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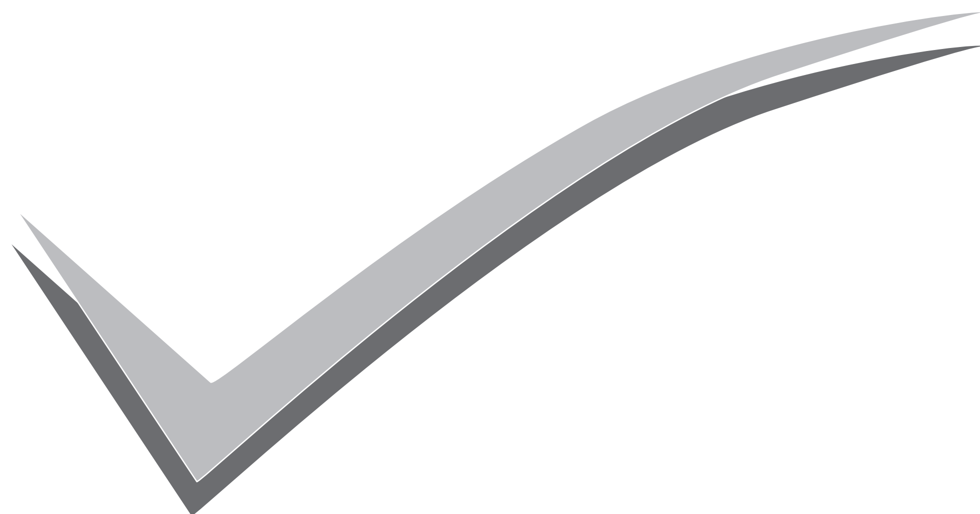

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Sensitive Thromboplastin for PT determination (ISI~1.0)

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INDEX

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3.	International Journal of Pharmacy and Pharmaceutical Sciences Volume 4, Suppl 3, 2012	109-112

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Assessment of PT, APTT, fibrinogen level among Sudanese patients with Systemic lupus erythematosus

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Abstract:

Objective: *To investigate the coagulation status among Sudanese patients with systemic lupus erythematosus by evaluating PT, APTT and fibrinogen level.*

Methods: *A total of 30 patients with SLE were included in the study …18...patients (60 %) had history of arterial and /or venous thrombosis and …12…patients (40 %) did not have such history. Platelet poor plasma from 30 healthy controls were examined. Prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen level were evaluated.*

Result: *Fibrinogen levels were significantly higher in patients with SLE than controls. The PT in patients with SLE (12.0 +/- 1.1%) was not significantly different in comparison with controls (12.1 +/-1.5 Seconds). Whereas APTT was significantly higher in patients than in controls (40.8+/- 5.4seconds).*

Conclusion: *In this study I confirmed the presence of disturbances of coagulation process and more common the developing of thrombosis rather than bleeding in patient with SLE.*

Key words: fibrinogen level, Sudanese patients, Systemic lupus erythematosus

1. INTRODUCTION

SLE is the most common multisystem connective tissue disease it is characterized by a wide variety of clinical features and presence of numerous auto-antibodies, circulating immune complexes and wide spread immunologically determined tissue damage (1, 2). The cause is believed to be environmental trigger which result in a misdirected immune response in people who are genetically susceptible(3).

Hematological abnormalities are common in SLE. All the cellular element of the blood &coagulation pathway can be affected in SLE patient.

The major hematological manifestation of SLE are: anemia, Leucopenia, thrombocytopenia and anti phospholipid syndrome.

Antibodies to a number of clotting factors including: VIII, IX, XI, XII and XIII have been noted in patient With SLE (3,4)

These antibodies may not cause abnormalities of in vitro coagulation test but may cause bleeding.

Much more common are APL (anti phospholipid Ab) the presence of which have been associated with prolongation of APTT and increase risk of arterial and venous thrombosis, thrombocytopenia and fetal loss (5, 6).

Antibody to other phospholipid and to phospholipid binding proteins (e.g. anti cardiolipin antibodies) in moderate or high level, may also be associated with these clinical phenomena. When Apl occur in association with one or more of these clinical features in patient with SLE it suggests the presence of Aps.

JUSTIFICATION & OBJECTIVES:

General objective:

To evaluate PT, APTT and fibrinogen level among Sudanese patients with SLE.

Specific objectives:

1. To assess PT, APTT and fibrinogen level by using coagulometer
2. To detect the disturbance in coagulation process
3. To correlate between this parameters and the clinical course of the disease

MATERIAL & METHODS:

Study population:

The study group comprised 30 Sudanese patients with SLE who were admitted for routine visit to our immunologic department of Omdurman hospital.

The main characteristic are:

1. All patients are female
2. The age (21 -45) years
3. Duration of the disease (3-5) years

None of the patients were receiving oral anticoagulants at the time of entry study.

The control group consisted of 30 age and sex –matched healthy Sudanese blood donor.

(18) Of the patients (60%) had history of thrombosis (deep venous thrombosis, pulmonary embolism and arterial thrombosis.

(2) Of the patients (7%) had a history of bleeding.

(10) Of the patients (33%) had history of recurrent mischarge.

All subjects gave informed consent before entering the study.

Laboratory testing:

All samples were coded and each specific test was performed in the same laboratory throughout the study.

Each session of laboratory tests included either study controls or laboratory control represented by pooled plasma.

Plasma Samples:

In all patients, blood samples were obtained using vacuum tubes containing 0.015ml buffered sodium citrate solution .plasma samples were obtained by centrifugation at 2000 g for 15 min.

*PT, APTT and fibrinogen were performed on the Bio base clot detection system (Asspanian Diagnostic Instrument).

PT:

Evaluated by using uniplastin R (Tulip diagnostics (P) LTD) is anoval, highly sensitive liquid calcified thromboplastin reagent which derived from rabbit brain.

*Normal Range: (11 - 15) seconds

APTT:

Evaluated by using liquicelin-E (Tulip diagnostics (P) LTD) is a liquid ready to use activated cephaloplastin reagent which is phospholipid prepration derived from rabbit brain with allagic acid as activator.

*Normal Range: (22 – 35) seconds

Fibrinogen level:

The evaluation of fibrinogen level was performed with Multi fibrin*U kits(Siemens Health care Diagnostics) which permits a quantitative determination of fibrinogen level in plasma by using the modification of the clauses method .

*Normal Range: 1.8 – 3.5 g/l

Statistical analysis:

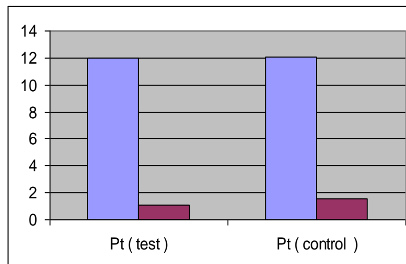
Statistical analysis was performed using Statistical package for social science (SPSS) software. Evaluation of patient's data was performed using the T- test. Result with P value less than 0.05 were considered statistically significant.

RESULTS:

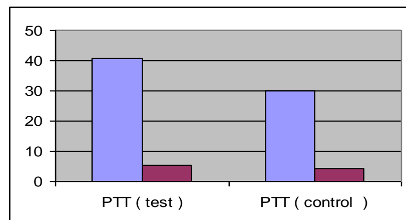
The PT in patient with SLE (12.0 +/- 1.1 %) Seconds was not significantly different in comparison with controls (12.1 +/- 1.5 %)Seconds (P : 0.00) whereas APTT was significantly higher in patients than in controls (40.8 +/-5.4seconds) (30 +/- 4.2%seconds), (P: 0.00).

Level of fibrinogen was significantly higher in patients than in controls (patients (6.0 +/-0.7 g/l), controls (2.9 +/- 0.4 g/l) (P: 0.00).

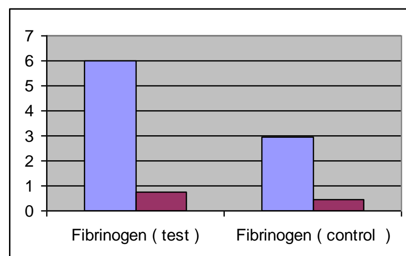
1- PT:
Seconds



2- APTT
Seconds



3- Fibrinogen:
g/l



DISCUSSION:

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown etiology that involves multiple organ systems. Arterial and venous thromboembolism is a well known clinical entity in SLE, with prevalence10% .This prevalence may even exceed 50% in high-risk patients (1, 2).

Coagulation disturbance is common manifestation in patient with SLE and these disturbance either hypercoagulability which clinically lead to thrombosis or hypocoagulability which clinically lead to bleeding that may cause severe life threating(7).

The identification of these risk factor clinically use full to predict the occurrence of thrombosis and bleeding (7).

In this study I was examined the presence of disturbance in coagulation process in patient with SLE with or without a history of bleeding or thrombosis.

I documented increased level of fibrinogen which is a marker of hypercoagulability. Patients (6.0+/- 0.4 g/l), control (2.9 +/- 0.4 g/l) (P : 0.00) .

Also I found prolongation of APTT which more common associated with anti phospholipid antibodies.

Patients (40.8+/- 5.4 seconds), controls (30+/- 4.2 % seconds) (P : 0.00).

The PT is not significantly increase in comparison with control.

Patients (12 +/- 1.1 %) seconds. Control (12.1 +/- 1.5 %) seconds. (P: 0.000).

This finding agreement with other previous study that obtained by a group of researcher (Anotetonella et all)

In American College of Rheumatology studied the thrombotic tendency in57 patient with SLE and also other previous study done by (Nahid et al) in Saudi Arabia, Studied the coagulation abnormalities in SLE patient.

So patient with SLE require good follow up for potential coagulation disturbances to avoid the risk factors of these disturbances complication.

CONCLUSIONS

This study confirm the presence of coagulation disturbance in patient with SLE by Assessing PT, APTT and fibrinogen level which more commonly developing of thrombosis, this hyper coagulability state associated with the presence of APL which is one of major factor responsible for thrombosis in patient with SLE , the risk of developing a thrombotic event in APL positive patients is likely to be enhanced by the presence of certain procoagulant alteration (7).

Acknowledgment
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ORIGINAL ARTICLE

ESTIMATION OF PROTHROMBIN TIME IN PREGNANCY COMPARED WITH NORMAL CONTROLS

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ABSTRACT: BACKGROUND: Normal pregnancy is associated with substantial changes in the tissue factor pathway and in the wider haemostatic system [1]. It is also characterized by impressive changes in the activating and inhibitory pathways of coagulation and fibrinolysis resulting in an accelerated, but well balanced, process of thrombin formation and resolution. These changes serve to protect the mother from the hazard of bleeding imposed by placentation and delivery, but they also carry the risk of an exaggerated response, localized or generalized, to coagulant stimuli [2]. Hemorrhage occupies an important position in the etiology of maternal mortality and therefore, remains a major problem [3]. To what extent normal pregnancy affects coagulation is not well known in our locality. Thus, our study aims to find out the changes that occur in the coagulation parameters in pregnancy as compared to that in normal controls.

METHODS: The study population included 25 healthy pregnant women, who visited the antenatal clinic of the KIMS Hospital in Narketpally, Nalgonda AP, between October 2008 and May 2009. 25 healthy age-matched non-pregnant women served as controls for the study. Both subjects and controls were randomly chosen from general population of pregnant women. Prothrombin Time test was done by using reagents bought from "UNIPLASTIN", TULIP DIAGNOSTICS (P) LTD. 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. **RESULTS**: The results showed that the control group has a mean Prothrombin Time of 9.16 sec with SD of ±1.10 where as the test group showed a mean Prothrombin Time of 14.32 Sec and SD of ±1.91, P <0.001 by the chi-square test is significant. Whereas the International Normalized Ratio INR for control group was 1.26 Sec and SD of ±0.15 Sec and the test group had INR of 1.67 Sec and SD of ±0.26 Sec, the p value is <0.96 by chi-square test is insignificant. The odds ratio for Prothrombin Time was 1.73 which indicates that the Prothrombin Time was significantly decreased in the test group. **CONCLUSION**: The fact that Prothrombin time is decreased in normal pregnancy should be interpreted with caution, and that INR is the better indicator of the coagulation profile of the individual, therefore Prothrombin Time should always be read with INR ratio.

KEY WORDS: PT - Prothrombin Time, **INR-** International Normalized Ratio

ORIGINAL ARTICLE

INTRODUCTION: Haemostasis in normal pregnancy involves a complex network of interactions with positive and negative feedback loops, integrating blood vessels; platelets, coagulation factors, coagulation inhibitors and fibrinolysis and has evolved to maintain the integrity of the vasculature. Normal pregnancy is associated with substantial changes in the tissue factor pathway and in the wider haemostatic system [1]. Normal pregnancy is a characterized by impressive changes in the activating and inhibitory pathways of coagulation and fibrinolysis resulting in an accelerated, but well balanced, process of thrombin formation and resolution. These changes serve to protect the mother from the hazard of bleeding imposed by placentation and delivery, but they also carry the risk of an exaggerated response, localized or generalized, to coagulant stimuli [2]. Hemorrhage occupies an important position in the etiology of maternal mortality and therefore, remains a major problem [3]. There is activation of blood coagulation and a simultaneous increase in fibrinolysis without signs of organ dysfunction during normal pregnancy. These changes increase as pregnancy progresses. During delivery there is consumption of platelets and blood coagulation factors including fibrinogen [4].

Pregnancy is a risk factor for venous thrombosis and the incidence of venous thromboembolism during normal pregnancy is 6-fold higher than in the general female population of child bearing age. Venous thromboembolism is an important cause of maternal morbidity and mortality [5].

The coagulation cascade is in an activated state in pregnancy. Activation includes increased concentrations of all clotting factors, except factors XI, XIII, with increased levels of High molecular weight fibrinogen complexes.

Changes in the haemostatic mechanism also involve decreased levels of anticoagulant proteins such as protein C and Protein S as well as enhanced thrombin generation and decreased fibrinolytic activity [6].

AIMS: To what extent normal pregnancy affects coagulation is not well known. A study like this is therefore necessary to assess the influence of normal uncomplicated pregnancy on Prothrombin time and also to know, the extent of relationship between pregnancy and Prothrombin time.

1. To study changes in Prothrombin time in normal pregnancy.
2. To study the Prothrombin time in normal age matched controls
3. To assess the changes in Prothrombin time between normal and pregnant females.

MATERIALS AND METHODS: SUBJECTS: The study population included 25 healthy pregnant women in third trimester, who visited the antenatal clinic of the KIMS Hospital in Narketpally, Nalgonda from October 2008 to May 2009.

25 healthy age-matched non-pregnant women served as controls for the study.
Both subjects and controls were randomly chosen from general population of pregnant women.

The study was approved by college ethics committee and all subjects gave written consent to participate in the study voluntarily.

INCLUSION CRITERIA:
Study is subdivided into two groups

1. **Controls**

a) Healthy females age above 20 and below 35 years
b) No history of significant medical illness.
c) Individuals who agreed to participate for the study voluntarily.

2. **Test group (pregnant).**

a) Healthy females age above 20 and below 35 years
b) 25 healthy pregnant women in third trimester were taken as study group.
c) No history of significant gynecological problems.

EXCLUDING CRITERIA:

i. Subjects < 20 years and > 35 years
ii. Cardiovascular diseases
iii. Renal diseases
iv. Liver diseases,
v. Endocrinal disorders

MATERIALS: Prothrombin time test was done by using reagents bought from "UNIPLASTIN", TULIP DIAGNOSTICS (P) LTD.

UNIPLASTIN is a novel, highly sensitive, low opacity, ready to use liquid Calcified Thromboplastin Reagent, which is derived from rabbit brain.

PRINCIPLE: Tissue thromboplastin in the 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.

SAMPLE COLLECTION: Patients were advised against vigorous exercises before sample collection, fasting (or after light fatty meals) was collected.

PROCEDURE:

- 1.8 ml of venous blood was collected under sterile conditions and immediately added to 0.2ml of 3.2% tri sodium citrate (anticoagulant).
- Centrifuge immediately for 15min at 1500-2000rpm and transfer the plasma into a clean test tube.
- It should be ensured that the plasma is free from platelets (PPP). Cap the test tubes to prevent deterioration of samples. Plasma must be tested preferably immediately.
- Bring the reagent vial to room temperature (20-30°C). Mix the contents of the vial to homogenize the suspension completely.
- Aspirate from the reagent vial enough reagents for immediate testing requirements in a thoroughly clean and dry test tube.
- Pre-warm the reagent and bring to 37°C before use in test procedure
- Recap the reagent vial and replace immediately to 2-8°C.
- To a 12 x75mm tube add 0.1 ml of plasma and place the tube in a water bath for 3-5minutes at 37°C.
- To the tube forcibly add 0.2ml of UNIPLASTIN reagent and simultaneously start a stopwatch. Shake the tube gently to mix contents.

- Gently tilt the tube back and forth and stop the stopwatch as soon as the first fibrin strand is visible and the gel/clot formation begins. Record the time in 'seconds'.
- Repeat steps above for a duplicate test on the same sample.
- Find the average of the duplicate test values. This is the Prothrombin Time (PT).

CALCULATION OF RESULTS

Manual Method

The results may be reported directly in terms of the mean of the double determination of PT of the test plasma in 'seconds'. Or as a ratio 'R':

$$R = \frac{\text{Mean of the patient plasma PT in seconds}}{\text{MNPT for the reagent}}$$

Or as International Normalized Ratio (INR), **INR = (R)** ^{ISI}, where ISI = International Sensitivity Index of the reagent

It is recommended by the WHO that MNPT should be established for each lot of PT reagents by each laboratory, since PT results are dependent on the combination of reagent lot, instrument and technique followed at each laboratory. Usually plasma from at least 20 normal healthy individuals should be used to establish the MNPT. The average of such PT results in seconds = MNPT.

EXPECTED VALUES

Normal values using UNIPLASTIN® are between 11-15 seconds.

RESULT PT: The results showed that the control group has a mean Pro thrombin Time of 9.16 sec with SD of ±1.10 where as the test group showed a mean Pro thrombin Time of 14.32 Sec and SD of ±1.91, P <0.001 by the chi-square test which is significant. Whereas the International Normalized Ratio INR for control group was 1.26 Sec and SD of ±0.15 Sec and the test group had INR of 1.67 Sec and SD of ±0.26 Sec, the p value is <0.96 by chi-square test is insignificant. The odds ratio for Pro Thrombin Time was 1.73 which indicates that the Pro Thrombin Time was significantly increased in the test group.

DISCUSSION: In the present study there is decreased Prothrombin time during normal pregnancy when compared with age matched control groups of non pregnancies. This decreased Prothrombin time may be because of change in haemostatic balance in the direction of Hypercoagulability in which increase concentration of all clotting factors except XI, XIII.

One similar done by Nihon Sanka et al; showed that in the third trimester, shortening of prothrombin time and activated partial thromboplastin time [11]. Hellgren M reported Increased endogenous thrombin generation, acquired activated protein C resistance and increased prothrombin complex level (PT) measured as international normalized ratio (INR) of less than 0.9 have been reported as well [4].

One similar study in which they also measured Prothrombin fragments, by Cerneca F et al has shown that the parameters showing the greatest variation during pregnancy were PT, FBG, PS, Prothrombin fragments F1+2. The existence of a hypercoagulable state in pregnancy was suggested by the increased levels of F1+2 [12].

According to a previous study prolonged Prothrombin time shows fetal weight gain from the 2nd & 3rd trimesters [1].

Lloyd R et al; showed that Prothrombin time was also decreased in pregnancy and Pregnancy was associated with a significant increase in the activity of factors VII, VIII, IX, and X and in the concentrations of fibrinogen, ⍺-1-globulin, and ⍺-1-antitrypsin [8]. Yet another study showed that term pregnant women were in a hypercoagulable state and had increased fibrinolysis [13].

In one study by Hui C, Lili M et al; the TEG Prothrombin time, activated partial thromboplastin time, thrombin time, international normalized ratio, and thrombomodulin and resistance index in uterine arteries showed a tendency to decrease in pregnant women [14].

Some comparative similar studies also shown, Prothrombin fragments are increased and Prothrombin time is decreased in pregnancy [7-10].

One study by Jørgensen M, Klajnbard A et al; has shown that Prothrombin time remains unchanged in pregnancy, which was contrary to our findings they also showed that the level of coagulation factors II, V, X, XI, XII and antithrombin, protein C largely remained unchanged [15].

The INR was not affected significantly in this study. The INR was introduced in the early 1980s when it turned out that there was a large degree of variation between the various Prothrombin time assays, a discrepancy mainly due to problems with the purity of the thromboplastin (tissue factor) concentrate. The INR became widely accepted worldwide, especially after endorsement by the World Health Organization. Since INR did not show any significant increase in the test group there is no any significant bleeding possibilities in the normal pregnancy.

SUMMARY AND CONCLUSION: Within the limitation of our study it is concluded that Prothrombin time is decreased in normal pregnancy but it should be interpreted with caution, and that INR is the better indicator of the coagulation profile of the individual, therefore Prothrombin Time should always be read with INR ratio.

The INR is a method of expressing the results of a Prothrombin time blood test. It is based on an international standard that automatically corrects for variations between labs.

Thus, using the INR, the PT measurement from one lab can be compared to a PT measurement from any other lab in the world, even if they use different methods to measure PT. the resulting measurement is often referred to as the INR/PT.

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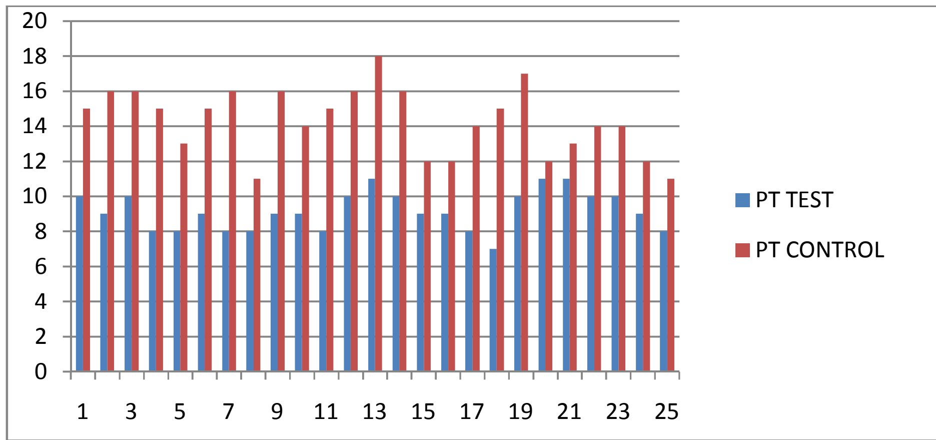
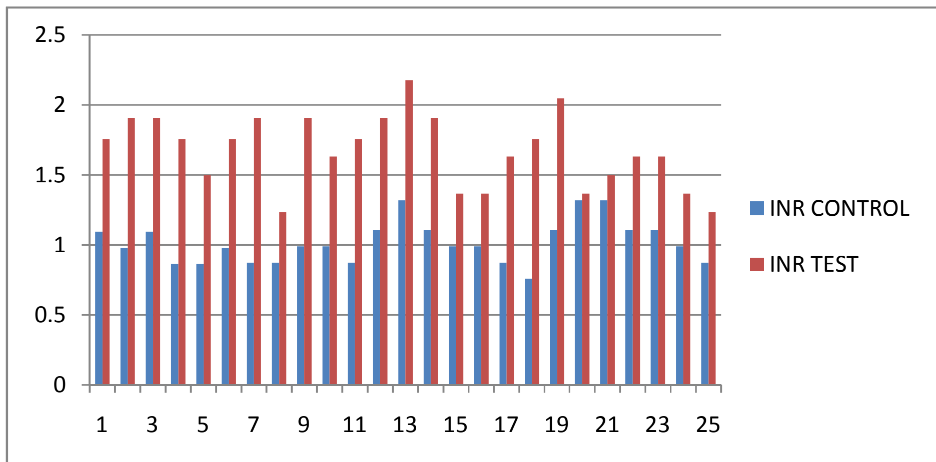
RESULT PT:

	CONTROL GROUP	TEST GROUP	t-VALUE	P-VALUE	RESULT
PROTHROMBIN TIME	9.16 ± 1.10	14.32 ± 1.91	2.05	<0.001	SIGNIFICANT
INTERNATIONAL NORMALIZED RATIO(INR)	1.26 ± 0.150	1.67 ± 0.26	9.55	<0.965	INSIGNFICANT





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Research Article

A BIOACTIVE COMPOUND FROM PIPER BETEL WITH ANTICOAGULANT ACTIVITY

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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[1]. The blood coagulation has been considered as the development of venous thrombosis also known as hypercoagulable state which significantly leads to thrombogenic process[2]. This leads the conclusion that the patient with Acquired or congenital antithrombin deficiency has increasing incidences of venous thrombo-embolism[3]. The administration of anticoagulant reduces thrombosis in post-operative or post traumatic patients as it prevents the formation of thrombus or embolus [4,5]. 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[6].

From more than 50years heparin a sulphated polysaccharide is the only the anticoagulant used for the thromboembolic processes[7]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[8].

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[6]. Heparin has been used from years as an 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 theraphy[9].

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[10].

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[13]. 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^{0}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.

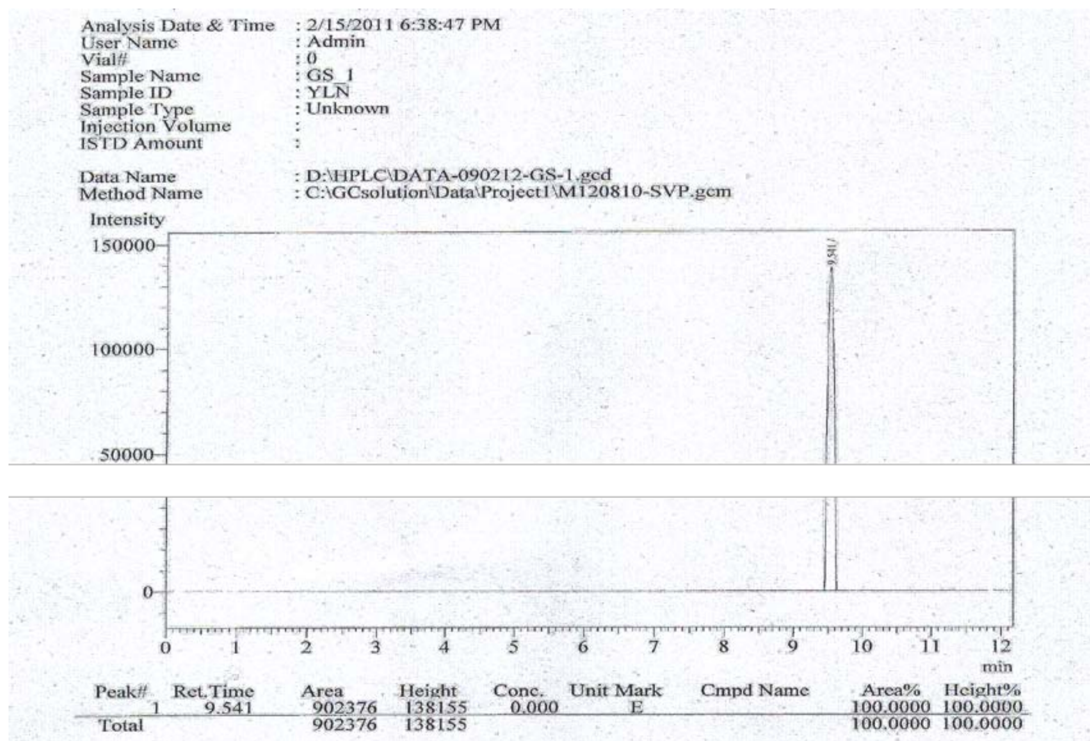

Fig. 1: HPLC studies exhibiting 99.99% purity

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 anticubital 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 2chambers 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 ore 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 throboplastin 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 thromboplatin 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.

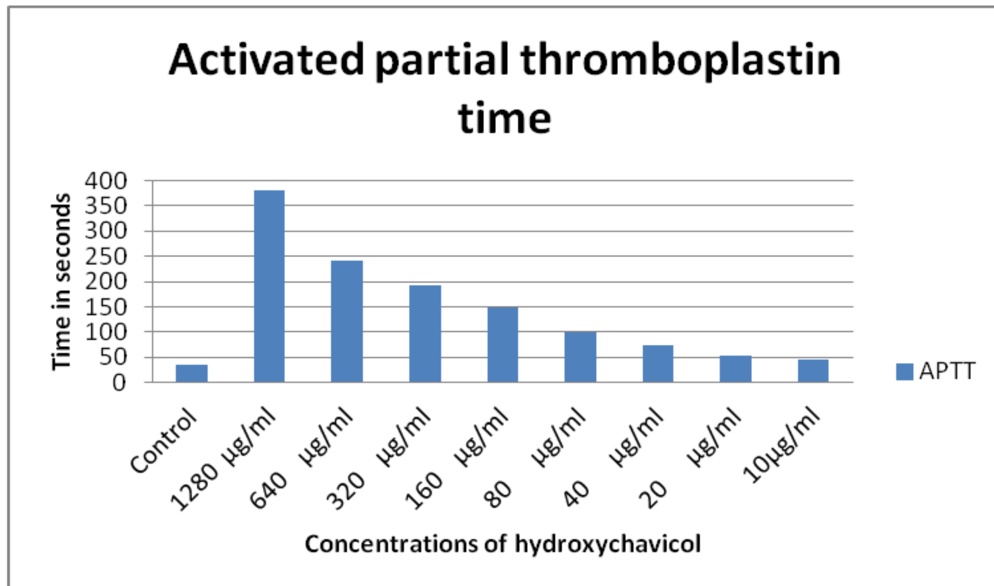

Fig. 3: Activated partial thromboplastin time studies of hydroxychavicol with different concentrations

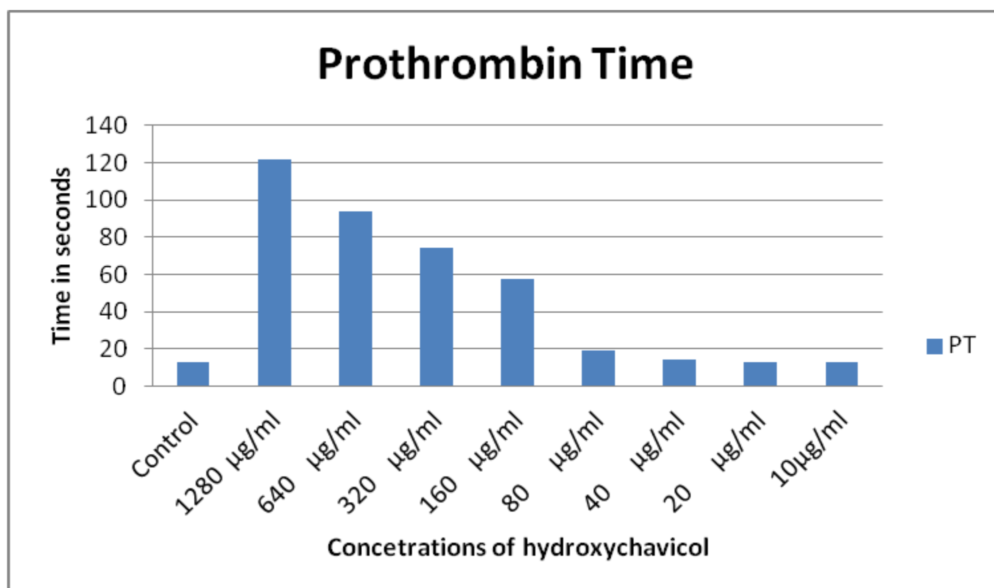

Fig. 4: Prothrombin Time studies of hyodroxychavicol with different concentrations

DISCUSSION

It is known that the hydroxychavicol has antibacterial activity[12]. The above test indicates that the piper betel extract posses the anticoagulant activity. Blood clotting is a complex procedure which involved 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[11]. 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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