitatively assayed by following the

procedure adopted by Koffuor and Amoateng (2011).

Aqueous methanolic extract solution of 2.5, 5 and 10% concentrations prepared. 20 sterilized test tubes were labeled and in respective labeled test tubes, 0.2 mL of each extract solution was taken except first five test tubes which were used as the control for each blood sample.

One milliliter of whole blood drawn either from the healthy volunteer (n=5) or rabbit (n=5) was added to the test tubes and placed in a water bath at 37°C . The time taken for the blood samples to clot was recorded with the help of stop watch (Alamgeer et al., 2016).

Effect on prothrombin time and activated partial thromboplastin time

Five labeled centrifuge tubes were taken and trisodium citrate was added. Three milliliters of blood sample was taken either from the human volunteer (n=5) or rabbit (n=5) and added to the centrifuge tube followed by centrifugation at 3,000 rpm for 5 min. With the help of micropipettes, plasma was separated and saved in Eppendorf tubes. The 250 μL of plant extract of varying concentrations (crude extract 2.5, 5 and 10%) and 250 μL of platelet poor plasma was mixed in an eppendorf tube and samples were incubated at 37°C for 5 min. 100 μL of test plasma and 200 μL of reagent was added and clotting time was measured as prothrombin time (Alamgeer et al., 2016; Jagtap et al., 2012; Klafke et al., 2012).

For the assessment of activated partial thromboplastin time, 100 μL of reagent was added to the 100 μL of test plasma (platelet poor plasma + extract). The resultant mixture was heated in incubator at 37°C for 1 min after which 100 μL CaCl_2 (25 mmol) was added and incubated for 15 sec and clotting time was recorded as activated partial thromboplastin time (Alamgeer et al., 2016; Klafke et al., 2012).

In vitro thrombolytic activity

Solutions of different concentrations of aqueous methanolic extract (25, 50 and 100 mg/mL) were prepared. From the healthy volunteer and rabbit, venous blood was drawn. The sterile eppendorff tubes were taken and weighed them. Blood was distributed (0.5 mL/tube) to each previously weighed sterile eppendorff tubes and incubated at 37°C for 45 min to form the clot. Serum was totally removed after clot formation without disturbing the clot and each tube having clot was again weighed to find out the clot weight. In each eppendorff tube containing pre-weighed clot, a volume of 100 μL of test solution was added. 100 μL of distilled water was separately added to the control tube numbered as a negative control. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. Fluid released was removed after incubation and tubes were again weighed to observe the difference in weight after

clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis (Prasad et al., 2006).

$$\% \text{Lysis} = \text{Weight of released clot} / \text{Weight of clot} \times 100$$

Where, weight of released clot = Weight of clot before lysis - Weight of clot after lysis

In vivo experiments

Effect on bleeding time and clotting time after single dose administration

In vivo anticoagulant activity of *B. orthobotrys* was measured at different doses as described by Elg et al. (2001). Rabbits were divided into three groups of 5 rabbit each. Group I, II and III received aqueous-methanolic extract (25, 50 and 100 mg/kg correspondingly). At 0, 30, 60 and 90 min, the bleeding time was recorded after administration of dose by pricking the small marginal ear vein. After every 5 sec, the pierced vein was gently blotted with the filter paper until the cessation of bleeding.

Meanwhile, capillary tube was placed horizontally on the bleeding vein to collect the blood in it. After every 30 sec, the tube was broken until the thread of clotted blood was appeared and clotting time was recorded (Alamgeer et al., 2016).

Effect on coagulation parameters after 7 days of treatment

By the administration of aqueous-methanolic extract, anticoagulant prospective of the plant was explored for seven days treatment. Rabbits were divided into 4 groups of 5 rabbits each. Group I served as control. All other groups were treated orally with aqueous methanol extract (25, 50 and 100 mg/kg). Animals were kept under observation and their laboratory changes were examined on day 7 of the study. From each animal of all groups, whole blood samples were collected in capillary tubes after vein puncture and clotting time was determined by the method of the capillary tube according to the method of Elg et al. (2001) and was followed for recording the bleeding time. From each animal of all groups, about 3 mL of whole blood samples were collected in dry clean tubes containing trisodium citrate and was subjected to centrifugation to separate plasma which was used for measuring the prothrombin time and activated partial thromboplastin time by consuming standard reagents.

Phytochemical investigation of B. orthobotrys crude extract using HPLC technique

The sample was prepared by adopting the method of Tokusoglu et al. (2003) with slight modifications. The sample preparation was attained by mixing 50 mg of crude aqueous methanolic extract with 16 mL of distilled water and 24 mL of methanol. Thereafter, 10 mL of 6 M HCl was added. The mixture was kept for 2

hours at 95°C in an oven to get aglycons of flavonol glycosides and then filtered via microfilter prior to injecting in HPLC. An HPLC analysis was performed using HPLC model LC-10A (Shimadzu, Japan), fitted with SCL-10A system control unit, rheodyne injector, two LC-10 AT pumps, CTO-10A column oven, SPD-10A UV-Visible detector and data acquisition class LC-10 software. A filtered sample (20 µL) was injected into an analytical Shim-Pack CLC-ODS reverse phase (C-18) column (25 cm × 4.6 mm; 5 µm particle size). Two solvent systems A (H₂O: Acetic acid- 94:6, pH= 2.27) and B (100% acetonitrile) were used. Graded elution of mobile phase was used to perform chromatographic separation (a mixture of solvent A and B (50:50 v/v), which was filtered using 0.5 µm membrane under vacuum before use) at a flow rate of 1 mL per min at 30° C. Detection was performed at a wavelength of 280 nm.

Statistical analysis

The results were expressed as means ± standard error of the mean (SEM). One-way ANOVA with posttest Dunnet's t-test was applied. In addition to this, Two-way ANOVA followed by Bonferroni test was also used for the analysis of results. The data with a confidence level of 95% (p<0.05) was considered as statistically significant.

Results

Effect of B. orthobotrys on in vitro clotting time, prothrombin time and activated partial thromboplastin time of human blood

Different concentrations (2.5, 5 and 10%) of crude extract produced dose-dependent and significant (p<0.001) increase in clotting time. Similarly, the addition of different concentrations (2.5, 5, 10%) of crude extract into human blood (n=5) caused significant (p<0.001) prolongation in prothrombin time of human blood sample as compared to control. The most profound increase in the prothrombin time was 80.6 ± 3.2 sec with 10% concentration of crude extract as compared to control i.e., 13.0 ± 1.0 sec. Moreover, crude extract (5 and 10%) significantly (p<0.001) prolonged the activated partial thromboplastin time. Moreover, 10% concentration of crude extract produced pronounced effects of activated partial thromboplastin time (340 ± 30.2) as compared to the control (23.0 ± 1.0) (Table I).

Effect on clotting time, prothrombin time and activated partial thromboplastin time of rabbit blood

The crude extract significantly (p<0.001) increase clotting time at of 5 and 10% concentrations with remarkable prolongation in clotting time of rabbit blood at 10% concentration (53 ± 2.6 min) as compared to control (7.8 ± 1.02 min). Similarly, aqueous methanol extract exhibited noteworthy (p<0.001) and dose-dependent (2.5, 5, 10%) increase in prothrombin time. How-

Table I								
<i>In vitro</i> effects of aqueous methanolic extract of <i>B. orthobotrys</i> on different coagulation parameters								
Parameters	Aqueous methanolic extract (%)							
	Human				Rabbit			
	0	2.5	5.0	10.0	0	2.5	5.0	10.0
Clotting time (min)	5.0 ± 0.5	14.2 ± 1.3 ^b	23.8 ± 1.8 ^a	46.6 ± 2.7 ^a	7.8 ± 1.0	14.4 ± 1.3 ^c	23.6 ± 1.3 ^a	53.0 ± 2.6 ^a
Prothrombin time (sec)	13.0 ± 0.9	43.6 ± 1.4 ^a	60.0 ± 1.7 ^a	80.6 ± 3.1 ^a	12.6 ± 0.7	45.0 ± 1.6 ^a	61.2 ± 1.9 ^a	73.2 ± 1.1 ^a
Activated partial thromboplastin time (sec)	23.0 ± 0.9	72.2 ± 2.6 ^{ns}	143.4 ± 10.1 ^a	340.0 ± 30.2 ^a	21.8 ± 1.2	49.4 ± 5.1 ^{ns}	109.6 ± 5.4 ^b	406.0 ± 30.1 ^a

Results are presented as Mean ± SEM. One-way ANOVA was applied to analyze the data where ns= non-significant, ^ap<0.001, ^bp<0.01 vs control

ever, increase in prothrombin time up to (73.2 ± 2.0 sec) was observed with 10% concentration as compared to control (12.6 ± 0.6 sec) (Table I). In the same manner, 2.5, 5, 10% of crude extract dose-dependently enhanced the activated partial thromboplastin time with most significant (p<0.001) increase in the activated partial thromboplastin time at 10% solution (Table I).

In vitro thrombolytic activity of *B. orthobotrys* on human and rabbit blood

Aqueous methanolic extract of *B. orthobotrys* showed considerable (p<0.001) and dose reliant increase in lysis of human blood clot at different concentrations of 2.5, 5 and 10%. Moreover, at the highest concentration (10%), extract showed more prominent lysis. Similarly, different concentrations (2.5, 5 and 10%) of crude extract also displayed the noteworthy and dose-dependent effect on the thrombolysis of rabbit blood (Figure 1).

Effect of *B. orthobotrys* on *in vivo* bleeding time and clotting time after single dose administration

Treatment with crude extract produced significant (p<0.001) increase in bleeding time at 25, 50 and 100 mg/kg doses after 30, 60 and 90 min of administration. (Table II). However, most prominent effects were produced at 100 mg/kg after 90 min of dose administration. Similarly, methanolic extract dose-dependently produced prolongation of clotting time with most pronounced effects at a dose of 100 mg/kg after 90 min of administration (Table II). The dose of 25 and 50 mg/kg produced prolongation of clotting time but the most pronounced effects were produced at the concentration of 100 mg/kg after 90 min of administration.

Effect of *B. orthobotrys* on different coagulation parameters after 7 days of treatment

There was significant (p<0.01-0.001) increase in bleeding time, clotting time, prothrombin time and activated partial thromboplastin time in animals treated with extract at doses of 25, 50 and 100 mg/kg, orally for seven days when compared to the control at day 7 of the study. On day 7, significant (p<0.05-0.001) rise in

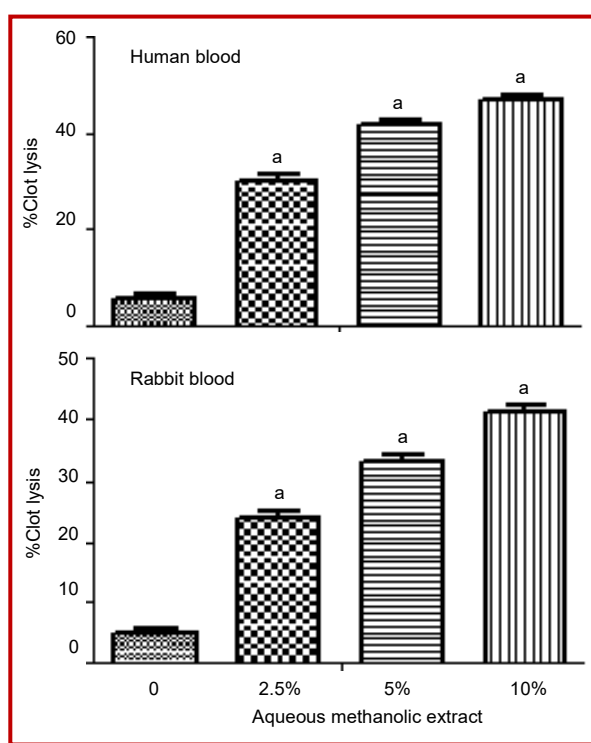


Figure 1: Effect of aqueous methanolic extract of *Berberis orthobotrys* on human (upper) or rabbit (lower) blood clot lysis done by One-way ANOVA where ^ap<0.001

bleeding time, clotting time, prothrombin time and activated partial thromboplastin time was observed at 50 and 100 mg/kg doses, but the dose of 25 mg/kg only prolong the clotting time and APTT significantly (p<0.001) when compared to control as depicted in (Table III).

Phytoconstituents in the aqueous methanolic extract of *B. orthobotrys* through HPLC

The HPLC analysis of *B. orthobotrys* crude extract revealed the presence of quercetin, gallic acid, p-coumaric acid, vanillic acid and 4-hydroxy-3-methoxy benzoic acid as illustrated in (Table IV).

Table II

Effects of *B. orthobotrys* extract on *in vivo* bleeding and clotting times in rabbit

Time (min)	Bleeding time (sec)			Clotting time (sec)		
	2.5%	5%	10%	2.5%	5%	10%
0	32.6 ± 2.7	32.6 ± 2.7	32.6 ± 2.7	242.0 ± 25.8 ^a	237.0 ± 23.8 ^a	260.0 ± 21.7 ^a
30	41.0 ± 1.7 ^a	44.8 ± 2.9 ^a	53.8 ± 2.5 ^a	323.0 ± 9.3 ^a	341.2 ± 3.5 ^a	359.8 ± 3.5 ^a
60	54.0 ± 1.5 ^a	60.4 ± 2.9 ^a	69.6 ± 1.9 ^a	348.8 ± 2.3 ^a	352.8 ± 7.1 ^a	378.0 ± 2.2 ^a
90	63.2 ± 1.1 ^a	67.2 ± 1.5 ^a	78.6 ± 1.1 ^a	362.0 ± 2.2 ^a	370.0 ± 1.7 ^a	411.4 ± 11.2 ^a

Results are expressed as means ± SEM. Two-way ANOVA was applied to analyze the data. Where, ^ap<0.001 vs control (0 min)

Table III

Effects of *B. orthobotrys* extract on different coagulation parameters after 7 days *in vivo* administration

Parameters	Treatment groups (aqueous methanolic extract)			
	Control	2.5%	5%	10%
Bleeding time (sec)	40.3 ± 2.9	52.0 ± 1.7 ^c	68.6 ± 3.8 ^a	77.0 ± 2.0 ^a
Clotting time (sec)	240.0 ± 34.6	358.0 ± 4.4 ^b	375.0 ± 2.5 ^b	392.3 ± 1.8 ^a
Prothrombin time (sec)	16.0 ± 1.7	27.3 ± 1.2 ^b	38.0 ± 1.7 ^a	52.7 ± 1.4 ^a
Activated partial thromboplastin time (sec)	32.0 ± 1.7	56.0 ± 4.4 ^b	82.3 ± 1.8 ^a	108.3 ± 6.1 ^a

Results are presented as mean ± SEM. One-way ANOVA was applied to analyze the data. Where, ^cp<0.05, ^bp<0.01 and ^ap<0.001

Table IV

Phytoconstituents identified in crude extract of *Berberis orthobotrys* using HPLC

Peak No.	RT (min)	Area count	Amount (ppm)	Compounds
4	2.9	28.6	1.5	Quercetin
7	4.9	319.9	1.1	Gallic acid
12	13.2	71.9	4.4	Vanillic acid
13	14.1	237.0	3.1	p-coumaric acid
14	17.7	246.0	26.0	4-hydroxy-3-methoxy benzoic acid

Discussion

In the current study, results of *in vitro* and *in vivo* studies illustrated that crude extract of *B. orthobotrys* produced a significant increase in prothrombin time as compared to control. Prolonged prothrombin time indicates blockade of the tissue factor pathway, which relies on the presence of factor VII, which in turn depends upon activation by vitamin K and formerly it has been reported that the warfarin and other vitamin K antagonists lead to prolonged prothrombin time. The anticoagulant effect may be endorsed to coumaric acid that has been noted in HPLC screening of crude extract of *B. orthobotrys* and antecedently coumarin has been

reported to inhibit vitamin K-dependent clotting factors activation such as clotting factors II, VII, IX and X (Rang et al., 2007). Moreover, some berberis species namely *B. aristata* and *B. lyceum* (Sabir et al., 2014) have been reported to possess anti-coagulant activity due to the presence of coumarins.

Activated partial thromboplastin time assay is used to assess intrinsic clotting index and coagulation factors such as VIII, IX, XI, XII (Azevedo et al., 2007). Administration of *B. orthobotrys* aqueous methanolic extract revealed a highly significant increase in activated partial thromboplastin time in human and rabbit's serum in both *in vitro* and *in vivo* assays. As intrinsic clotting activity is measured by activated partial thromboplastin time test and usually increased values of activated partial thromboplastin time represents a deficiency in factors VII, IX, XII, XIII and von Willebrand's factor (Laffan and Bradshaw, 1995) it could be proposed that *B. orthobotrys* has inhibitory effects on these factors.

Thrombosis results when activated platelets from platelet to platelet bonds and also bind to leukocytes and form a complex process of plaque formation and growth. By pharmacological means, thrombolysis is the breakdown of blood clots. For this reason, it is collectively referred to as 'clot bursting'. Fibrinogen and fibrin contained in a clot is lysed by plasmin which is a fibrinolytic agent (Das et al., 2013). Thus in the present study, *in vitro* thrombolytic activity showed that *B. orthobotrys* aqueous methanolic extract possesses notable ability to break down blood clot thus strengthening

its anticoagulant potential.

Polyphenolic compounds are a large group of organic secondary plant metabolites which have been divided into several classes including hydroxy benzoic acid, hydroxy cinnamic acid, coumarins, xanthenes, stilbenes, anthraquinones, lignans and flavonoids (Manach et al., 2004). Formerly, the polysaccharide phenolic protein complex has been found to exhibit both anticoagulant and procoagulant effects on blood coagulation system (Pawlaczyk et al., 2011). Also, Pawlaczyk et al. (2011) proved phenolic polysaccharide isolated from *Erigeron canadensis* as a potentially useful anticoagulant and antiplatelet agents. HPLC screening of *B. orthobotrys* revealed the presence of quercetin, gallic acid, 4-hydroxy-3-methoxy benzoic acid and p-coumaric acid.

Coumarin is derived from a variety of plants used as an anticoagulant. In the blood coagulation cascade, coumarin works by inhibiting the calcium activity. Previously, it has been reported that coumarins have antithrombotic and thrombolytic activity, therefore antithrombotic activity of test plant extract might be due to its phenolic coumarins and marked increase of prothrombin time and activated partial thromboplastin time is apparently caused by the interference of procoagulant action of calcium by phenolic compounds found in crude extract of *B. orthobotrys*. Previously, it has also been reported that flavonoids have thrombolytic activity (Adiba et al., 2013) and quercetin, a flavonoid that is also a strong free radical terminator and can aid to reduce atherosclerosis that leads to heart attack and stroke (Koffuor and Amoateng, 2011). Thus, the anticoagulant activities exhibited by *B. orthobotrys* may be due to the presence of these constituents thus supporting its traditional use in cardiac patients.

Conclusion

The aqueous methanolic extract of *B. orthobotrys* shows antithrombotic potential.

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University of Sargodha

Ethical Issue

All experimental procedures were approved by the Institutional Animal Ethical Committee, College of Pharmacy, and University of Sargodha and were performed according to the standard procedures of National Research Council (NRC, 1996).

Conflict of Interest

Authors declare no conflict of interest

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