

**A BIOACTIVE COMPOUND FROM PIPER BETEL WITH ANTICOAGULANT ACTIVITY****J. JESONBABU<sup>1</sup> N. SPANDANA<sup>2</sup> M.SREENIVAS REDDY<sup>3</sup> AND DR. K. ARUNA LAKSHMI<sup>4</sup>**<sup>1,2,4</sup>Department of Biotechnology, GIT, GITAM University, Visakhapatnam-530045, A.P, <sup>3</sup> Sneha Diagnostic Clinical Reference Laboratories, Ongole-523001. Email: jesonmicro@gmail.com*Received: 16 Nov 2011, Revised and Accepted: 25 Jan 2012***ABSTRACT**

An attempt has been made to isolate an anticoagulant compound from the piper betel leaves. In vitro coagulation assays such as activated partial thromboplastin time (APTT), prothrombin (PT) and the studies performed on healthy volunteer blood samples which are mixed with different concentrations of extract (10-1280µg/ml). The results showed that the plasma sample mixed with the plant extract showed the marked prolong in anticoagulation assays. The anticoagulant activity of the compound was assayed by the activated partial thromboplastin time (APTT), prothrombin time (PT), and these assays were compared with the anticoagulant heparin. Therefore, the isolated compound showed its action on the intrinsic and as well as extrinsic pathways of the blood coagulation systems.

**Keywords:** PT, APTT, Thromboembolism.**INTRODUCTION**

The increasing in the incidence of deep venous thrombosis and its closely related pulmonary embolism during the post-operative and post traumatic management of the patients has lead the investigators to examine the aetiology and prevention of the procedure. It was estimated that 2 to 4 individual out of 1000 require anticoagulant therapy every year for the symptomatic deep vein thrombosis and pulmonary embolism<sup>1</sup>. The blood coagulation has been considered as the development of venous thrombosis also known as hypercoagulable state which significantly leads to thrombogenic process<sup>2</sup>. This leads the conclusion that the patient with Acquired or congenital antithrombin deficiency has increasing incidences of venous thrombo-embolism<sup>3</sup>. The administration of anticoagulant reduces thrombosis in post-operative or post traumatic patients as it prevents the formation of thrombus or embolus<sup>4,5</sup>. Other main cause of thrombosis is vessel wall damage due to atherosclerotic lesions. Thus thrombosis of acute atherosclerosis likely contributes to the evolution of atherosclerotic lesions<sup>6</sup>.

From more than 50years heparin a sulphated polysaccharide is the only the anticoagulant used for the thromboembolic processes<sup>7</sup> a part from this activity this compound is employed during extracorporeal circulation, such as major vascular surgery and haemodialysis. Its potentiality is achieved through the major plasma protein inhibiting coagulation enzymes i.e. thrombin and factor Xa or Stuart power factor. As per the natural therapeutic agent heparin will be followed by insulin<sup>8</sup>.

The most of the leading deaths were of disorders in cardiovascular system. Cardiovascular diseases remain the cause of mortality and morbidity, especially in the developed world. But even in developing countries the incidence of cardiovascular diseases is still increasing<sup>9</sup>. Heparin has been used from years as a commercial anticoagulant and recently it was found that there were side effects of heparin such as development of thrombocytopenia, hemorrhagic effect, ineffectiveness in congenital or acquired antithrombin deficiencies, incapacity to inhibit thrombin bound to fibrin and availability of anticoagulants in low concentrations has been arisen the demand for safer anticoagulant therapy<sup>9</sup>.

Blood coagulation pathways were divided into two types one is of intrinsic pathway and extrinsic pathway. Anticoagulation is mainly due to the coagulation serine proteases, thrombin and factor Xa by the activity of serine protease inhibitor antithrombin III<sup>10</sup>.

So in the present study we undertook a systematic analysis of the anticoagulant activity and we made an attempt to test the plant extract of Piper betle and its compound in the routine coagulation

assays. Nature is the best combinatorial chemist and till now natural compounds were discovered from medicinal plants<sup>13</sup>. This study provided useful insights into the modulation actions of the new anticoagulants. Anticoagulant activity in the piper betle plant was not studied and till now there were no reports of the anticoagulant activity of the piper betle.

**MATERIALS AND METHODS**

Healthy matured green leaves of piper betel were collected fresh from its natural habitat. Young shoots and stems were not included in the sampling.

**Preparation of Chloroform extract**

Fresh leaves of P.betel (1kg) were washed under running tap water and shade dried for 2days and the leaves were powdered. Ten grams of the powder was subjected to soxhlet apparatus by using a150ml of chloroform as a solvent for 2days. The plant extracts were filtered through what man No.1 filter paper into vials and stored at 4°C for further use.

**Salivary stimulant extraction**

Leaves of piper betel were washed under tap water, air dried and shade dried for 3 days and they were pulverized to powder. Approximately 10g of the powdered leaves were extracted with one litre of salivary stimulant for 5hours at 100°C under reflux. The hot liquid extract was cooled to the room temperature i.e 25°C prior to filtration in a glass-sintered vacuum filter using what man grade no 1 filter papers to remove the plant debris. The filtrate was then concentrated to dryness in vacuo in a rotary evaporator, later it is freeze-dried and stored at -20°C until further use.

**Extraction of Hydroxychavicol by Column chromatography and Thin layer Chromatography**

The chloroform extract and the salivary stimulant extract was passed through the column chromatography to separate the compound present in it by using the 1% of methanol in chloroform as eluting solvent and the samples collected at different time intervals were subjected to the thin layer chromatography. The thin layer chromatography showed the detection of hydroxychavicol from the chloroform and salivary stimulant extract of piper betel leaves by using methanol and chloroform 1:19 ratio mobile phase and spraying Folin ciocalteu (Phenol) reagent over the silica gel plate for the detection of hydroxychavicol. The fractions containing the pure hydroxychavicol were pooled and the desire compound was crystallized from benzene petroleum ether. And the purity of the hydroxychavicol was estimated by the HPLC and found 98% pure.

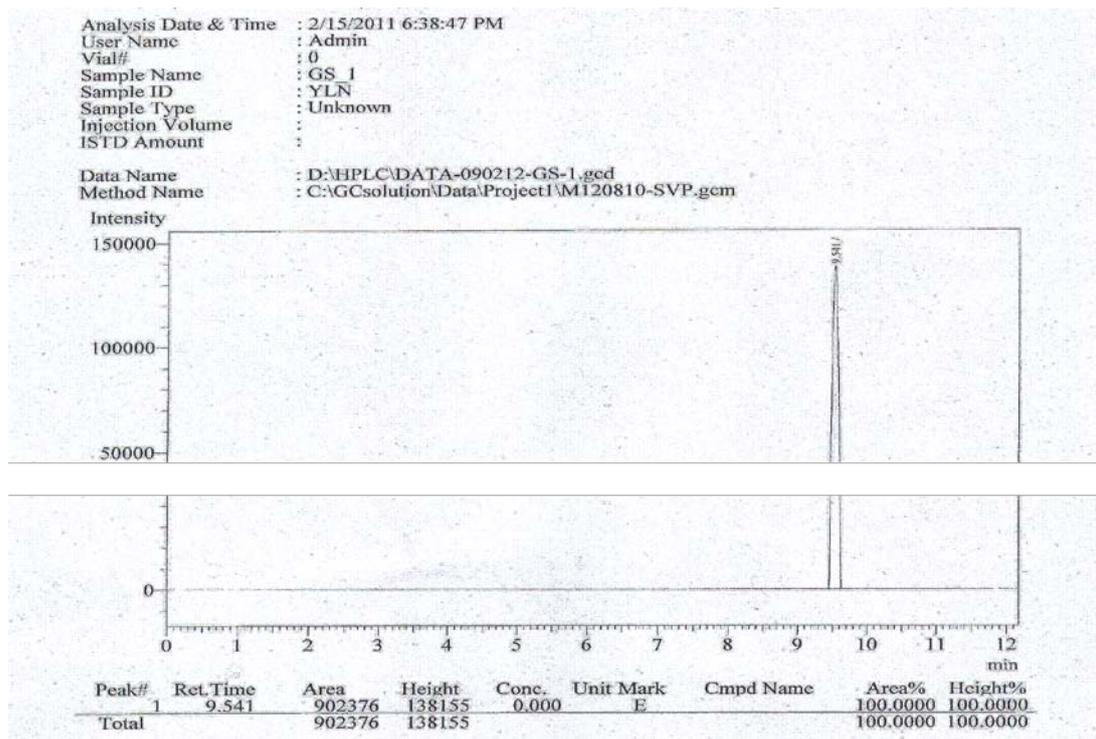


Fig. 1: HPLC studies exhibiting 99.99% purity

As the chloroform extract was not able to mix with the plasma only the salivary stimulant extract was used to detect the anticoagulation activity.

#### Blood sample collection

Blood samples were drawn from healthy volunteer donors (n=25) both genders age between 18- 50 years old after screening the forms for the family history of cardiovascular diseases and other coagulopathies. Blood samples of short listed donors were subjected to routine haematological parameters to exclude the others having abnormal results.

Blood samples were drawn via vein puncture at antecubital fossa of fore arm of donors (n=10). Blood sample mixed with the tri sodium citrate (0.109 M) in ratio 9:1. Later samples were mixed properly and subjected to centrifuge at 2000g for 30min at room temperature (25°C) to obtain platelet poor plasma and plasma was separated from cellular components and stored at -20°C until use.

#### Assay of anticoagulant activity

Activated partial thromboplastin time (APTT), prothrombin time (PT) clotting assays were performed using normal human plasma. The clotting times were recorded in a coagulometer (coag 2 chambers coagulometer). Three separate assays measuring APTT, PT were carried out to investigate at which stage blood clotting pathways were inhibited. The anticoagulant activity was expressed as clotting time or IU/mg using a parallel standard curve based on the Heparin Standard (193 IU/mg).

#### Determination of prothrombin time

Determination of prothrombin time (PT) was done using Uniplastin reagent (Tulip, India) containing ready to use liquid calcified thromboplastin reagent derived from rabbit rain. Thromboplastin in presence of calcium activates the extrinsic pathway of human blood coagulation mechanism. When uniplastin reagent is added to normal citrated plasma, the clotting mechanism is initiated, forming a solid gel clot within a specified period of time. The time required for clot formation would be prolonged if there is acquired or congenital deficiency of factors/factor activity in the extrinsic pathway of the coagulation mechanism or reduction in the activity of vitamin K dependent clotting factors during oral anticoagulant therapy.

Aspirate the reagent vial enough reagent for immediate testing requirements in a thoroughly clean and dry reaction cuvette. Pre warm the reagent and bring to 37°C before use in test procedure (5-10min may be required depending on the reagent volume to attain 37°C before testing). 100ul of plasma should be placed in the reaction cuvette at 37°C for 3-5min. To the cuvette forcibly add 200ul of uniplastin reagent and automatically the coagulometer will read the clot formation begins with in seconds. Each test is repeated 3 times and the mean value is recorded. All experiments were carried out threefold, using Coag 2, 2chambers coagulometer (Helena, Japan)

#### Determination of Activated partial thromboplastin time

Liquicelin-E reagent (Tulip diagnostics, India) is a liquid ready to use activated cephaloplastin reagent for the determination of Activated Partial Thromboplastin Time. It is a phospholipids preparation derived from rabbit brain with ellagic acid as an activator. Cephaloplastin activates the coagulation factors of the intrinsic pathway of the coagulation mechanism in the presence of calcium ions. APTT is prolonged by a deficiency of one or more of these clotting factors of the intrinsic pathway and in the presence of coagulation inhibitors like heparin. Reagent liquicelin-E and Calcium chloride solution should be brought to 37°C. Add 100ul of test plasma and liquicelin-E. Shake briefly to mix the reagent and plasma and incubate it at 37°C for 3 to 5min. Following incubation add forcibly 0.1ml of prewarmed calcium chloride in to the plasma and liquicelin-E mixture simultaneously stop watch will start in coagulometer. Repeat the test for 3times average test values from the mean were recorded.

#### RESULTS

The in vitro anticoagulant activity of Piper betle compound was obtained by the column chromatography was studied. It has been found that the phenolic compound present in the piper betle was responsible for the anticoagulant activity. Blood coagulation system consists of the intrinsic and extrinsic pathways, where the series of the factors plays a vital role in the mechanism. Anticoagulants inactivate or restrict the activity of factors that affect either one or both the pathways. APTT is a measure of intrinsic pathways depending clotting time and PT is the extrinsic pathways depending clotting time. The FT fibrinogen time revealed the fibrin clot forming

time. The anticoagulant activity of the compound was measured by activated partial thromboplastin time test (APTT) prothrombin time test (PT) and fibrinogen time (FT). The human plasma from the healthy donors was used as reservoir of coagulation cascade enzymes.

**Extraction and screening of Piper betel leaf extract for anticoagulant activities**

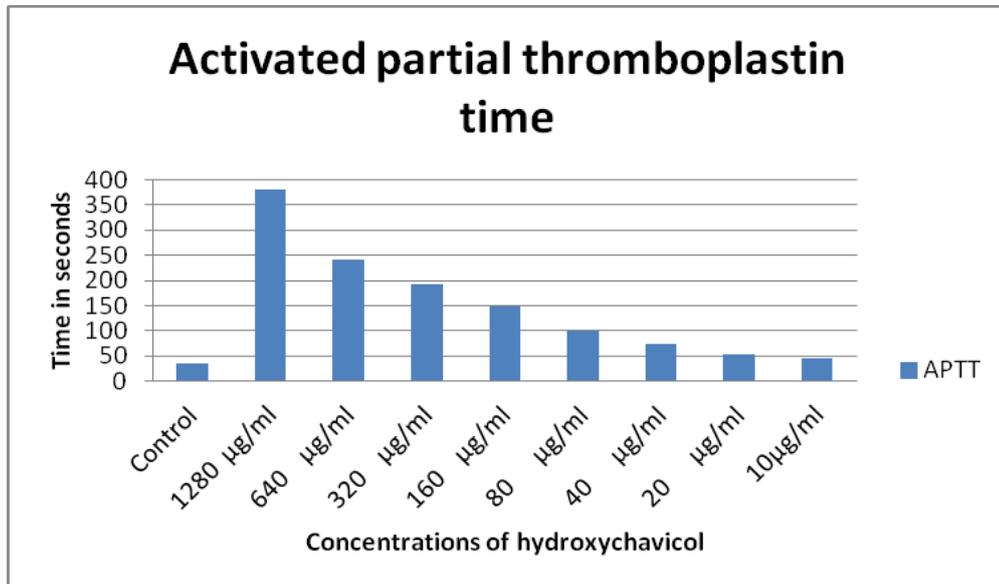
Salivary stimulant was employed to extract the fresh leaves of Piper betel in this study as the chloroform extract was hard to mix with the plasma samples. The blood coagulation assays routinely used in screening procedures consisted of APTT, PT.

In reference to Table 1, all three coagulation parameters were significantly prolonged in plasma with salivary stimulant extract compared to the normal control plasma. The APTT and PT measurements were 380.1 s and 121.6 s, respectively in comparison to the control plasma of 35.2s and 13.1s.

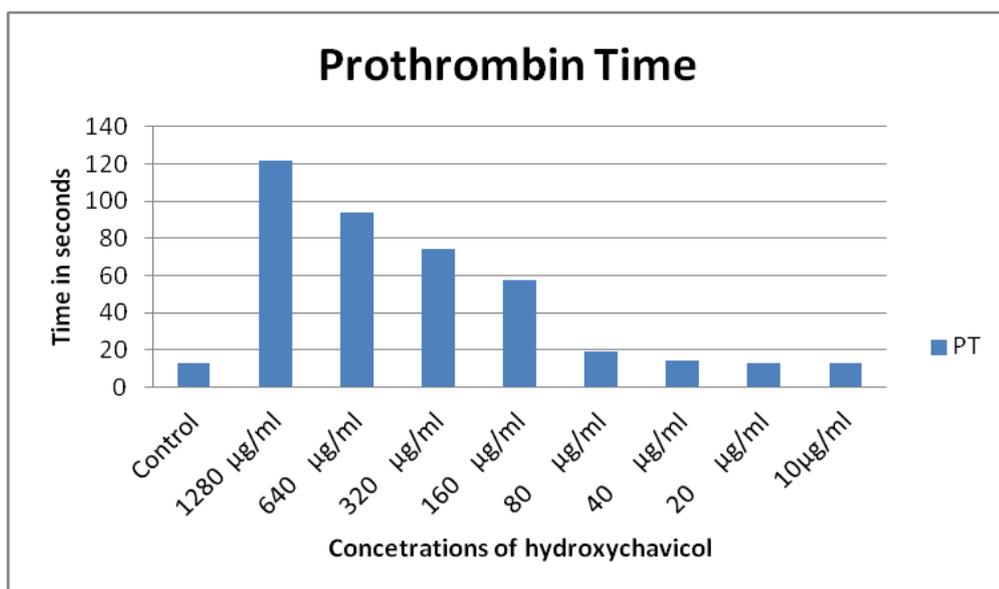
**Table 1: Results of Activated partial thromboplastin time and prothrombin time**

Concentrations	APTT	PT
Control	35.2	13.1
1280 µg/ml	380.1	121.6
640 µg/ml	240.2	94.1
320 µg/ml	193.5	74.2
160 µg/ml	149.2	57.9
80 µg/ml	100.2	19.4
40 µg/ml	73.2	14.7
20 µg/ml	54.5	13.3
10 µg/ml	46.2	13.2

Extract was measured in different concentrations in order to evaluate the biological activity of the compound. The compound showed the complete inhibition of plasma clot formation in vitro experiments i.e. in APTT as well as in PT tests.



**Fig. 3: Activated partial thromboplastin time studies of hydroxychavicol with different concentrations**



**Fig. 4: Prothrombin Time studies of hydroxychavicol with different concentrations**

**DISCUSSION**

It is known that the hydroxychavicol has antibacterial activity<sup>12</sup>. The above test indicates that the piper betel extract possesses the anticoagulant activity. Blood clotting is a complex procedure which involves numerous factors in the plasma and tissues. Both the intrinsic and extrinsic pathways play a vital role. Inhibitors of the blood coagulation affect any factors in the blood. The both PT and APTT test were used to distinguish the effects of test agent between the extrinsic and intrinsic pathways<sup>11</sup>. The above result shows the presence of the anticoagulant agent in the piper betel plant. While examining the PT and APTT test the anticoagulant indicates that it affects both the tests in vitro. At this stage there is no indication of the anticoagulant where it was acting exactly since it affected the PT and APTT in similar manner cases.

**CONCLUSION**

Plants belong to *Piperaceae* families are known to use in folk medicine in India. These plants are also known to be rich in phenols. The aim of the present work was to obtain heparin like compound. Our work was directed for the isolation of the compound from the piper betel by the salivary stimulant. But the anticoagulant activity was not reported earlier except of other plants. The low costs of manufacturing of plant medicines were having a very good advantage of usage the plant materials.

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