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Antithrombotic potential of *Berberis calliobotrys* extract

Alamgeer, Hira Asif and Shahid Rasool

Department of Pharmacology, Faculty of Pharmacy, University of Sargodha, Sargodha, Punjab, Pakistan.

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Abstract

This study was focused with the aim to investigate the antithrombotic potential of *Berberis calliobotrys*. Aqueous-methanolic extract and various fractions showed significant ($p<0.05$-0.001) increase in prothrombin, activated partial thromboplastin and clotting time while only aqueous methanolic extract caused clot lysis when added to the blood samples of rabbit and human. *In vivo* study in rabbits, butanolic fraction (100 mg/kg) produced more significant prolongation in bleeding, prothrombin, activated partial thromboplastin and clotting time. Interestingly butanolic fraction had shown more pronounced effects among all tested extracts both *in vivo* and *in vitro* studies. Hence, it was subjected to antilipid peroxidation and phytochemical studies (total falvonoid contents, HPLC-DAD profile, FTIR). In conclusion, *B. calliobotrys* induce transient changes in the coagulation parameters, may it possess active constituents responsible for its antithrombotic potential.

Introduction

Thrombosis is a grievous disorder that is typified by the evolution of a thrombus (blood clot) within the vascular system (Nicolini et al., 1992). Over 150 year ago, German pathologist Rudolf Virchow first postulated that a triad of conditions predispose to thrombus formation (Kyrle and Eichinger, 2009). Thrombosis was identified as one of the momentous fons et origo of cardiovascular disorders (Wang et al., 2006) while, currently used antithrombotic agents (anticoagulants, antiplatelet and thrombolytic agents) are linked with many side effects and limitations such as bleeding, thrombocytopenia, drug-drug interactions, unpredictable dose response relationship, need of intense monitoring, allergic reactions, cerebral hemorrhage, and reocclusion may occur in some cases due to these limitations and increased mortality and morbidity associated with thrombotic complications there is a need for the development of new antithrombotic agents.

As the time dwindle medicinal plants have been proposed as an important source of novel therapeutics. Current appraise has propounded that, in many emerging countries, about 2/3rd of the population reckons laboriously on medicinal plants to encounter their basic health care needs (Farnsworth and Soejarto, 1991). Lavish endeavors have been made for revelation and development of natural medicines from diverse animal and plant sources which possess antithrombotic poten-tial (Rajapakse et al., 2005), such as aspirin a well known and commonly used antiplatelet drug is also derived from bark of medicinal plant namely *Salix alba* (Vickers and Zollman, 1999).

As the medicinal plants are reservoir of many cheap and safe therapeutic agents thusly, a traditional medicinal plant namely, *Berberis calliobotrys* Bienert ex Aitch (Family: Berberidaceae) commonly known as chowenj used for the treatment of cardiovascular diseases was selected for the current research work and an effort made for the validation of possible antithrombotic effects so that it could be helpful in the cure of thrombotic disorders.

Materials and Methods

Collection of plant, preparation of extract and fractions

Plant was collected from Quettain in the month of July. Aqueous-methanolic extract of *B. calliobotrys* was concocted by using cold maceration process. Activity directed fractionation of aqueous methanolic extract was carried out using different organic solvents so that the relative fraction with desired activity could be obtained (Williamson et al., 1998). Briefly; 100 g of aqueous-methanolic extract was dissolved in distilled water and was mixed with equal volume of n-butanol in a separating funnel with vigorous shaking and periodically removed air. Mixture was allowed to separate for about 20-30 min into two layers. Upper layer was removed, same procedure was repeated twice more and all fractions were combined and finally concentrated under reduced pressure to obtain the corresponding fraction. Finally, remaining lower layer was also evaporated and considered as aqueous fraction.

Chemicals

Following chemicals were used in current investigation: Prothrombin reagent, activated partaial thromboplatin reagent, DMSO, methanol, n-butanol, NaOH, thiobarbituric acid (TBA), trichloroacetic acid (TCA), qurecetin, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gum acacia and hydrochloric acid, all were obtained from Sigma-Aldrich.

Experimental subject

In vitro pharmacological studies were carried out on blood sample of human as well as rabbit while only rabbits were utilized for the *in vivo* experiments. The rabbits were purchased from local supplier and kept in university animal house. The animals were housed in stainless steel cages at standard laboratory conditions (light period from 8 AM to 8 PM, room temperature of 21 ± 2°C, relative humidity 55%). Animals were provided with to a balanced rabbit's diet consisting of green fodder and water *ad libitum*.

In vitro experiments on human and rabbit blood

Preparation of extract solutions: Briefly, solutions of following concentrations were prepared by dissolving the extract and fractions in 10% DMSO (V/V). Aqueous methanolic extract: 5, 10, 20%, n-butanol fraction: 2.5, 5, 10% and aqueous fraction: 5, 10, 20%.

Effect on clotting time

The anticoagulant propensity was quantitatively assessed by adopting the procedure of Koffuor and Amoateng, (2011). Shortly, 0.2 mL of each extract solution was taken in respective test tubes except control test tubes. The blood sample was drawn from healthy volunteers (5) and rabbits (5). One milliliter of whole blood was added to the respective test tube and placed immediately in a water bath at 37°C. The time taken for the blood sample to clot was recorded with the help of stop watch as shown in **Video clip**.

Effect on in vitro prothrombin time and activated partial thromboplastin time

Trisodium citrate was added in all centrifuge tubes. 3 mL of blood sample from human volunteers (n=5) and rabbits (n=5) was added to the centrifuge tubes and subjected to centrifugation at 3,000 rpm for 5 min. Plasma was separated with the help of micropipettes and saved in appendorff tubes. 250 uL of each, plant extract and fractions were mixed with 250 uL of plasma in an eppendorff tube. For evaluation of prothrombin time, samples were incubated at 37°C for 5 min then 200 uL of prothrombin time reagent was added to 100 uL of test plasma and clotting time was measured as prothrombin time. For evaluation of activated partial thromboplastin time, 100 uL of activated partial thromboplastin reagent was added to the 100 uL of test plasma (platelet poor plasma + extract) and the mixture was incubated for 1 min after which 100 uL calcium chloride (25 mmol) was added and incubated for 15 sec and clotting time was recorded as activated partial thromboplastin time (Jagtap et al., 2012). **Video clip**

In vitro thrombolytic activity

Thrombolytic activity was carried out according to the method described elsewhere (Prasad et al., 2006). In brief, the venous blood drawn from the healthy volunteer and rabbit was distributed to previously weight eppendorff tube (0.5 mL/tube) and incubated for 45 min at 37°C to form the clot. The serum was completely removed after the clot and weighed the tube again. 100 µL of each test solution of extract was added to each eppendorff tube containing pre-weighed clot. Distilled water (100 µL) was added separately to the numbered tube (control). All the tubes were then incubated at 37° C for 90 min and observed for clot lysis. After incubation, fluid released was aspirated and tubes were again weighed. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis.

% lysis = Weight of released clot/ Weight of clot x 100.

Where, weight of released clot = weight of clot before lysis – weight of clot after lysis

Effect on different coagulation parameters after seven days in vivo treatment

Rabbits were divided into ten groups of 3 rabbits each. Group I served as control. All other groups were treated intraperitoneally with aqueous-methanol extract (50, 100, 200 mg/kg), butanolic fraction (25, 50, 100 mg/kg) and aqueous fraction (50, 100, 200 mg/kg) dissolved in 2% acacia and normal saline respectively. At day 7 blood samples were collected from each animal of all

groups and were used for the determination of prothrombin time, activated partial thromboplastin time and platelet count by following standard methods (Jagtap et al., 2012). In addition to this clotting time and bleeding time was also measured by capillary tube and filter paper method respectively (Elg et al., 2001).

Anti-lipid peroxidation assay (TBARS)

A modified thiobarbituric acid-reactive species (TBARS) assay (Ohkowa et al., 1979) was used to measure the lipid peroxide formed, using egg yolk homogenate as lipid rich medium. Egg homogenate (0.5 mL of 10% v/v) and 0.1 mL of extract were added to a test tube and made up to 1 mL with distilled water. 0.005 mL of FeSO4 (0.07M) was added to induce lipid peroxidation and incubated for 30 min. Then 1.5 mL of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 mL of 0.8% (w/v) Thiobarbituric acidin 1.1% sodium dodecyl sulfate and 0.5 mL 20%, trichloroacetic acid were added and the resulting mixture was vortexed and heated at 95°C for 60 min. 5.0 mL of butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. Inhibition of lipid peroxidation (%) by the extract was calculated according to

(1-E/C) x 100

where, C is the absorbance value of the fully oxidized control and E is the absorbance of sample

Total flavonoids content of butanolic fraction

Total flavonoid content in butanolic fraction was estimated by using the method of Chang et al. (2002) with some modifications. Quercetin was used to obtain the calibration curve. Total flavonoids content in terms of mg QE/gram was calculated by using the curve equation:

Y= mX + b

where, Y= absorbance of sample, m= slope of standard curve, X= mg of qurecetin, b= Y axis intercept

Phenolic profile of butanolic fraction with HPLC-DAD

In short, the sample was prepared by mixing 50 mg of butanolic fraction with 16 mL distilled water and 24 mL of methanol. After mixing 10 mL of 6 M hydrochloric acid was added and kept in oven for 2 hours at 95°C. A 20 µL volume of the filtered sample was injected and chromatographic separation was performed by isocratic elution of the mobile phase at a flow rate of 1.0 mL per min at 30°C. Detection was performed at a wavelength of 280 nm (Sultana et al., 2008).

Fourier transform infraredanalysis of butanolic fraction

1 mg of butanolic fraction was mixed with 2.5 mg of dry potassium bromide and grind in pestle mortar the powder so obtained was filled in 2 mm internal diameter microcup and loaded onto FTIR, and set at 26 ± 1°C. Samples were scanned using infrared in the range of 4000-400 cm^{-1} using FTIR spectrophotometer. Spectral data obtained were compared with reference chart to identify the functional groups present in the butanolic fraction (Naumann, 1991).

Statstical analysis

Data was presented as mean ± SEM and statistical analysis were performed by analysis of variance (ANOVA) by using Graph Padprism version 5.

Results

Effect of extracts on in vitro clotting, prothrombin and activated partial thromboplastin time

Aqueous methanolic extract butanolic and aqueous fractions of *B. calliobotrys* produced the significant ($p<0.05$-0.001) increase in clotting, prothrombin and activated partial thromboplastin time of human as well as rabbit blood. However, the effect of butanolic fraction was more profound (Table I).

In vitro thrombolytic activity on human and rabbit blood

Aqueous methanolic extract of *B. calliobotrys* produced significant ($p<0.001$) and dose dependant increase in lysis of human and rabbit blood clot. Moreover, at higher concentration of 20%, extract showed more pronounced lysis (Figure 1). The butanolic and aqueous fractions did not produce remarkable clot lysis both in human as well as in rabbit blood.

Effect of butanolic fraction on lipid peroxidation

Butanolic fraction caused the inhibition of lipid peroxidation in a dose dependant manner. Different concentrations of 62.5, 125, 250, 500, 1000 ug/mL produced significant ($p<0.001$) decrease in lipid peroxidation (56.3 ± 0.0, 63.5 ± 0.0, 65.8 ± 0.0, 68.3 ± 0.0, 68.6 ± 0.0% LPO respectively) and the results were comparable to the same concentrations of qurecetin with % lipid peroxidation of 64.3 ± 0.0, 69.3 ± 0.0, 70.7 ± 0.0, 74.7 ± 0.0, 74.9 ± 0.0 respectively.

Effect on different coagulation parameters after seven days of treatment

There was significant ($p<0.01$-0.001) escalation in bleeding time, clotting time, prothrombin time and activated partial thromboplastin time in rabbits, treated with different doses of aqueous methanolic extract, butanolic and aqueous fraction when compared to the control at day 7 of study, but the lowest dose of 50 mg/kg of crude extract only prolonged the bleeding time significantly ($p<0.001$). In addition to this profound ($p<0.001$) decrease in the platelet count at all given doses was also observed (Table II).

Table I

Effect of different extracts on *in vitro* clotting, prothrombin and activated partial thromboplastin time

Parameters	Effect of crude extract							
	Human blood				Rabbit blood			
	Control	5%	10%	20%	Control	5%	10%	20%
CT (min)	8.8 ± 0.4	13.6 ± 1.1[a]	18.4 ± 0.6[a]	22.2 ± 1.2[a]	9 ± 0.5	13.4 ± 0.9[a]	16 ± 0.6[a]	20.4 ± 0.5[a]
PT (s)	13 ± 1.2	47.6 ± 1.2[a]	107.3 ± 3.7[a]	170 ± 5.7[a]	11.3 ± 0.9	44.0 ± 2.1 [a]	83.7 ± 3.4[a]	204.6 ± 1.4[a]
APTT (s)	25.3 ± 0.3	49.6 ± 0.3[a]	107.6 ± 7.8[a]	183.3 ± 3.3[a]	28 ± 1.5	61.6 ± 1.6[a]	121.6 ± 1.6[a]	237.6 ± 1.4[a]
Parameters	Effect of butanolic fraction							
	Human blood				Rabbit blood			
	Control	2.5%	5%	10%	Control	2.5%	5%	10%
CT (min)	8 ± 0.0	10.3 ± 0.3[c]	14 ± 0.4[a]	44 ± 1.4[a]	6.8 ± 0.5	9.8 ± 0.3[b]	12.8 ± 0.5[a]	40.5 ± 0.5[a]
PT (s)	13 ± 1.15	49.6 ± 1.3[a]	72.6 ± 1.5[a]	127.6 ± 1.5[a]	11.3 ± 0.9	49 ± 2.1[a]	73.6 ± 0.7[a]	138.3 ± 2.7[a]
APTT (s)	25.3 ± 0.3	44.6 ± 2.6[a]	74.6 ± 0.3[a]	202.3 ± 2.3[a]	28 ± 1.5	43.3 ± 3.4[a]	74 ± 2.1[a]	202.3 ± 1.5[a]
Parameters	Effect of aqueous fraction							
	Human blood				Rabbit blood			
	Control	5%	10%	20%	Control	5%	10%	20%
CT (min)	8.2 ± 0.2	10.8 ± 0.2[a]	12.6 ± 0.2[a]	14.8 ± 0.4[a]	5 ± 0.5	8.4 ± 0.7[a]	11.2 ± 0.7[a]	16.4 ± 0.4[a]
PT (s)	13 ± 1.2	28 ± 0.6[a]	30 ± 0.0[a]	40.3 ± 0.9[a]	11.3 ± 0.9	25.6 ± 0.9[a]	30.3 ± 0.9[a]	39.3 ± 0.7[a]
APTT (s)	25.3 ± 03	35 ± 0.0[a]	43.3 ± 0.3[a]	66.3 ± 0.9[a]	28 ± 1.5	36.3 ± 1.8[a]	40.6 ± 1.3[a]	61.6 ± 1.2[a]

Results are expressed as Mean ± SEM where, [a]p<0.001, [b]p<0.01 and [c]p<0.05; CT=Clotting time, PT= Prothrombin time, APTT= Activated partial thromboplastin time

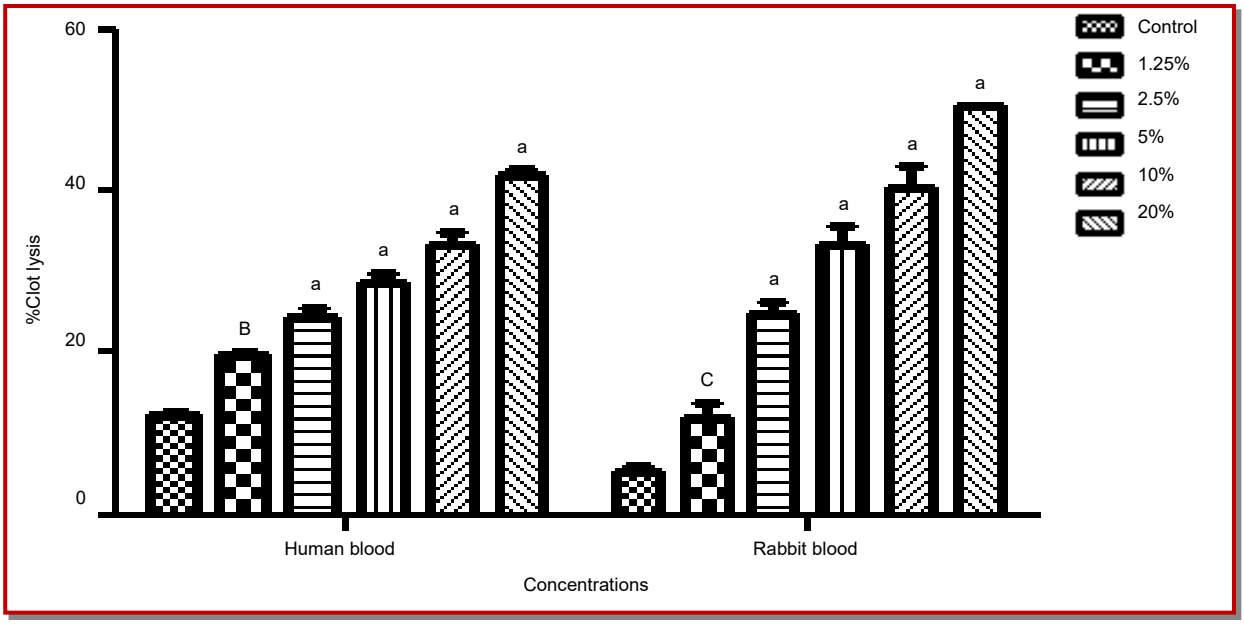

Figure 1: Effect of aqueous methanolic extract on *in vitro* clot lysis of human and rabbit blood. [a]p<0.001, [b]p<0.01 and [c]p<0.05

Table II

Effect of aqueous methanolic extract, butanolic and aqueous fractions on different coagulation parameters after *in vivo* administration

Parameters	Treatment groups (Aqueous methanolic extract)				Treatment groups (Butanolic fraction)				Treatment groups (Aqueous fraction)			
	Control	50 mg/kg	100 mg/kg	200 mg/kg	Control	25 mg/kg	50 mg/kg	100 mg/kg	Control	50 mg/kg	100 mg/kg	200 mg/kg
Bleeding time (s)	33.3 ± 2.4	47.6 ± 1.5^{b}	55 ± 2.9^{a}	66 ± 2.1^{a}	32.3 ± 1.45	47.6 ± 1.5^{b}	55 ± 2.9^{a}	66 ± 2.1^{a}	33.6 ± 2.7	36.3 ± 3.1^{ns}	44.3 ± 0.7^{c}	52.3 ± 1.5^{a}
Clotting time (s)	180 ± 0.0	185 ± 2.9^{ns}	234.3 ± 2.3^{a}	300.6 ± 2.3^{a}	180 ± 0.0	189 ± 0.6^{c}	232.6 ± 2.6^{a}	301 ± 2.0^{a}	180 ± 0.0	191.6 ± 0.8^{a}	197.3 ± 1.2^{a}	212.6 ± 1.5^{a}
PT (s)	11.6 ± 0.7	17.7 ± 1.5^{ns}	20.6 ± 2.7^{b}	26.3 ± 0.3^{a}	12 ± 0.57	14.37 ± 0.7^{c}	17 ± 0.6^{a}	22.3 ± 0.3^{a}	11.6 ± 0.7	14 ± 0.6^{ns}	16 ± 0.6^{b}	18.6 ± 0.9^{a}
APTT (s)	18.6 ± 0.9	30.3 ± 2.4^{ns}	47.3 ± 5.6^{a}	55 ± 2.5^{a}	20 ± 0.57	44.3 ± 2.9^{b}	53.3 ± 3.3^{a}	78.3 ± 4.4^{a}	18.6 ± 0.9	28.6 ± 0.9^{a}	34.6 ± 0.3^{a}	40.6 ± 0.7^{a}
Platelet count ($10^{3}mm^{-3}$)	793 ± 0.0	613 ± 2.0^{a}	525 ± 5.0^{a}	477 ± 1.0^{a}	793 ± 0.0	6.20 ± 0.5^{a}	586 ± 1.0^{a}	473.5 ± 3.5^{a}	793 ± 0.0	747.5 ± 2.5^{a}	729.5 ± 0.5^{a}	715 ± 1.0^{a}

Results are proposed in Mean ± SEM where, ^{ns}=non significant, ^{a}=p<0.001, ^{b}=p<0.01 and ^{c}=p<0.05. PT= Prothrombin time, APTT= Activated partial thromboplastin time, s= seconds

Phytochemical investigations of butanolic fraction

Preliminary qualitative phytochemical screening and determination of total flavonoid contents

The observation and results of initial screening confirmed presence of saponins, flavonoids, tannins, phenolic compounds, steroids, anthraquinones, alkaloids and phlobatanins in butanolic fraction. Butanolic frcation of *B. calliobotrys* is a rich source of total flavonoid contents that is 300 mg QE/g.

FTIR analysis of butanolic fraction

The comparison of FTIR spectrum of butanolic fraction to reference chart revealed the presence of alkane, amine, amide, carboxylic acid and halide functional groups in sample.

Phenolic profile of butanolic fraction with HPLC-DAD

Analysis of phenolic compounds by HPLC-DAD system of butanolic fraction revealed the presence of quercetin, gallic acid, caffic acid, 4-hydroxy-3-methoxy benzoic acid, p-coumeric acid and ferulic acid in butanolic fraction of *B. calliobotrys*.

Discussion

In present appraisal possible effect of *B. calliobotrys* on platelet activity was assessed by measuring bleeding time which is significantly increased after administration of plant extracts simultaneously, significant decrease in platelet count was also observed thus prolongation of bleeding time may be attributed to the observed decrease in platelet count and antilipid peroxidation which leads to the inhibition of platelet aggregation (Akiba et al., 1998). This is in agreement with a previous finding that inhibition of platelet aggregation prolongs the bleeding time (Gadi et al., 2009) as well as in accordance with the findings of De Caterina et al. (1994) who had proposed an inverse relationship between platelet number and bleeding time. Like *B. calliobotrys*, *Exacum bicolor* shows thrombolytic activity (Ashwini et al., 2015).

The present investigation showed significant ($p<0.05$-0.001) delay in clotting time that may be due to the decrease in activity of any of the clotting factor, involved in intrinsic pathway. As blood contains about a dozen clotting factors that can be called into action when tissues or blood vessels are damaged (Alesci et al., 2008). Prothrombin time and activated partial thromboplastin time are hematological index that gives an insight into the coagulation status of suspected coagulopathy patients (Furlanello et al., 2006). Above mentioned results revealed that *B. calliobotrys* produced significant increase in prothrombin and activated partial thromboplastin time as compared to control.

Coumarins are well-known to suppress the extent of coagulation (Pochet et al., 2004). Warfarin is a coumarin that has ability to inhibit vitamin K-depen-dent clotting factors (Rang et al., 2007). Thus, it could be deduced that this plant has inhibitory effects on these factors that may be due to the presence of specific active principles like coumarins. One possibility to explain these effects is that *B. calliobotrys* belongs to the *Berberis* genus and previously the presence of coumarins has been evinced in plants of same genera (Lamichhane et al., 2014).

The present study also signifies the role of aqueous methanolic extract in thrombolysis while aqueous and butanolic fraction shows insignificant effect on lysis of clot. Previously, Hoque et al. (2011) has reported that clot lysis effects are exerted by the richness of alkaloids, flavonoids, tannins and terpenoids. Phytochemical studies showed that butanolic fraction is rich in flavonoids. Still it has not caused clot lysis. Thus, it could be proposed that there are some other active constituents that are responsible for this exerted thrombolytic effect of aqueous methanolic extract. The provoking process of thrombus development is platelet aggregation which is initiated by the deposition of lipid peroxides and thromboxane A2 in platelet (Kubatiev and Andreev, 1979). Butanolic fraction exhibited pronounced antilipid peroxidation effect that might be linked with antithrombotic effect of plant by decreasing the platelet aggregation or by affecting the cyclo-oxygenase pathway of arachidonic acid metabolism as malondialdehyde is one of the end products (Rattan, 1998).

Furthermore the results of total flavonoids contents assay stipulated that very high quantity of flavanoids is present in butanolic fraction i.e 300 mg/g QE which strengthen its antithrombotic potential because previous findings postulated that flavonoids exhibit anticoagulant activity by inhibition of NAD(P)H: quinine acceptor oxidoreductase (Chen et al., 1993), an enzyme inhibited by oral anticoagulants, or by interfering with phosphatidylserine exposure (Bucki et al., 2003). In addition to this, antiplatelet effect of flavonoids has been reported (Pearson et al., 2002). Qurecetin a well known flavonoid blocks the glycoprotein pathway thus, inhibits the response of platelets to collagen (Hubbard et al., 2003). HPLC profile of butanolic fraction in current experiments showed the presence of qurecetin, ferulic acid, p-coumaric acid and caffeic acids, It could be proposed that antithrombotic effect of *B. calliobotrys* is possibly due to the presence of qurecetin and other active constituents, which is in accordance with the results of previous finding of Fuentes et al. (2013) who linked the possible antithrombotic effect of tomato with the presence of chlorogenic, caffeic, ferrulic and p-coumaric acids.

Conclusion

B. calliobotrys may exhibit the antithrombotic potential.

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University of Sargodha

Ethical Issue

The study protocol was approved by the Institutional Animal Ethics Committee, Faculty of Pharmacy, University of Sargodha. All the experiments comply with the declarations of National Research Council (NRC, 1996).

Conflict of Interest

Authors declare no conflict of interest

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Author Info
Alamgeer (Principal contact)
e-mail: alam_yuchi@yahoo.com