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Antiplatelet activity of *Phyllanthus niruri* Linn

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ABSTRACT

Phyllanthus niruri L belongs to family Euphorbiaceae is a field weed. Effect of aqueous and ethanolic extract of *P.niruri* was investigated on *in vitro* human blood platelet aggregation in terms of PT (prothrombin time) and APTT (activated partial thromboplastin time). In PT activity was observed maximum in aqueous extracts of aerial parts at 1000 µgmL^{-1}, which was 28 times higher than the standard value. Among the plant parts, aerial parts gave better activity than roots. APTT activity was higher in aqueous extract of roots at 750 µgmL^{-1} and aerial parts at 1000 µgmL^{-1} that was 10.75 and 9 times higher, respectively over the standard values. The study revealed that *P. niruri* has potent anti-platelet aggregation properties.

Introduction

Blood platelets are involved in haemostasis. The normal haemostatic system limits blood loss by precisely regulated interactions between components of vessel wall, circulating blood platelets and plasma proteins. Platelets can adhere to the walls of the blood vessels, release bioreactive compounds and then aggregate to each other. These properties increase to a well established level in conditions of arterial thrombosis and atherogenesis that may cause life-threatening disorders such as unstable angina, heart attack and reclusions after angioplasty (Guyton, 1991). Therefore, inhibition of platelet aggregation is important in the prevention and treatment of cardiovascular diseases (Jin *et al*, 2007; Lee *et al*, 2006). Several drugs have been developed to block the different steps in platelet activation pathways. Inhibition of platelet function by Aspirin has been very well established (Patrono, 1994). Besides this anticoagulant drugs used like heparins, vitamin K-antagonists and their derivatives have shown deleterious and life-threatening side effects (Stone *et al*, 2007; Bounameaux, 2009). Thus the focus is being laid on potent phyto-origin anticoagulants to reduce the risks of thrombo embolism (Matsubara *et al*, 2001).

Phyllanthus niruri L belongs to family Euphorbiaceae is an annual and field weed that is widespread in temperate and tropical climates (Iizuka et al., 2006). *P. niruri* is a small erect annual herb growing up to 30 - 40 cm in height and indigenous to the Amazon rainforest and other tropical areas, including South East Asia, Southern India and China (Girach et al., 1994). It is popularly used in Asia, Africa and South America (Mellinger et al., 2005) as a stomachic, aperitive, antihyperglycemic, antispasmodic, anti-hepatotoxic, antiviral, antibacterial, laxative, diuretic, carminative, management of diabetes, constipation, fever including malaria, jaundice, hepatitis B, dysentery, gonorrhea, syphilis, tuberculosis, cough, influenza, diarrhea, vaginitis, tumors and dissolving the kidney stones (Syamasundar et al.,1985; Olive-Bever, 1986; Chopra et al., 1986; Unanderet al., 1995; Paranjape, 2001; Lin et al., 2003). Studies on extracts from various parts of the plant have also revealed the antioxidant (Tasaduq et al., 2003) and antimalarial (Cimanga et al., 2004) properties. However, there are no reports on analysis on antiplatelet aggregation activity from this plant.

Materials and Methods

Preparation of Extract

Ethanolic (EtOH 50 %) and aqueous extracts were obtained by macerating 100 g of dried and powdered aerial parts plant (leaves and stem) and roots, separately, kept on a rotary shaker for 24 h. The extract was filtered, centrifuged at 5000 rpm for 15 min and was dried under reduced pressure. The extract diluted to different concentrations and stored at 4ºC in airtight bottles.

Blood samples were collected from SMS Medical College, Jaipur and subjected to centrifugation at 10,000 rpm for 5.5 min. 0.2 ml platelet rich plasma was taken from the sample, dissolved in isotonic $CaCl_2$. Various haemostatic parameters; Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) were measured (Perlick, 1960). Amelung /Sigma Amax CS400 Coagulant Analyzer, New York. USA was used for the experimental work

Determination PT

Principle

The coagulation process gets triggered by incubation of plasma with the optimum amount of thromboplastin and calcium. The time to formation of a fibrin clot is then measured.

Reagents used

- Fresh Standard Human Plasma for determining the reaction time
- PT Reagent (Thromborel)
- Sodium Citrate solution

Procedure

1 part of sodium citrate (0.11 mol L^{-1}) with 9 parts of venous blood were mixed with care and formation of foam was avoided. The blood specimen was centrifuged at 1500rpm for 15 min at room temperature and stored in a capped tube at room temperature for not more than 5 min. Plasma was tested within 24 h of blood collection. 100µl of citrated plasma samples were pipette out in pre sterilized test tube at room temperature and different concentrations of plant extracts (100 to 2000µgmL^{-1})

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were added. Stop watch was used on the coagulation analyzer to determine the coagulation time.

Determination of APTT

Principle

The deficiency of one or the more clotting factors of the intrinsic pathway and also the presence of heparin (coagulation inhibitor) prolongs APTT of the plasma. The time taken for the clot to form is measured and used to determine the anticoagulant status.

Reagents used

- APTT Reagent (Activated Cephaloplastin)
- Calcium Chloride ($CaCl_2$)
- TSC solution (3.2% buffered Tri-Sodium Citrate solution)

Procedure

Fresh plasma was used for the entire tests because it performed best when tested immediately after collection as compared to stored plasma. 9 parts of whole blood from veins was poured in a clean test tube containing 1 part of TSC solution (3.2% buffered tri-sodium citrate solution) and mixed immediately with an anticoagulant to avoid foam formation. Later, it was centrifuged for 15 min at 3000rpm and the plasma was collected in a separate test tubes. Haemolysed, lipaemic turbid samples were avoided. APTT reagent was added gently swirling before use. 100µl each of APTT reagent and 0.025 mol L^{-1} $CaCl_2$ reagent was used as standard, in separate test tubes that was pipette out and incubated on water bath at 37^0C for 3 min.

After 3 min, 100µl of well mixed $CaCl_2$ reagent was added to the 37^0C maintained temperature test tube containing APTT reagent and plasma and simultaneously the stop watch was started. The contents of the tube were mixed back and forth and the stopwatch was put to stop as soon as fibrin strand were visible through high powered lens in coagulation analyzer that initiated gel clot formation. The time taken for clot formation was measured to the nearest 0.1 sec. Thus the APTT value was calculated in seconds. The control was also run in parallel where plant extracts in different concentrations (100 to 2000 µgmL^{-1}) were added replacing the APTT reagent to evaluate the APTT activity of the test samples.

Results

Antiplatelet Activity

Thrombin Time (PT)

All the extracts prolonged the clotting time as compared to control. In general, the activity was higher in aqueous extracts than the 50% EtOH extracts. Significant activity was observed at 100 µgmL^{-1} (19.3 times), which increased slowly up to 500 µgmL^{-1} (26 times) and maximum at 1000 µgmL^{-1} (28 times) as compared to the standard. Both the extracts prolonged the clotting time as compared to standard and control run in parallel (Table 1). The activity was highly significant over both standard and control in all the cases.

Activated Partial Thromboplastin Time (APTT)

All the extracts prolonged the clotting time in terms of APTT as compared to control (+$CaCl_2$) and standard run in parallel (Table 2). The aqueous fractions were found to be more potent than EtOH fraction. The APTT activity was higher in aqueous extract of roots (9 & 10.75 times higher at 500 & 1000 µgmL^{-1}, respectively) than aerial parts and EtOH extracts in comparison to standard and control run parallel.

The aqueous extracts of roots and aerial parts prolonged the clotting time maximum up to 430 and 362 sec (+ 10.75 & 9 fold higher), respectively at 1000 µgmL^{-1} and remained marginally at par on further increase in dose concentration.

Discussion

During the initial stage of thrombosis, damage in blood vessels causes the production of adhesive proteins and soluble agonists at the injury site. This event then stimulates platelet adhesion, activation and aggregation, resulting in the formation of a platelet-rich thrombus (Jackson *et al*, 2003). Activated platelets facilitate thrombin formation by providing a catalytic surface on which coagulation activation can occur. Thrombin not only is responsible for the formation of fibrin but also acts an extreme platelet activator. The growing aggregation of activated platelets is eventually stabilized by cross-linked fibrin and results in the formation of a platelet-rich thrombus (Ross, 1993).

Increased platelet aggregation (PA), as a result of increased platelet sensitivity to agonists *in vivo*, contributes to the initiation and progression of atherosclerosis and occurrence of thrombotic events (Lusis, 2000 & Ross, 1999). Platelet aggregation, which is associated with an increased release of reactive oxidative species (Freedman *et al*, 1999) and platelet-vessel wall interactions (Adams *et al*, 2000) results in damage to the vascular endothelium (Cai & Harrison, 2000).

The atherosclerotic disease process is thought to begin with the impairment of endothelial function (Adams *et al*, 2000) followed by the increased accumulation of oxidized LDL (ox-LDL) within the wall underlying the damaged endothelium. Unregulated uptake of oxi-LDL by tissue macrophages leads to the transformation of these macrophages into foam cells that build up within the arterial wall causing intimal thickening and the formation of fatty streak lesions (Nordestgaard *et al*, 1995). In addition to atherosclerosis the platelets also contribute to the progression of the cardiac disease by releasing various growth and chemotactic factors that accelerate the proliferation and migration of smooth muscle cells (Ross, 1999). Therefore, reducing the activity of platelets could potentially reduce the development as well as progression of CAD.

Hong *et al* (2008) while screening anti-platelet aggregation activities advocated for a new approach, which could be used to analyze and evaluate the binding of drug receptors under a laboratory physiological environment. On the other hand, growing fascination for discoveries of natural anticoagulants stemming up because of overwhelming consumer response seeking remedies devoid of unfavorable side effects (Low, 2008). Therefore, the inhibition of aggregation of platelet formation and anticoagulants using phytoceuticals and nutraceuticals can be promising approach for the prevention of thrombosis.

Pierre *et al* (2005) reported the effect of aqueous extract of several herbs on human platelet aggregation *in vitro*. Out of 28 herbs investigated by them *Camomile*, *Nettle* and *Alfalfa* were found to be more potent and effective in inhibiting platelet aggregation *in vitro*. They concluded that herbs are potential for inhibiting platelet activation and platelet mediated events in CVD.

Manicam *et al* (2010) worked on leaves extract of *Melastoma malabathricum* using water, hot water and MeOH as solvents. The results highlighted the biochemical nature of the active compounds might contribute to anticoagulation. It was evident in their findings that hot water extract gave maximum anticoagulant activity prolonged maximum up to 180 sec, may be due to phytochemicals responsible for the activity,

presumably the active compounds extracted at this temperature were thermally labile.

The extraction of phytoceuticals involves the use of various solvents based on their ability to extract bioactive compounds of different solubility and polarities (Liu, 2003). Cognizant to this aspect, extractions using both water and 50% EtOH extraction were carried out in the present study, was in agreement with the above findings.

In the present investigation extracts of different plant parts of *P. niruri* gave significant antiplatelet activity. Among these two plant parts the aerial parts extract in PT and roots in APTT event exhibited the more potent anticoagulant properties for all parameters tested in the *in vitro* screening, may be due to accumulation of certain bioactives in particular organ.

It was observed that aqueous extracts were more potent than EtOH extract and prolonged clotting about 28.66 times in PT Assay when compared with standard and 9.35 times when compared with APTT Assay. The minimum aqueous treatment doses at 100 μgmL^{-1} was found to be quite significant in PT Assay and a linear increase in APTT Assay was observed with increment of treatment dose up to 1000μgmL^{-1}, which may be due to release of some metabolite with time and alcoholic concentration used, needs further investigations on identification of alleged bioactive/s. Very slow progression in clotting time in seconds was observed in PT assay beyond 1000μgmL^{-1} dose was directly proportional to higher doses indicated that minimum dose was equally efficient.

Presents study highlighted that the anticoagulant activity of *P.niruri* aerial parts and roots aqueous extracts affect the intrinsic pathway of the coagulation cascade by causing clotting factor(s) deficiency. Consequently, *P.niruri* extracts proved a potential herbal-based anticoagulant candidate and demonstrated remarkable activities when subjected to a series of *in vitro* coagulation screening procedures.

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Table – 1

Clotting Time of various extracts of plant parts at different concentrations assayed by prothrombin time (PT) in *P.niruri*

Extracts	Control	Clotting time in seconds PT Assay 100	250	500	750	1000	1500	2000 (μgmL^{-1})	Standard PT (plasma+PT reagent)
A. 50 % EtOH extracts	37	**200**	**228**	**282**	**300**	**305**	**308**	**308**	
	*s	*13.3*	*15.2*	*18.8*	*20*	*20.3*	*20.5*	*20.5*	
Aerial Parts (leaves+stem)	**c	*5.4*	*6.16*	*7.6*	*8.10*	*8.24*	*8.26*	*8.26*	
		180	**240**	**280**	**318**	**320**	**323**	**324**	
Roots	*s	12	16	18.6	21.2	21.3	21.5	21.5	
	**c	4.8	7.89	18.6	8.59	8.60	8.72	8.72	15
B. Aqueous extracts	90	**290**	**320**	**390**	**384**	**420**	**423**	**424**	
	*s	19.3	21.3	26	25.6	28	28.2	28.2	
Aerial parts (leaves+stem)	**c	3.2	3.5	4.3	4.26	4.66	4.70	4.70	
		268	**288**	**310**	**352**	**360**	**361**	**362**	
Roots	*s	17.8	19.2	20.6	23.4	24	24	24.1	
	**c	2.9	3.2	3.4	3.9	4.	4	4.1	

*Standard value for PT [plasma + thromborel (PT reagent); 1:2] -=15 sec ;Control A- (plasma+50 % EtOH)); Control B =(plasma+distilled water); *s times higher than standard;; ** c times higher than control*

Table – 2

Clotting time of various extracts of plant parts at different concentrations assayed by activated partial thromboplastin time (APTT) in *P.niruri*

Extracts	Control	Clotting time in Seconds APTT Assay 100	250	500	750	1000	1500	2000 (μgmL^{-1})	Standard APTT (plasma+APTT reagent + $CaCl_2$)
A 50 % EtOH extracts		**20.1**	**25.5**	**32**	**39.8**	**42.5**	**47**	**47**	
	47	*s 0.50	0.63	0.8	0.99	1.06	1.17	1.17	
Aerial parts (leaves+stem)		**c 0.42	0.54	0.68	0.84	0.90	1.00	1.00	
		26.5	**30.0**	**30.8**	**44.0**	**44.5**	**44.9**	**44.9**	
Roots		*s 0.66	0.75	0.77	1.11	1.1	1.12	1.12	
		**c 0.56	0.63	0.65	0.93	0.94	0.95	0.95	40
B Aqueous extracts									
	70	**280**	**292**	**320**	**343**	**360**	**362**	**362.5**	
Aerial parts (leaves+stem)		*s 7.0	7.3	8.0	8.5	9.0	9.05	9.05	
		**c 4	4.17	4.57	4.9	5.14	5.15	5.15	
		220	**228**	**340**	**389**	**430**	**438.3**	**438.5**	
Roots		*s 5.5	5.7	8.5	9.7	10.75	10.9	10.9	
		**c 3.14	3.25	4.85	5.5	6.14	6.26	6.26	

*Standard value for APTT plasma+activated cephaloplastin (APTT Reagent) +$CaCl_2$; 1:1:1] - 40 sec; Control A- (plasma+50 % EtOH+$CaCl_2$); Control B- (plasma+distill water+$CaCl_2$) *s times higher than standard;; ** c times higher than control*