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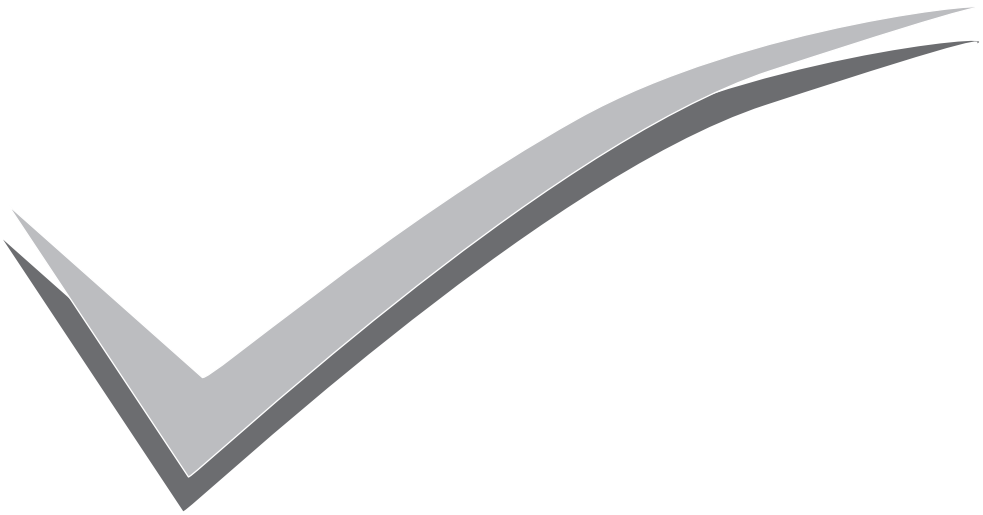

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A pilot study of the association of pharmacokinetic and pharmacodynamic parameters of warfarin with the dose in patients on long-term anticoagulation

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Warfarin is a widely used anticoagulant with a low therapeutic index.
- There is wide interindividual variation in the pharmacokinetics and pharmacodynamics of warfarin which is also reflected in the warfarin dose requirement.
- CYP2C9 and VKORC1 polymorphisms have been shown to affect warfarin dose requirement. However a large amount of the variation in warfarin dose remains unaccounted for.

WHAT THIS STUDY ADDS

- Our findings suggest that in patients who are on long-term warfarin therapy, INR : plasma 7-hydroxywarfarin concentration correlates well with warfarin requirement and also accounts for a large amount of variation in warfarin dose.

AIMS

To assess the correlation between plasma total warfarin concentration, plasma 7-hydroxywarfarin concentration and INR and the weekly doses of warfarin in patients on long-term anticoagulation.

METHODS

Twenty-five patients on long-term anticoagulation with warfarin were studied. Plasma total warfarin and 7-hydroxywarfarin concentrations and INR were determined. Equations were derived with the weekly warfarin dose as the dependent variable and plasma total warfarin concentration : plasma 7-hydroxywarfarin concentration, INR : plasma total warfarin concentration and INR : plasma 7-hydroxywarfarin concentration as independent variables.

RESULTS

There was a good correlation between INR : plasma total warfarin concentration and the weekly dose of warfarin ($y = 46.73e^{-0.30x}$, $r^2 = 0.65$). There was a better correlation between INR : plasma 7-hydroxywarfarin concentration and the weekly dose of warfarin ($y = 156.52x^{-0.63}$, $r^2 = 0.74$)

CONCLUSIONS

Pharmacokinetic parameters along with INR seem to correlate with the weekly doses of warfarin in patients on long-term anticoagulation. These parameters may therefore be useful for predicting warfarin doses.

Introduction

Warfarin is a widely used anticoagulant with a low therapeutic index. There is wide interindividual variation in the pharmacokinetics and pharmacodynamics of warfarin. Polymorphisms of CYP2C9 and VKORC1 have been shown to affect warfarin dose requirement. Previous studies have evaluated the utility of genotyping for CYP2C9 and VKORC1 in predicting warfarin dose in patients on long-term anticoagulation, but have found that these genotypes account for only about 60% of variation in dose [1]. Hence genotyping for CYP2C9 and VKORC1 is not used currently for optimizing warfarin therapy. Many factors other than CYP2C9 and VKORC1 polymorphisms also affect warfarin requirement including polymorphisms in other genes, concomitant drug intake, ethnicity and vitamin K content of the diet [2]. Therefore, models for prediction of warfarin doses using parameters other than gene polymorphisms are necessary. Hence the present study was undertaken to assess the correlation between plasma total warfarin concentration, plasma 7-hydroxywarfarin concentration and INR and the weekly dose of warfarin in patients on long-term anticoagulation.

Methods

Twenty-five patients on long-term warfarin therapy were studied as per a protocol approved by the Institutional Ethics Committee, after obtaining written informed consent. At screening, two INR measurements 15 days apart were done. If these were within the therapeutic range without any change in the dose, then the patient was eligible for recruitment to the study. As per the treatment protocol followed in the hospital, the target range for INR was between 2 and 3 for all indications except for patients with prosthetic mitral valves where the range was 2.5–3.5. Venous blood (7 ml) was collected from all patients 12 h after the last dose of warfarin. This sample was used to perform a third INR as well as estimate the concentration of total warfarin and 7-hydroxywarfarin by high performance liquid chromatography (HPLC) using a C-18 column. Briefly, isopropanol with phosphate buffer was the mobile phase, flow rate was 1 ml min^{-1}, pressure range up to 5000 psi, and wavelength (λ) of the UV lamp was as 308 nm at 30°C. The standard concentrations for the drug and metabolite were 0.05, 0.1, 0.5, 1.0, 2.5 and 5 μg ml^{-1}. The assay was linear in the range of 0.05–5.0 μg ml^{-1}. The inter-day coefficient of variation was between 8.75% and 12.85%. The intraday coefficient of variation was between 4.23% and 7.14%. Carbamazepine was used as an internal standard. PT-INR (prothrombin time-International Normalized Ratio) was determined using the Liquiplastin reagent obtained from Tulip Diagnostics (P) Ltd, Goa, India. Equations were derived with the weekly warfarin dose as the dependent variable and plasma total warfarin concentration : plasma 7-hydroxywarfarin concentration, INR : plasma total warfarin concentration and INR : plasma 7-hydroxywarfarin concentration as independent variables. The third INR value was used for deriving the equations.

Results

The mean age of the patients (18 males and seven females) was 37.28 ± 10.23 (SD) years (range 22–56 years). The indication for warfarin therapy was mitral valve replacement ($n = 8$), deep vein thrombosis ($n = 6$), aortic valve replacement ($n = 3$), aortic and mitral valve replacement ($n = 3$), atrial fibrillation ($n = 2$), venous sinus thrombosis ($n = 2$) and arterial embolism ($n = 1$). The mean weekly dose of warfarin was 34.58 ± 14.76 (SD) mg (range 7–70 mg $week^{-1}$). The mean INR at recruitment was 2.20 ± 0.67 (SD) (range 1.3–3.7). The mean total warfarin concentration was 3.01 ± 2.48 (SD) μg ml^{-1}. The mean 7-hydroxy warfarin concentration was 0.20 ± 0.13 (SD) μg ml^{-1}.

There was an extremely poor correlation between the plasma total warfarin : plasma 7-hydroxywarfarin ratio and the weekly dose of warfarin ($y = 26.67e^{0.01x}$, $r^2 = 0.02$). There was a better correlation between INR : plasma total warfarin concentration and the weekly dose of warfarin ($y = 46.73e^{-0.30x}$, $r^2 = 0.65$) (Figure 1). There was a good correlation between INR : plasma 7-hydroxywarfarin concentration and the weekly dose of warfarin ($y = 156.52x^{-0.63}$, $r^2 = 0.74$) (Figure 2).

The concomitant drugs taken by patients during the study were furosemide (6), diltiazem (3), digoxin (3), KCl (3), aspirin (2), salbutamol (1), enalapril (1), pentoxyphylline (1), vitamin B complex (1), amlodipine (1), phenytoin (1), benzathine penicillin (1), folic acid (1), spironolactone (1) and chlorpromazine (1). In the patient receiving phenytoin, the

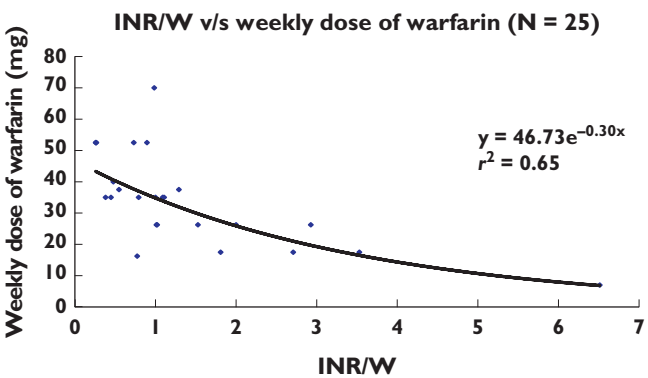

Figure 1

INR : plasma total warfarin concentration *vs.* weekly dose of warfarin ($n = 25$). INR = International Normalized Ratio; W = Plasma warfarin concentration (μg ml^{-1})

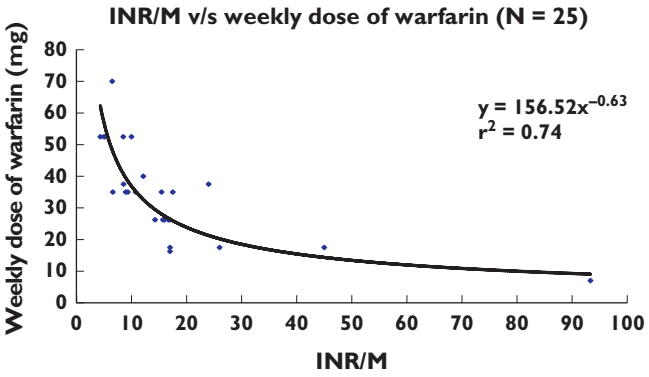

Figure 2

INR : plasma total 7-hydroxywarfarin concentration *vs.* weekly dose of warfarin ($n = 25$). INR = International Normalized Ratio; M = Plasma 7-hydroxywarfarin concentration (μg ml^{-1})

7-hydroxywarfarin concentration was 0.2 μg ml^{-1} (this was comparable with concentrations seen in other patients), the plasma total warfarin concentration was 6.4 μg ml^{-1} and the weekly warfarin dose was 52.5 mg.

Discussion

The ratio of the plasma concentration of a drug to that of its metabolite, also known as the metabolic ratio, has been used to quantify metabolism by the hepatic cytochrome enzyme system and thus delineate activity of various CYP isoforms. Several probe drugs, i.e. those that undergo metabolism predominantly by a single pathway have been used to determine the metabolic ratio and classify populations into poor and rapid metabolizers. These include omeprazole (CYP2C19), dextromethorphan (CYP2D6) and flurbiprofen (CYP2C9). Warfarin, too, is metabolized to its major metabolite 7-hydroxywarfarin by CYP2C9. Therefore the ratio of plasma warfarin and plasma 7-hydroxywarfarin concentration should provide an index of the rate of metabolism of warfarin. It would not only take into account polymorphism in the CYP2C9 genotype, but will also account for acquired factors affecting warfarin metabolism like liver function and concomitant use of drugs competing for the same metabolic pathway. However, we found an extremely poor correlation between the plasma total warfarin : 7-hydroxywarfarin concentration and the warfarin dose required to achieve adequate anticoagulation.

This is not surprising, because plasma total warfarin concentrations alone will not have a predictable anticoagulant effect in every individual. Large differences in its pharmacodynamic effect may occur due to variables like VKORC1 haplotype, concomitant drugs and the dietary content of vitamin K. We therefore postulated the ratio of INR : plasma total warfarin concentration as a pharmacodynamic index of warfarin and found that the warfarin dose correlated well with the INR : plasma total warfarin ratio ($y = 46.73e^{-0.30x}$, $r^2 = 0.65$).

The dose of warfarin required to achieve adequate anticoagulation would be lower in patients with a higher metabolic ratio as well as those with a higher INR : plasma total warfarin concentration ratio. Thus both INR : plasma total warfarin concentration ratio and the metabolic ratio would have an inverse relationship with the dose. To simplify things, we combined the pharmacodynamic index and the metabolic ratio:

Equation 1

$$\text{Warfarin dose} = K1 \times 1/(\text{INR} : \text{plasma total warfarin concentration}) \text{ or}$$

$$\text{Warfarin dose} = K1 \times \text{plasma total warfarin concentration} : \text{INR}$$

Equation 2

$$\text{Warfarin dose} = K2 \times 1/(\text{plasma total warfarin concentration} : \text{plasma 7-hydroxywarfarin concentration}) \text{ or}$$

$$\text{Warfarin dose} = K2 \times \text{plasma 7-hydroxywarfarin concentration} : \text{plasma total warfarin concentration}$$

Combining the two equations,

$$\text{Warfarin dose}^2 = K \times \text{plasma 7-hydroxywarfarin concentration} : \text{INR}$$

$$\text{where } K = K1 \times K2,$$

i.e.

$$\text{Warfarin dose} = K' \times (\text{INR} : \text{plasma 7-hydroxywarfarin concentration})^a$$

Our results revealed that the value of K′ was 156.52 and that of the constant 'a' was −0.63.

Numerous factors including CYP2C9 genotype, VKORC1 genotype, diet, age, liver function, BMI and interacting drugs influence the warfarin dose [3]. Some factors may affect the pharmacodynamic index, others may affect the metabolic ratio of warfarin; liver dysfunction can affect both. Previous studies have evaluated the usefulness of genotyping for CYP2C9 and VKORC1 in predicting warfarin doses in patients on long-term anticoagulation, but are not used in optimizing warfarin therapy since these genotypes account for only about 60% of variation in the anticoagulant effect of warfarin [1]. Since our index is a combination of warfarin sensitivity and metabolic ratio of warfarin, this index could possibly adjust for the effects of several other nongenetic factors that could affect warfarin dose. This would explain the high degree of correlation between INR : plasma 7-hydroxywarfarin and the warfarin dose obtained in our study.

One limitation of our study is the small sample size. Secondly, in the present study we only enrolled patients who were on maintenance therapy with warfarin. The real value of our findings would be to predict the therapeutic dose of warfarin in subjects in the initial dose titration phase of therapy based on 7-hydroxywarfarin and INR. A larger study with this objective is in progress.

In conclusion, the ratio of INR : 7-hydroxywarfarin correlates better with the weekly dose of warfarin in patients on long-term anticoagulation than the metabolic ratio (ratio of warfarin : 7-hydroxywarfarin) or the pharmacodynamic index (ratio of INR : warfarin). Phenotypic parameters may therefore be useful for predicting warfarin doses.

Competing interests: None declared.

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ORIGINAL ARTICLE

Influence of virgin coconut oil on blood coagulation factors, lipid levels and LDL oxidation in cholesterol fed Sprague–Dawley rats

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KEYWORDS
Atherosclerosis;
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Thrombosis;
PUFA;
Risk factors;
Copra oil;
Sunflower oil;
Vitamins

Summary
Background & aims: Experimental and epidemiological studies indicate an association between dietary saturated fatty acids and thrombosis, but the effects of individual fatty acids on haemostasis are still controversial. The purpose of this research is to evaluate the effect of feeding virgin coconut oil (VCO) on blood coagulation factors, lipid levels and in vitro oxidation of LDL in comparison with copra oil (CO) and sunflower oil (SFO) in cholesterol (1%) and oil (10% w/w) fed rats.
Methods: Rats were given the test oils along with cholesterol for 45 days. After the experimental period, serum cholesterol and triglyceride levels, thrombotic risk factor levels *viz*. fibrin, fibrinogen, factor V, 6-ketoPGF1α and prothrombin time were measured. In vitro Cu^{2+} induced oxidation of LDL, erythrocyte membrane and LDL TBARS content and plasma antioxidant vitamins (A and E) were also evaluated.
Results: Administration of VCO showed significant antithrombotic effect compared to copra oil and the effects were comparable with sunflower oil fed animals. The antioxidant vitamin levels were found to be higher in VCO fed animals than other groups. LDL isolated from VCO fed animals when subjected to oxidant (Cu^{2+}) in vitro showed significant resistance to oxidation as compared to the LDL isolated from other two groups. Dietary administration of VCO reduced the cholesterol and triglyceride levels and maintained the levels of blood coagulation factors. Results also indicate that VCO feeding can prevent the oxidation of LDL from oxidants. These properties of VCO may be attributed to the presence of biologically active unsaponifiable components *viz*. vitamin E, provitamin A, polyphenols and phytosterols.

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Introduction

The formation of thrombus in the coronary or cerebral arteries is one of the major causes of morbidity and mortality throughout the world. The role of coagulation system in the process of coronary heart disease is increasingly recognized.[1] Identified risk factors for thrombosis include genetics, diet, life style, smoking, lipids and cholesterol levels, molecular and circulating signals of chronic vascular inflammation.[2] Animal models of thrombosis are critical to mimic the human diseases and have been widely implemented in antithrombotic drug development.[3,4]

Numerous studies have examined the relationship between dietary fat and cardiovascular disease (CVD), but the effects of individual fatty acids on haemostasis are still controversial.[5,6] Experimental and epidemiological studies indicate an association between dietary saturated fatty acids and venous thrombosis, but the chain of evidences lack documentation from prospective clinical studies.[7] However, it is now thought that some saturated fatty acids may not deserve this reputation.[8] Previous reports suggest that saturated fatty acids and cholesterol each independently elevate blood cholesterol and low-density lipoprotein (LDL) concentrations.[9] Hypercholesterolemia and atherosclerotic disorders have been associated with a low-grade inflammation that involves not only the intrinsic cells of the artery wall, but also circulating cells, *viz* platelet, WBC, Hb and LDL.[10]

Free radical mediated oxidation of circulating LDL also plays a critical role in the progression of atherosclerosis.[11,12] Oxidized LDL stimulates platelet adhesion and aggregation by decreasing endothelial production of nitric oxide (NO) and increasing prostacyclin (PGI2) production.[13] It also stimulates coagulation, reduces fibrinolytic activity of endothelium and may also contribute to its dysfunction and plaque disruption.[14] During the course of normal metabolism, reactive oxygen species (ROS) and free radicals are formed, which induce oxidative damage to biomolecules and play an important role in the pathological conditions such as atherosclerosis, aging and inflammatory diseases and variety of other disorders.[15] Oxidatively modified lipids cause both direct and indirect toxic effects on the vascular endothelium. The antioxidant system (AOS) that includes the enzymatic and non-enzymatic mechanisms for lipid peroxide (LPO) product inactivation confers protection from endothelial dysfunction.[16]

Virgin coconut oil (VCO), unlike the coconut oil obtained from dried copra, is extracted directly from coconut meat under mild temperature. This extraction process avoids the loss of minor components like provitamin A and vitamin E and polyphenols due to UV irradiation from sunlight during drying of copra. Sunflower oil is reported to have beneficial effect which is associated with coronary heart disease (CHD).[17] We recently reported that VCO is more beneficial than copra oil (CO) and groundnut oil in lowering lipid levels and preventing the oxidation of LDL by physiological oxidants.[18] In this context we proposed to examine the effect of virgin coconut oil on blood coagulation factors that contribute towards thrombosis, lipid levels and LDL oxidation compared to copra oil (CO) and sunflower oil (SFO) in cholesterol fed rats.

Material and methods

Chemicals

α-Tocopherol, retinol, 6-ketoPGF1α, and prothrombin were purchased from Sigma Chemicals SA. All other chemicals used were of high analytical grade.

Test oils

Mature coconuts (West Coast Tall variety), grown at the Kerala University Campus were used for the extraction of copra oil and virgin coconut oil. (a) *Virgin coconut oil*: the solid endosperm of mature coconut was crushed, made into viscous slurry and squeezed through cheesecloth to obtain coconut milk which was refrigerated for 48 h to separate fat and water layer. Fat layer was carefully removed and subjected to mild heating (50 °C) in a thermostat oven. The obtained virgin oil was filtered through cheesecloth and was used for the present study.[18] (b) *Copra oil*: coconut meat was dried in sunlight continuously for 4 days to remove moisture and the resulted copra was pressed in the mill to obtain copra oil. (c) *Sunflower oil*: sunflower oil (Gold Winner brand, Chennai) was purchased from the local market.

Fatty acid analysis of VCO and CO

The fatty acid composition of VCO and CO was analyzed by gas chromatography. Fats were methylated with trimethylsulfonium hydroxide.[19] Fatty acid methyl esters were separated by gas chromatography using a system (HP 5890, Hewlett Packard GmbH, Waldbronn, Germany) equipped with an automatic on-column injector, a polar capillary column (30 m FFAP, 0.53 mm I.D., Macherey and Nagel, Düren, Germany) and a flame ionisation detector.[20] Helium was used as carrier gas at a flow rate of 5.4 ml/min. Fatty acid methyl esters were identified by comparing their retention times with those of individually purified standards (Table 1).

Table 1 Fatty acid composition of the test oils

Fatty acids	VCO	CO	SFO[45]
8:0	8.05	8.15	<0.1
10:0	5.42	5.56	0.0
12:0	45.51	43.55	0.0
14:0	19.74	18.38	0.1
16:0	7.83	8.25	6.5
18:0	3.14	2.65	4.2
18:1	4.70	6.70	18.7
18:2	1.88	1.49	68.6
20:0	0.086	0.086	0.3
20:1	0.027	0.042	0.3
22:0	0.016	0.018	0.7
24:0	0.032	0.065	0.2

Values are mean of three estimations and are expressed as percentage amount.

Animals

All the animal cares and procedures were according to the guidelines of the Institutional Animal Ethical Committee (IAEC). One-month-old male Sprague—Dawley rats (100—130 g) bred in our department animal house was used for the study. The animals (6/group) were housed individually in polypropylene cages in a room maintained at 25 ± 1 °C with a 12 h light and 12 h dark cycle.

Experimental protocol

A total of 18 rats were used to perform the present study. Rats were divided into three groups with six animals each as follows. (I) Copra oil (10% w/w) + 1% cholesterol, (II) virgin coconut oil (10% w/w) + 1% cholesterol, and (III) sunflower oil (10% w/w) + 1% cholesterol. Oils were fed along with the normal laboratory diet (10 g/rat) (Amrut Laboratory Animal Feed, Mumbai) for 45 days. Gain in body weight was recorded weekly. After 45 days, animals were fasted overnight and sacrificed by sodium pentothal injection, blood and tissues were collected for various estimations.

Estimation of hematological parameters

Blood was drawn in WBC pipette followed by WBC diluting fluid (Turke's fluid). Mixture was mixed well and transferred into a counting chamber. WBC was counted uniformly in four large corner squares. For counting platelets, blood was drawn in an RBC pipette and diluted with 1% ammonium oxalate and charged the chamber and platelet counted using 40× objective lens in the 80 smaller squares of the chamber. Hemoglobin was estimated using cyanomethemoglobin. For RBC count blood was mixed with RBC diluting fluid and counted as described earlier.[21]

Thrombotic risk factor levels

Fibrinogen was estimated as described by Fearnley and Chakrabarti[22] using a Fibroquant kit from Tulip Diagnostics (P) Ltd, Goa, India. Fibrin was estimated as described by King and Wootten.[23] Plasma (0.05 ml) was diluted with 2 ml of isotonic saline and 0.2 ml of 2.5% calcium chloride solution was added. The mixture was kept at 37 °C for overnight until a clot was formed. The fibrin was carefully collected, pressed to remove liquid and washed with water. Sixty percent of perchloric acid (0.2 ml) was added and digested until it become colorless. Solution is cooled, mixed with 5 ml of water and 1 ml of Nesslers reagent. The color developed was read against reagent blank at 620 nm. Pure dry ammonium chloride (4 mg/100 ml water) was used as the standard.

Prothrombin time was determined using liquiplastin kit from Tulip Diagnostics (P) Ltd, Goa, India. To 25 μl plasma at 37 °C forcibly added 0.2 ml of liquiplastin reagent (prewarmed at 37 °C for 3 min), simultaneously started a stopwatch and stopped as soon as the first fibrin strand was visible and the clot formation begins. Time is recorded in seconds and was taken as prothrombin time. Factor V was assayed by the method of Daniel.[24] The following cold reagents were taken in a small tube, 0.2 ml of prothrombin (200 U/ml), 0.2 ml of fibrinogen solution (0.5%) and 0.2 ml plasma diluted to 5% and allowed to stand at 37 °C for 4 min. Added 0.2 ml of $CaCl_2$ solution (25 mM) and the coagulation time was recorded.

Isolation and estimation of 6-ketoPGF1α by HPLC

Lipids were extracted from the serum with chloroform:methanol (1:1). It was then filtered through a Whatman No 1 filter paper. The residue collected was evaporated to dryness under N_2, after protein estimation. Residue was redissolved in 5 ml of ethanol and 15 ml of water was added, acidified to pH 3 with formic acid and kept for 24 h at 4 °C with constant shaking at 2 h interval. Sample was applied to a C18 Seppak catridge, and washed with 5 ml of water followed by 5 ml of 15% ethanol and 5 ml of hexane. The eicosanoids were eluted with 2.5 ml ethyl acetate.[25] 6-ketoPGF1α was detected using a C18 column (isocratically with acetonitrile:water:H3P04, 30:70:0.01, v/v, pH 2.95, flow rate 0.5 ml/min detection at 192 nm) in a Shimadzu S PDA 10 chromatograph.[26]

Serum vitamins A and E

Vitamins A and E content of serum were determined simultaneously by HPLC.[27] Briefly, 100 μl serum was added to 10 ml methanol containing 0.01% butylated hydroxytoluene (BHT) and hexane. Mixture was centrifuged and hexane layer collected, dissolved to dryness with N_2 and added 2 ml 10% methanolic KOH. After keeping for 2 h at room temperature 4 ml hexane was added. Hexane layer was collected, evaporated under N_2 and dissolved in 100 μl methanol and injected into HPLC column (C18 silica column, 100% methanol as mobile phase with 1.5 ml/min flow rate) and detected at 292 nm.

Erythrocyte membrane isolation

Blood from rats was drawn into tubes with potassium EDTA as anticoagulant. Plasma and platelets were removed by differential centrifugation (15 min, 1000×*g*). The erythrocytes were dispersed in isotonic phosphate buffer (0.119 mol PO_4/L, pH 7.4) and washed two or three times by centrifugation (20 min, 1000×*g*).[28] Erythrocyte membranes were prepared by hypotonic lysis in 7.6 mmol PO_4/L (pH 7.4) according to the procedure of Dodge.[29] Membrane preparations were washed in the 7.6 mmol PO_4/L until the supernatant was clear to remove hemoglobin and other cytoplasmic components. From this aliquots were removed and TBARS content was measured.[30]

Isolation of LDL

Blood from rats was collected into tubes containing potassium EDTA as anticoagulant. Plasma and platelets were removed by differential centrifugation (15 min, 1000×*g*). A volume of 3 ml plasma was centrifuged at $d = 1.006$ kg/l in an ultracentrifuge (Sorvall Ultra 80) at 40,000 rpm using a T-865 rotor at 14 °C for 10 h.[31] After ultracentrifugation floating VLDL and chylomicrons were removed and LDL was separated by precipitation from

Table 2 Hematological parameters of VCO, CO and SFO fed animals

Groups	Hb (g/100 ml)	WBC ($\times 10^3$/c.mm)	RBC ($\times 10^6$/c.mm)	Platelet count (/c.mm)
Copra oil	14.03 ± 0.12	7900 ± 115.47	6.03 ± 0.09	$26{,}500.00 \pm 288.67$
Virgin coconut oil	13.37 ± 0.18[a]	6600 ± 57.73[a]	5.30 ± 0.06[a]	$24{,}000.00 \pm 57.74$[a]
Sunflower oil	12.23 ± 0.17[a]	6800 ± 57.74[a]	5.17 ± 0.09[a]	$24{,}066.67 \pm 88.19$[a]

Values are mean $\pm$ SEM of six rats.
[a] $p < 0.05$ vs Group I.

the solution.[32] Forty milliliters of 4% phosphotungstic acid in 1 M NaOH was added, stirred and 10 μl of 2 M $MgCl_2 \cdot 6H_2O$ was added and cenrtifuged at $1500 \times g$ for 30 min at 4 °C. The supernatant was discarded and the precipitated LDL was redissolved in 0.4 ml 0.5 M Na_2CO_3, kept in ice overnight and dialyzed against three changes of PBS for 12 h.[18]

Estimation of serum lipid levels

Serum total cholesterol was estimated by the method as described by Abell et al.[33] Triglycerides were estimated by the method of Van Handel and Zilversmit.[34]

Statistical analysis

Analysis was done using SPSS 10. All values are mean $\pm$ SEM calculated by one-way ANOVA. Duncan's variance was applied to assess significant differences of continuous variables among groups.

Results

There were no differences in weight gain pattern of rats between groups. Table 2 represents the levels of hematological parameters. In VCO fed animals the WBC, RBC and Hb levels were lower than CO but the result was similar to that obtained in SFO fed animals. Platelet count was reduced in Groups II and III animals fed VCO and SFO than Group I animals fed CO, which was found to be significantly increased.

Prothrombin time (PT) was significantly lowered in CO treated animals (10.16 ± 0.16). VCO and SFO fed animals showed higher PT (11.25 ± 0.14 and 11.37 ± 0.08, respectively) (Table 3). Fibrin and fibrinogen levels were also found to be lower in VCO and SFO fed animals. In the case of CO fed animals, the levels of these parameters were significantly greater. The levels of factor V were found to be increased in CO fed groups (29.52 ± 0.66) but there were no significant changes in VCO and SFO fed groups (30.28 ± 0.11 and 30.01 ± 0.24, respectively) (Table 3). 6-ketoPGF1α was slightly reduced in VCO fed animals. But their levels were similar in other two groups (Table 3).

Vitamin A in the serum of VCO fed animals showed a significant change than SFO and CO fed animals. Vitamin E levels were greater in SFO group than VCO and CO fed animals with CO fed group showed a significantly lower level compared to VCO fed animals (Fig. 1).

Serum cholesterol levels in CO fed animal were significantly greater compared to CO and SFO fed animals. The values were similar in VCO and SFO fed animals. Serum triglyceride levels in CO fed animal were also significantly greater compared to CO and SFO fed animals. The values were similar in VCO and SFO fed animals (Fig. 2).

Erythrocyte membrane and LDL TBARS content of VCO fed animals were significantly lower than CO and SFO fed animals (Fig. 3). In SFO fed animas, LDL and erythrocyte membrane TBARS content were found to be significantly greater than other two oil fed groups.

LDL isolated from VCO fed animals when subjected to oxidation by Cu^{2+} was found to prevent oxidation than the LDL isolated from SFO and CO fed animals. In all the three cases the oxidation of LDL was found to increase with respect to time but the increase was slower in the case of LDL isolated from VCO fed animals compared to SFO and CO fed animals. The oxidation level was higher in LDL from SFO fed animals after 6 h indicated by high TBARS content. Oxidation level was lower in CO but not significant compared to VCO fed animals (Fig. 4).

Discussion

The present study was conducted to evaluate the effect of virgin coconut oil (VCO) on blood coagulation factors, lipid levels and LDL oxidation as compared to copra oil (CO) and sunflower oil (SFO) in cholesterol co-administered rats. Supplementation of VCO diet showed significant beneficial effects on blood coagulation when compared to CO and SFO. The lipid levels, and thrombotic risk factors *viz.*

Table 3 Blood coagulation factor levels of VCO, CO and SFO fed animals

Groups	Fibrin (mg/dl)	Fibrinogen (mg/dl)	Prothrombin time (s)	Factor V (s)	6-ketoPGF1α (pg/dl)
Copra oil	13.57 ± 0.53	296.67 ± 7.26	10.16 ± 0.16	29.52 ± 0.66	16.88 ± 0.25
Virgin coconut oil	10.50 ± 0.50	232.93 ± 4.3[a]	11.25 ± 0.14[a]	30.28 ± 0.11[a]	14.05 ± 0.47[a]
Sunflower oil	11.70 ± 0.82	225.13 ± 4.9[a]	11.37 ± 0.08[a]	30.01 ± 0.24[a]	15.94 ± 0.26

Values are mean $\pm$ SEM of six rats.
[a] $p < 0.05$ vs Group I.

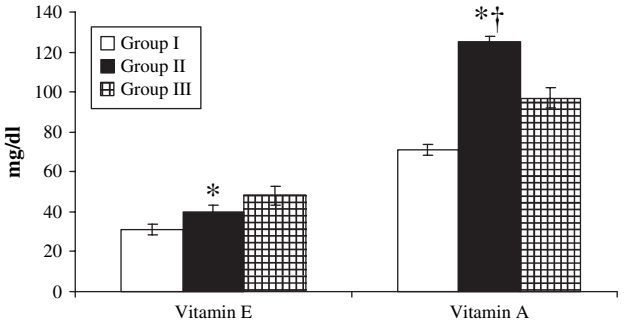

Figure 1 Levels of vitamins A and E levels of serum from test animals. Group I — copra oil (10%) + 1% cholesterol, Group II — virgin coconut oil (10%) + 1% cholesterol, and Group III — sunflower oil (10%) + 1% cholesterol. Bars represent mean values ± SEM of six rats. *$p < 0.05$ vs Group I; and †$p < 0.05$ vs Group III.

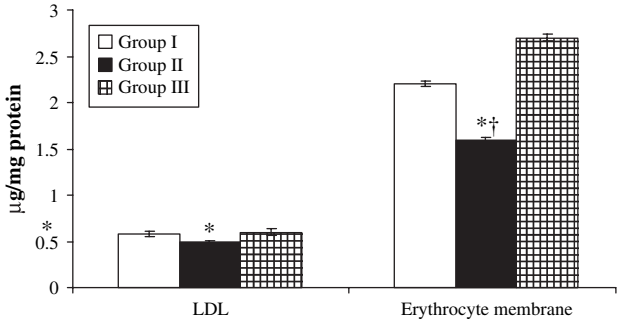

Figure 3 Levels of LDL and erythrocyte membrane TBARS content of test animals. Group I — copra oil (10%) + 1% cholesterol, Group II — virgin coconut oil (10%) + 1% cholesterol, and Group III — sunflower oil (10%) + 1% cholesterol. Bars represent mean values ± SEM of six rats. *$p < 0.05$ vs Group I; and †$p < 0.05$ vs Group III.

platelets, fibrin, fibrinogen, and factor V were lower in rats fed VCO. Hematological factors *viz*. WBC, Hb and RBC were also lower in this group. Blood coagulation is the host mechanism involved in perfecting the integrity of the vascular system in which platelets are known to play an active role. Elevated platelet count also contribute to the circulation and progression of coronary artery narrowing by atherosclerotic plaques resulting in occlusive coronary arterial thrombosis and finally to unstable angina and myocardial infarction. WBC is suggested to promote myocardial ischemia by release of toxic oxygen metabolites. There are substantial evidence that dietary factors, particularly fatty acids may affect platelet function.

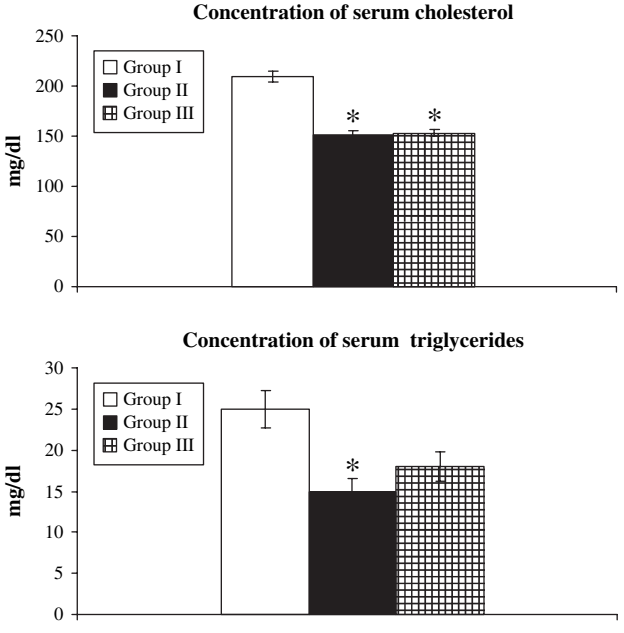

Figure 2 Levels of serum total cholesterol and triglyceride levels of test animals. Group I — copra oil (10%) + 1% cholesterol, Group II — virgin coconut oil (10%) + 1% cholesterol, and Group III — sunflower oil (10%) + 1% cholesterol. Bars represent mean values ± SEM of six rats. *$p < 0.05$ vs Group I.

The lower levels of fibrinogen and fibrin observed in VCO and SFO fed rats reflects the decreased blood-clotting tendency. The rate of conversion of fibrinogen to insoluble product fibrin is a key factor in haemostasis.[35] Fibrinogen, an acute-phase protein, becomes elevated as a consequence of inflammatory reactions that occur during the development of atherosclerotic plaques.[36] Previous studies have identified fibrinogen as a risk factor as powerful as cholesterol in producing ischemic events. Interaction of platelets with fibrinogen mediates a variety of responses including adhesion, platelet aggregation and fibrin clot retraction. We have also found decreased levels of factor V in VCO and SFO fed animals compared to CO fed group. Factor V, a large single chain plasma glycoproteins is an essential component of blood coagulation cascade and also an independent risk factor for myocardial infarction. During coagulation, factor V is converted to active co-factor, factor Va which combines with Xa and assemble to form prothrombinase complex, which converts prothrombin to thrombin.[37]

Concentration of PGF1α was found to be lower in VCO fed animals. 6-ketoPGF1α is a stable metabolite of PGI2, which is a critical local regulation of a variety of cellular processes. 6-ketoPGF1α is found to possess cardioprotective effects in animal model of myocardial infarction. During platelet activation, arachidonate is released from

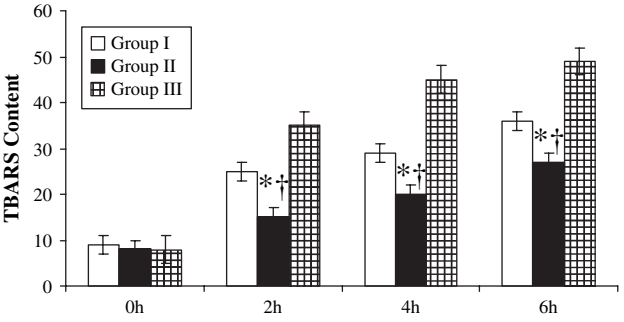

Figure 4 Cu^{2+} induced oxidation pattern of LDL isolated from test animals. Bars represent mean values ± SEM of six rats. TBARS content is expressed as nM/mg protein. *$p < 0.05$ vs Group I; and †$p < 0.05$ vs Group III.

the phospholipids and then converted into prostaglandin H2 and thromboxane A2, which strongly potentiate the activation process.[38] The low levels of 6-ketoPGF1α in VCO fed animals may be due to the lower level of linoleic acid which serves as the substrate for arachidonic acid synthesis.

In the regulation of primary haemostasis, the interaction of blood vessels with platelet plays an essential role and its in vivo evaluation involves bleeding time measurement.[39] Secondary haemostasis is another extremely important factor determining thrombus formation in vessels. Blood clotting can be inhibited by attenuation of plasma prothrombin activation system.[40] Here we have observed an increase in prothrombin time (PT) in VCO and SFO compared to CO fed groups. Serum total cholesterol (TC) was found to be decreased in VCO fed animals and was comparable with SFO fed group. The TBARS content of isolated LDL and erythrocyte membrane of VCO fed animals were also significantly decreased compared to other two groups. The lower levels of LDL and VLDL cholesterol in VCO fed animals may be the reason for the above effect (unpublished report). Feeding cholesterol rich diet caused significant increase in the plasma, erythrocyte and liver TC, plasma TG and TBARS levels. Plasma and erythrocyte TBARS levels are markers of oxidative stress.

Circulating lipid peroxides may promote lipid peroxidation of other circulating lipids and lipoproteins, resulting in disseminated endothelial dysfunction.[41] MDA, a breakdown product of spontaneous fragmentation of peroxides form from polyunsaturated fatty acids (PUFA) mainly from the oxidation of cell membranes.[42] The observed lower level of plasma and erythrocyte TBARS content may be due to the higher amount of antioxidant vitamins in the serum and lower amount of PUFA of VCO. SFO contain very high amount of unsaturated fatty acids (Table 3)[43] and is more susceptible to oxidation. Feeding of oils rich in polyunsaturated fatty acids (PUFA) results in their accumulation in cell membranes and increased the oxidative stress, since PUFAs are highly susceptible to peroxidation compared to monounsaturated and saturated fatty acids. This may be the reason for the higher TBARS formation in the plasma and erythrocyte membrane of SFO fed animals.[44,45]

It is generally believed that the highly saturated nature of coconut fatty acids (Table 3) increases cholesterol synthesis in our body and thus contributes to higher incidence of heart disease. This contention, however, has been refuted scientifically.[46] Available scientific reports showed that it is not hypercholesterolemic and atherogeneic.[47] Medium chain triacyglycerols (MCTs) of coconut oil fed rats reduced storage fat accumulation, serum and tissue cholesterol and linoleate requirement.[48] Compared to CO, VCO contain appreciable amounts of many biologically active minor components viz. polyphenols (80 mg/100 g oil) and antioxidant vitamins (30 μg/100 g oil), which may have an effect on blood coagulation. In vitro studies have proved that polyphenols inhibit platelet aggregation, increases the Ca^{2+} influx and mobilization of Ca^{2+} in endothelial cells.[49] These compounds also reported to suppress adhesion molecules and inhibit experimental atherosclerosis. Diet supplemented with polyphenolic compounds improved the lipid metabolism and increased the plasma antioxidant potential especially in rats fed with added cholesterol.[50] Recently we have reported that VCO polyphenols can prevent the oxidation of LDL in in vitro conditions.[18] They can trap reactive oxygen species from aqueous series such as plasma and interstitial fluid of arterial wall thereby inhibiting oxidation of LDL and showing atherosclerotic activity.[51]

We have observed an increase of serum vitamins A and E in VCO fed animals than CO fed animals and is comparable to SFO fed animals. The lower levels of these vitamins in CO fed animals may be due to the low content in CO (Table 3) that might have lost during the exposure of copra to UV radiation of sunlight during extraction process. Vitamin E has been implicated in the body's protective armory against diseases and is the major chain breaking lipophilic antioxidants in tissue and plasma.[52] Vitamin E decrease platelet aggregation by dephosphorylating protein kinase Cα (PKCα) and might thus affect thrombotic tendencies.[53,54] Plasma vitamin E decreases the TBARS content in hypercholesterolemic rats by trapping the chain-propagating peroxyl radicals.[55] It also suppresses the expression of adhesion molecules and chemokines by endothelial cells/monocytes in culture. Studies showed that antioxidant vitamins A and E were found to be decreased in the plasma of patients with angina and myocardial infarction.[56,57]

LDL isolated from VCO treated animals showed a reduced TBARS content when treated with $CuSO_4$. This may de due to the higher levels of antioxidants *viz*. vitamins E and A and polyphenols in the LDL which protect it from physiological oxidants. Oxidized LDL has been shown to be highly cytotoxic for vascular cells, to activate endothelial recruitment of leukocytes, macrophage cytokine production and stimulate smooth muscle cells (SMCs) proliferation.

Oxysterols are responsible for most of the cytotoxic effects of oxidized LDL.[58] During the oxidation of LDL, the LDL molecule undergoes a large number of structural changes that alter its metabolism.[59] Vascular thrombogenicity is induced by progressive LDL oxidation and that alterations of the antioxidant/oxidant balance of the LDL particle in favor of the antioxidant tone are protective against the thrombotic response triggered by oxidative stress.[60] It is generally accepted that the primary generation of lipid hydroperoxides in our body initiates a reaction cascade leading to rapid propagation and to amplification of the number of reactive oxygen species formed; this ultimately leads to extensive fragmentation of the fatty acid chains,[61] and conversion of the LDL to a more atherogenic form.[62] Peroxidation of LDL with Cu^{2+} produced cholesteryl ester core aldehydes, such as 9-oxononanoylcholesterol (9-ONC) and 5-oxovaleroylcholesterol (5-OVC), as the major oxidized cholesteryl esters.[63] Oxidized LDL reduces the fibrinolytic activity of endothelium by decreasing secretion of tissue-type plasminogen activator (tPA) and increasing release of plasminogen activator inhibitor-1 (PAI-1)[64] also stimulates coagulation by reducing thrombomodulin (TM) transcription.[65]

In conclusion, the results indicate that consumption of VCO supplemented diet exerts a significant antithrombotic effect, which is associated by suppression of platelet aggregation and low levels of cholesterol and triglycerides. VCO also prevented the formation of lipid peroxides in both erythrocyte membrane and LDL of experimental animals.

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Original Article

Haemostatic efficacy: Exploration of *Azadirachta indica* Bark

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ABSTRACT

The haemostatic potential of alcoholic extracts of *Azadirachta indica* bark has been evaluated systematically. An attempt has been made to elucidate the modes of action of the cold and hot alcoholic extracts, using platelet aggregation test, prothrombin time test, activated partial thromboplastin time test and whole blood clotting for a range of concentrations. HPTLC fingerprint of alcoholic bark extract has been obtained as a quality control parameter. Presence of tannins and saponins in the extract of bark has been confirmed by HPTLC profiling. These metabolites are known to help in clot formation. The study indicates that alcoholic bark extract of *Azadirachta indica* can be used effectively as an excellent topical haemostatic agent.

Key words: *Azadirachta indica*, topical haemostatic agent, HPTLC fingerprint, HPTLC profile for tannins, saponins

INTRODUCTION

Azadirachta indica belongs to the family Meliaceae. It is an evergreen, medium sized tree. Bark of this tree is usually dark grey externally and reddish inside [1]. It is often used as antibacterial, anti-inflammatory, wound healer, antiulcer, immunostimulant, analgesic and antitumor agent in the folk medicine [2].

Loss of blood in humans can be due to injury, surgical bleeding, pathological conditions or genetic disorders related to blood coagulation. It is one of the leading causes of death worldwide [3]. The methods used to stem severe bleeding include blood transfusion, surgery, angiographic embolization and use of haemostatic agents. There are several topical haemostatic products available which include haemostatic gauze, matrix proteins, fibrin polymer, thrombin, ε-aminocaproic acid, feracrilum, aprotinin, human recombinant factor VIIa, to name a few [4, 5]. These agents differ in their modes of action and cost per application. However, they might give rise to certain complications such as allergic reactions or thromboembolism and the risk of contracting bovine spongiform encephalitis or hepatitis [6]. Therefore there is always a need for a new, effective as well as economical haemostatic agent.
The ethnobotanical literature inquiry is a cost-effective means of locating new and useful plant compounds. Many important drugs from indigenous medicine practised by people throughout the world have been brought to use in the modern medicine through their scientific investigation. Several plants have been recorded to have medicinal properties, many of them are yet to be validated. The present paper focuses on the haemostatic potential of bark of *Azadirachta indica*, which is widely used in traditional medicine for other indications [2]. Bark is an excellent renewable source of secondary metabolites which often function as useful resources of plant based medicines [7]. The secondary metabolites tannins and saponins, are reported as effective contributors to the haemostatic activity [8, 9]. In the present study, HPTLC analysis has confirmed the presence of both these metabolites in the alcoholic bark extract of the bark of *Azadirachta indica.*

MATERIALS AND METHOD

Collection and Identification of plant material: The bark of *Azadirachta indica* was collected in the village of Padghavali, district Raigad, Maharashtra, India. The bark was identified and deposited as a voucher specimen in the Department of Biological Sciences, Ramniranjan Jhunjhunwala College, Ghatkopar, Mumbai, India.

Preparation of Extract: The bark of the plant was shade dried for 8 days and crushed into a coarse

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powder using electrical grinder. Cold and hot extraction of the coarse powder was carried out using alcohol. For the cold extract, 30 g coarse bark power was soaked in 350 ml alcohol for 72 hours. Hot extraction was carried out for 8 hours, using soxhlet apparatus [10]. Filtered extracts were dried using rota evaporator. The alcohol free powder was used for efficacy studies.

Phytochemical evaluation: The secondary metabolites are the compounds responsible for the medicinal properties of plants [11]. During the present investigation, the alcoholic bark extracts of *Azadirachta indica* were screened for presence of carbohydrates, alkaloids, tannins, saponins, gums and resins using protocol described by Kokate [12]. Total phenols in the alcoholic bark extracts of *Azadirachta indica* were determined using Folin-Ciocalteau method [13]. A calibration curve for gallic acid (µg/ ml) was prepared. The equation $y = 0.0031x$ with regression $R^2 = 0.9964$ was used to calculate the concentration of total phenols present the cold and hot bark extracts, in terms of gallic acid equivalent.

High Performance Thin layer Chromatography (HPTLC): Chromatographic fingerprint was obtained for the methanolic bark extract of *Azadirachta indica* using CAMAG HPTLC (Switzerland) comprising of Linomat IV Spotter, Scanner II, CAMAG CATS 3 software. HPTLC was also used to confirm the presence of saponins and tannins in the alcoholic bark extract of *Azadirachta indica.* 20 µl of sample was applied with CAMAG Limnomat IV Spotter on HPTLC plate of Silica gel 60 F254. Toluene: Ethyl acetate: Formic acid (3:7:0.8) and Chloroform: Acetic acid: Methanol: Water (6.4:3.2:1.2:0.8) were used as mobile phase for tannins and saponins respectively [14, 15]. 10% alcoholic $FeCl_3$ was used as derivatising agent for tannins and acid anhydride, for saponins. The plates were visualised under UV 254 nm, visible 550 nm and fluorescence 366 nm.

Collection of Blood: The blood was collected from volunteers with their due consent, by a clean venipuncture using a plastic disposable syringe with 20 SWS needle. Adequate precautions were taken before collecting blood. Freshly collected blood was transferred to plastic vials containing 3.2% tri-sodium citrate.

Preparation of PPP and PRP: Platelet poor normal plasma (PPP) and platelet rich normal plasma (PRP) was obtained by differential centrifugation of fresh citrated blood collected from volunteers. To obtain PPP the citrated fresh blood was centrifuged at 1500g for 15 minutes. Platelet rich normal plasma was obtained by centrifuging citrated blood of volunteers at 150-200 g for 15 minutes. The plasma samples were removed and transferred to tubes. The average platelet count was more than 400,000 for PRP and less than 1000 for PPP [16].

Determination of Clotting Time: Method described by Lee- White was used to determine the clotting time [17]. Cold and hot extracts of bark of *Azadirachta indica* at 10 mg/ml, 50 mg/ml, 100 mg/ml and 250 mg/ml of saline were used. 0.2ml of extract was mixed with 0.2 ml of whole blood in a test tube and swirled gently. The temperature was maintained at 37ºC in a water bath. The time required for clot formation was noted. Control tube contained 0.2 ml of blood and 0.2 ml of saline. The process was monitored for 15 minutes. If the blood did not coagulate within 15 minutes the extract was considered ineffective and was discarded.

Platelet Aggregation Test (PAT): The test was carried out using Tulip Diagnostics kit following recommended protocol. Briefly, the standard solution contained, adenosine diphosphate sodium (ADP) salt at the concentration of 200 µg/ml with 0.2 ml of PRP. Test solution contained 0.2 ml of extract at concentrations ranging from 10 mg/ml to 250 mg/ml saline with 0.2 ml of PRP. All the tests were carried out at 37ºC in a water bath. Control tube contained 0.2 ml saline with 0.2 ml of PRP. Time required to form coagulum was noted.

Prothrombin Time (PT) Test: PT measures the activity of extrinsic pathway of coagulation. It is performed by using PPP. Liquiplastin, which is a calcium thromboplastin reagent (Tulip Diagnostics Pvt. Lt., Goa), was used as standard. 0.2 ml of liquiplastin was added to 0.2 ml of PPP for standard reaction. To 0.2 ml of PPP, 0.2 ml of test extract at concentration of 10 mg/ml, 50 mg/ml, 100 mg/ml and 250 mg/ml of saline was added. The procedure was carried out at 37ºC using a water bath. Control tube contained 0.2 ml PPP and 0.2 ml of saline. Coagulation time was recorded.

Activated Partial Thromboplastin Time (APPT) Test: APPT is associated with intrinsic pathway of clotting. The term 'partial' means phospholipid is present without tissue factor. Liquicelin E, an activated cephaloplastin reagent and $CaCl_2$ was procured from Tulip Diagnostics. Liquicelin E was used as a standard. To a tube containing 0.2 ml of PPP, 0.2 ml of extract at concentrations indicated above, was added and the tubes were kept in water bath at 37ºC for 3 minutes. Pre-warmed 0.2 ml $CaCl_2$ solution was added to these tubes forcefully and placed in water bath at 37ºC. Control tube contained 0.2 ml PPP and 0.2 ml of saline. Time

required to gel plasma was noted. Each reaction was carried out in triplicates.

RESULTS

The results of the general qualitative phytochemical tests are presented in Table 1. The hot and the cold extracts of *Azadirachta indica* have been denoted by AIH and AIC, respectively. The total phenol content of the cold and hot bark extract was found to be 4.83 mg/ml and 5.48 mg/ml, respectively. The HPTLC fingerprint of methanolic bark extract of *Azadirachta indica* yielded 12 peaks as seen in Fig. 1. The maximum Rf and area under curve for the peaks are listed in Table 2. The HPTLC analysis of bark tannins after derivatisation with 10% $FeCl_3$ showed presence of two blue colour bands which confirmed the presence of tannins. Fig. 2 represents the HPTLC plates before and after derivatisation and the corresponding HPTLC profile. The details of the maximum Rf and area under curve can be found in Table 3. HPTLC profile for saponins and the corresponding HPTLC plates before and after derivatisation have been shown in Fig. 3. The presence of two violet colour bands ascertain existence of saponins in methanolic bark extract of *Azadirachta indica*. Details of maximum Rf and area under the curve for which are presented in Table 4.

The complete haemostatic profile of cold (AIC) and hot (AIH) alcoholic bark extract of *Azadirachta indica* is presented in Table 5. It was observed that AIC and AIH exhibited excellent haemostatic activity by formation of a solid clot. The extracts showed concentration dependant activity. The optimum activity was at 250 mg/ml with clotting time 14.32 s and 17.32 s for AIC and AIH respectively. Prothrombin Time was almost instantaneous for both the extracts at 250 mg/ml. Both the extracts formed solid clot almost instantaneously for APPT as well as for PAT. However, AIC and AIH extract at 10 mg/ml did not exhibit activity with PRP.

DISCUSSION

The haemostatic system comprises of platelet aggregation, coagulation and fibrinolysis which are termed as primary, secondary and tertiary haemostasis [18]. However the pathological changes brought by trauma lead to breakdown of this system. This may result in severe blood loss, which may be fatal.

A topical haemostatic agent imitates or bypasses specific steps in coagulation cascade. Topical haemostatic agents of herbal origin, despite of being effective, have not been explored extensively. Though, Ankaferd Blood Stopper, a traditional Turkish preparation has been successfully used to stop the bleeding [19].

In the present study, *Azadirachta indica* bark extracts were evaluated for their haemostatic efficacy using whole blood, platelet rich (PRP) and platelet poor (PPP) plasmas [17]. These fractions were subjected to whole blood clotting, prothrombin time test, activated prothrombin test and platelet aggregation test (see Table 5). It was observed that *Azadirachta indica* bark extracts at the concentration of 250 mg/ml exhibited excellent haemostatic efficacy by forming instantaneous clots in all the systems, thereby indicating that the extracts not only affected intrinsic and extrinsic pathways of coagulation but also platelet aggregation effectively.

The biological activity of plants is attributed to the secondary metabolites. These secondary metabolites confer various properties like anti-microbial, analgesic, anti-inflammatory on the plant [20]. The concentration of secondary metabolites varies from one plant to another as well as in different parts of the same plant. Bark is known to be rich in secondary metabolites. In the present study, general phytochemical tests confirmed the presence of several secondary metabolites in the hot and cold alcoholic bark extracts of *Azadirachta indica* (see Table 1). The differential total phenol content found in AIC and AIH did not alter the extent of haemostatic efficacy of the two extracts.

Further, chromatographic fingerprint, unique to the bark of *Azadirachta indica*, was obtained by HPTLC technique and has been used in quality control of the bark as a drug. Presence of tannins and saponins as seen in the HPTLC profile can be major contributory factors in the haemostatic property of the extract. Many researchers have explored the extracts of different plant components as haemostatic agents [21]. Gao *et. al*. have attributed the initiation of mechanism of clotting to saponins influencing platelet aggregation. Tannins are known to precipitate plasma proteins there by initiating or adding to the mechanism of clot formation. These results differ from those obtained for the extracts of bark of *Garuga pinnata Roxb.*, where the extract induced coagulation exclusively through protein precipitation reaction without affecting intrinsic or extrinsic pathways [17].

CONCLUSIONS

The alcoholic extract of the bark of *Azadirachta indica* influences all the four reactions, namely,

whole blood clotting, PT, APTT and PAT, leading to clot formation. The time of clotting reaction is significantly low when the drug is applied at 250 mg/ml concentration, and hence these extracts can be effectively used to stop traumatic surface bleeding. The bark is an inexpensive renewable resource, thus the anti-haemorrhagic preparation made using the bark will become useful drug to treat bleeding wounds in rural as well as urban settings.

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Table 1. General Qualitative Phytochemical Tests

Sr. No.	Constituent	Test	Inference	
			AIH	AIC
1	Alkaloids	Dragendroff	+	+
		Wagner	-	-
		Hager	-	-
2	Gums	Lead Acetate	+	+
3	Phenols	$FeCl_3$	+	+
4	Tannins	Gelatin	+	+
5	Starch	Iodine water	-	-
6	Flavones	Dilute H_2SO_4	+	+
7	Carotenoids	Antimony Trichloride	+	+
8	Proteins	Ninhydrin	-	-

The above table indicates that alkaloids, gums, phenols, tannins, flavones and carotenoids are present in cold as well as hot bark extracts of *Azadirachta indica.*
'+' indicates presence of a phytoconstituent
'-' indicates absence of a phytoconstituent

Table 2. Chromatographic fingerprint of *Azadirachta indica* bark.

Peak	Maximum Rf	% Area	Peak	Maximum Rf	% Area
1	0.08	2.69	7	0.50	18.12
2	0.17	1.16	8	0.56	13.14
3	0.28	2.01	9	0.62	19.81
4	0.33	6.51	10	0.70	20.32
5	0.38	5.36	11	0.77	3.31
6	0.41	4.08	12	0.84	3.21

Table 3. Maximum Rf values and areas under curves for *Azadirachta indica* bark tannins.

Peak	Maximum Rf	% Area
1 2	0.47 0.56	55.49 44.51

Table 4. Maximum Rf values and areas under curves for *Azadirachta indica* bark saponins at 540 nm.

Peak	Maximum Rf	% Area
1 2	0.72 0.83	79.21 20.79

Table 5. Haemostatic evaluation of *Azadirachta indica* alcoholic bark extracts. Concentration is reported in mg/ml and time is expressed in seconds.

Conc.	Time required for Clot formation (seconds)							
	Whole Blood		PT		APTT		PAT	
	AIC	AIH	AIC	AIH	AIC	AIH	AIC	AIH
10	901 ± 6	1213 ± 1	> 900	> 900	14.16 ± 0.14	15.2 ± 0.2	11.26 ± 0.25	10.0 ± 0.1
50	663 ± 9	682 ± 2	20.00 ± 0.05	30.3 ± 0.2	10.0 ± 0.2	7.4 ± 0.4	5.16 ± 0.15	5.03 ± 0.05
100	405 ± 4	463 ± 2	1.43 ± 0.02	3.06 ± 0.05	2.13 ± 0.15	3.03 ± 0.05	3.13 ± 0.11	3.16 ± 0.15
250	14.32 ± 0.02	17.32 ± 0.11	< 1	< 1	2.2 ± 0.2	2.0 ± 0.1	< 1	< 1
Control	> 900		> 900		> 900		> 900	
Standard			Liquiplastin 15.08 ± 0.07		Liquicelin-E 13.85 ± 0.06		ADP 20.28 ± 0.02	

Note: *All the values in the table are average of three readings. The error bars indicated above are standard deviations.*

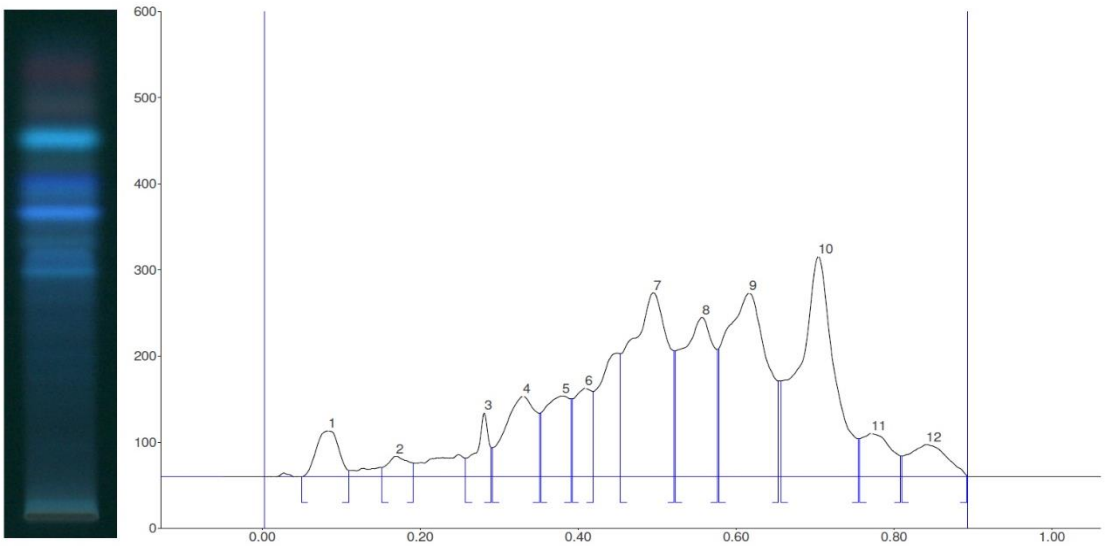

Fig 1. HPTLC fingerprint of methanolic bark extract of *Azadirachta indica* visualised at 360nm.

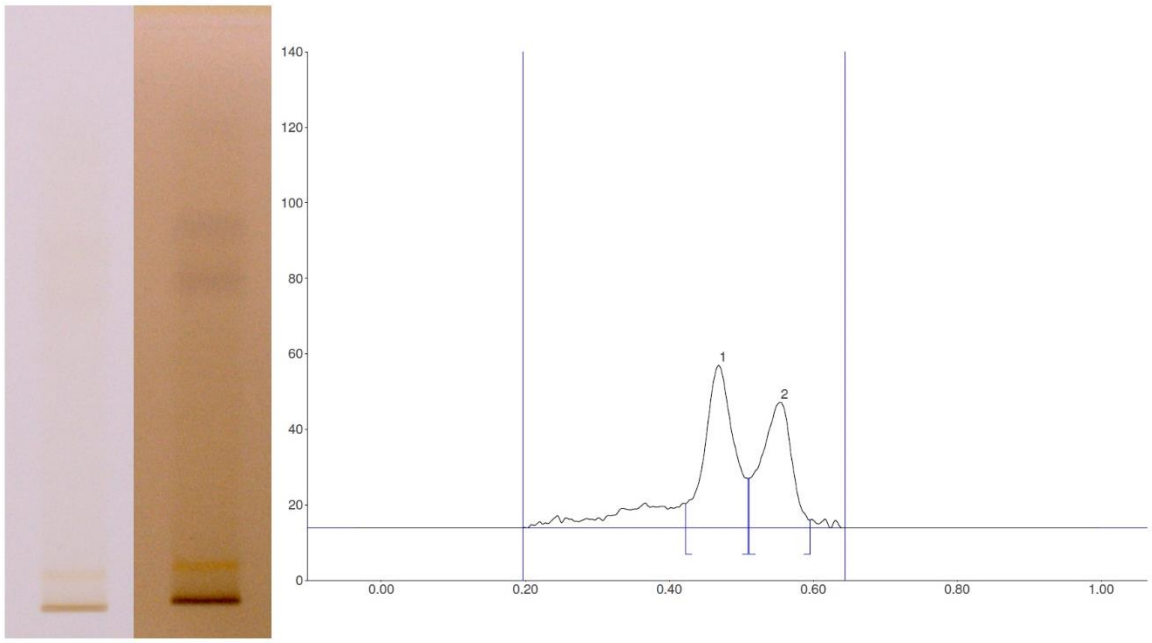

Fig 2. HPTLC profile of bark tannins of *Azadirachta indica.* The plate before derivatisation and after derivatisation.

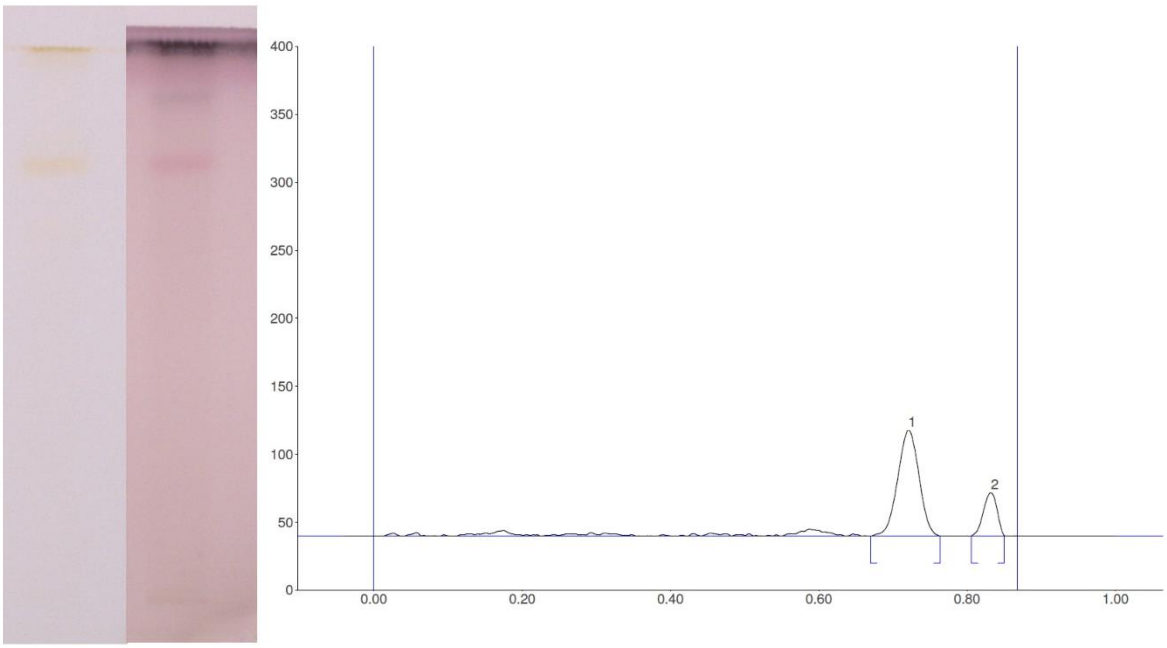

Fig. 3. HPTLC profile of saponins of *Azadirachta indica* bark. The plate before derivatisation and after derivatisation.

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Original Article

Efficacy of Vitamin E supplementation in patients with alcoholic liver disease: An open-label, prospective, randomized comparative study

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ABSTRACT

Background: The evidence that oxidative stress is involved in the pathogenesis of ALD and Vitamin E deficiency being well documented in patients of ALD, an antioxidant like Vitamin E could likely be beneficial in patients with ALD. **Methods:** This is a prospective, open labeled, randomized comparative study of eight weeks duration, involving a total of 30 adult patients diagnosed with ALD who were randomized into two groups of 15 each and were designated as group A who received standard treatment and group B who received vitamin E along with standard treatment. Biochemical parameters like Liver Function Tests, De Ritis Ratio, Hb and TLC; prognostic parameters like Child Pugh Score and Model for End-Stage Liver Disease score were recorded before and after the treatment period in each group and compared. **Results:** In group A, the change observed in total protein and child pugh score were significant ($P < 0.05$) whereas that seen in PT was highly significant ($P < 0.001$). In group B, the changes observed in total protein, A:G ratio, bilirubin, PT, MELD score, Hb and TLC were significant ($P < 0.05$) whereas those seen in albumin, PT-INR, Child Pugh Score were highly significant ($P < 0.001$). When the differences observed in various parameters in Group A were compared with those seen in Group B, the changes in albumin, globulin and A:G ratio observed in Group B were statistically significant compared to their respective changes observed in Group A. **Conclusion:** These findings suggest that Vitamin E given in adequate dose will be a useful addition for treating alcoholic liver disease, although larger studies involving more number of patients should be done.

Key words: Antioxidant, Child-Pugh score, liver function tests, model for end stage liver disease

INTRODUCTION

Alcoholic liver disease (ALD) is a term that encompasses the liver manifestations of alcohol overconsumption, including fatty liver, alcoholic hepatitis, and chronic hepatitis with liver fibrosis or cirrhosis.[1] It remains as a major cause of liver disease worldwide and is responsible for approximately 25% of deaths due to alcohol consumption.[2]

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Despite significant advances in the understanding of the pathogenesis of alcohol-related liver injury and many drugs such as corticosteroids, pentoxifylline being used in this condition, until now there are no Food and Drug Administration-approved treatments for ALD[3] and so the search for effective and safe drugs is continuing.

Oxidative stress plays a key role in the pathogenesis of ALD.[4] Alcohol metabolism includes increased synthesis of reduced form of nicotinamide adenine dinucleotide (NAD) hydrogen and suppression of mitochondrial β oxidation and increased lipid peroxidation in liver. This liberates oxygen-free radicals and decrease in mitochondrial glutathione and S-adenosyl-L-methionine levels, thus depleting the endogenous antioxidant capabilities.[5,6]

Vitamin E deficiency has been well documented in ALD.[7,8] Alcoholics with cirrhosis often have low Vitamin E levels in the liver.[9] Some observational studies suggest that Vitamin E deficiency increases the liver's vulnerability to alcohol.[10] Vitamin E has experimentally (in rats) proven hepatoprotective capabilities including membrane stabilization, reduced nuclear factor-kappa B (NF-κB) activation, reduced tumor necrosis factor (TNF) production, and inhibition of hepatic stellate cell activation[7,11-13] which are the primary pathological factors involved in the development of ALD.

Based on this background, it was thought that an antioxidant like Vitamin E could likely be beneficial in patients with ALD. As Vitamin E is economical and having least adverse effects, if its usefulness can be proved in patients with ALD, it can be utilized in these patients in a resource poor country like India and also in other developing countries of the world. In two previous studies which tested the efficacy of Vitamin E in ALD patients, there were no significant results in liver indices as one employed Vitamin E as monotherapy alone[14] and the other used a lower dose.[15] Hence, this study was aimed to evaluate whether Vitamin E supplementation in adequate doses for 8 weeks will have any advantage over standard treatment regarding biochemical parameters and prognostic indicators in patients with ALD.

METHODOLOGY

The study is an open label, prospective, randomized, comparative study of 8 weeks duration that extended from May 17 of 2014 to July 16 of 2014 conducted at our institute which is a Tertiary Care Teaching Hospital. The study includes thirty patients clinically diagnosed with ALD and all these patients are in-patients of Department of Medicine of our hospital. Patients who are stable and conscious, above 18 years of age, of either sex who met the clinical and biochemical criteria of severe alcoholic hepatitis characterized by a history of chronic and heavy alcohol intake (>80 g/day for the previous 5 years), rapid onset of jaundice in the absence of a biliary tract obstruction, painful hepatomegaly and ascites, transaminases ≥2 times above the normal value, an aspartate aminotransferase/alanine transaminases (AST/ALT) ratio ≥1.2, neutrophilia and a total bilirubin >2.5 mg/dL admitted in the Department of Medicine, were included in this study. The sample size was determined based on prevalence of ALD in this part of the region and a simple random sampling was done. Patients who did not abstain from alcohol consumption and those who did not consent for the treatment prescribed were excluded. Patients with renal failure, lung or any other organ disease, severe hypertension, malignancy, sepsis, bleeding diathesis and those with poor prognostic factors are excluded from this study. Pregnant and lactating patients were excluded from this study. A written informed consent to participate in the study was obtained from all the patients. The study protocol confirmed to the ethical guidelines of the 1975 Declaration of Helsinki and ethical clearance was obtained from Institutional Ethical Committee before commencing the study. All the patients were included in the study after explaining the patient's diagnosis, the nature and purpose of the proposed treatment, the risks and benefits of the proposed treatment, alternative treatment and the risks and benefits of the alternative treatment.

All the patients included in this study were requested to abstain from alcohol consumption. A total of thirty patients were randomized into two groups each of 15. One group (of 15 patients) designated as Group A, received the standard treatment for ALD and the other group (of 15 patients) designated as Group B, received Vitamin E as a capsule (one capsule-twice daily) along with the standard treatment. The standard treatment for the patients with ALD in our institute includes hepatoprotective drugs such as ursodeoxycholic acid or Liv 52 or both together, a diuretic like spironolactone or furosemide for treating ascites, an antibiotic like cephalosporin or metronidazole, an anti-ulcer drug like pantoprazole or ranitidine, an intravenous fluid like 25% dextrose or ringer lactate, drugs like lactulose or l-ornithine l-aspartate for prevention/treatment of hepatic encephalopathy, a beta blocker like

propranolol for prevention/treatment of variceal bleeding and chlordiazepoxide for treating symptoms of alcohol withdrawal. Vitamin E (Evion®-400 IU) capsules were used for this study.

Patient's details were collected and verified. Their present clinical severity and features of alcohol-induced liver disease were noted. The following demographic details of age, sex, present and history, duration of alcohol consumption were obtained, and the presence of co-morbid factors such as hypertension, diabetes, cerebrovascular disease, coronary artery disease, peptic ulcer disease, and chronic pulmonary disease was noted. The body mass index, body temperature, blood pressure, heart rate, and respiratory rate were recorded for all the patients. Details of clinical examination were duly noted down. Use of concurrent allopathic and alternative medications for other systemic issues was noted and excluded based on their reported interactions. Patient's clinical data are maintained confidentially.

Laboratory parameters such as total protein, albumin, globulin, albumin: Globulin ratio (A:G ratio), total bilirubin, conjugated and unconjugated bilirubin, ALT, AST, alkaline phosphatase (ALP) (i.e., patient's liver function test [LFT]), De Ritis ratio (AST: ALT ratio), prothrombin time (PT), hemoglobin (Hb), total leukocyte count (TLC) and blood urea and serum creatinine (i.e., patient's-renal function test [RFT]) were measured before and 8 weeks after treatment.

Venous blood samples were collected from the patients. Becton Dickinson-franklin lakes NJ USA vacutainer tubes (buffer - 3.2% sodium citrate) with capacity of 2.7 ml were used for collecting blood samples for PT/PT-international normalized ratio (INR) estimation. CDRICH, China vacutainertubes (ethylenediaminetetraacetic acid – K_3) with capacity for 3 ml (lavender) were used for collecting blood samples for hematological investigation PT, TLC.

Empty glass tubes were employed for serum collection and the serum obtained is used for performing LFT and RFT. Semi auto-analyzer BTS 350 (Biosystems) is used for laboratory analysis. Genius CA 5I Coagulometer is used for PT and PT-INR estimation.

The total protein level was measured using Biuret method colorimetry using Biuret reagent. The albumin level was calculated using bromocresol green (BCG) albumin assay colorimetry using BCG reagent. The direct and the indirect bilirubin levels were measured using dimethylsulfoxide method-colorimetry using total/direct bilirubin reagent and activator kit. The ALP was measured by p-nitrophenyl phosphate (pNPP) ALP assay-colorimetry using pNPP substrate and 2-amino-2-methyl-1-propanol buffer. The ALT/serum glutamate pyruvate transaminase (SGPT) was calculated using ALT/SGPT test calorimetry with SGPT enzyme and substrate reagent. The AST/SGOT was calculated using AST/SGOT test colorimetry with serum glutamate oxaloacetate transaminase enzyme and substrate reagent. The blood urea level was measured using Berthelot method-colorimetry using urease enzyme reagent and chromogen reagent. The serum creatinine level was analyzed using alkaline picrate method (Jaffe's method) using creatinine buffer and creatinine picrate reagent. All the above mentioned investigational reagents were manufactured by Beacon Diagnostics Pvt. Ltd., India. The PT and PT-INR were calculated using LIQUIPLASTIN®-liquid calcified thromboplastin reagent which was manufactured by Tulip Diagnostics Pvt. Ltd., India [Table 1].

Table 1: Laboratory parameters and their standard investigation techniques with the reagents used

Parameter	Name of the standard technique employed	Reagents used	Reagent manufacturing company's name
Total protein	Biuret method-colorimetry	Biuret reagent	Beacon Diagnostics Pvt. Ltd., India
Albumin	BCG albumin assay-colorimetry	BCG reagent	Beacon Diagnostics Pvt. Ltd., India
Bilirubin (direct and indirect)	DMSO method-colorimetry	Total/direct Bilirubin reagent and activator kit	Beacon Diagnostics Pvt. Ltd., India
ALP	pNPP (ALP) assay-colorimetry	pNPP substrate and AMP buffer	Beacon Diagnostics Pvt. Ltd., India
ALT/SGPT[a]	ALT/SGPT test-colorimetry	SGPT enzyme and substrate reagent	Beacon Diagnostics Pvt. Ltd., India
AST/SGOT[b]	AST/SGOT test-colorimetry	SGOT enzyme and substrate reagent	Beacon Diagnostics Pvt. Ltd., India
Blood urea	Berthelot method-colorimetry	Urease enzyme reagent and chromogen reagent	Beacon Diagnostics Pvt. Ltd., India
Serum creatinine	Alkaline picrate method (Jaffe's method)	Creatinine buffer and creatinine picrate reagent	Beacon Diagnostics Pvt. Ltd., India
PT[c] and PT-INR[d]	Liquiplastin®	Liquiplastin®-liquid calcified thromboplastin reagent	Tulip Diagnostics Pvt. Ltd., India

[a]SGPT: Serum glutamate pyruvate transaminase; [b]SGOT: Serum glutamate oxaloacetate transaminase; [c]PT: Prothrombin time; [d]PT-INR: Prothrombin time-international normalized ratio; ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; pNPP: p-Nitrophenyl phosphate; DMSO: Dimethylsulfoxide; BCG: Bromocresol green

Child-Pugh score and model for end-stage liver disease (MELD) were used to monitor prognosis for these patients. Both are calculated at the time of admission and 8 weeks after the treatment. Child-Pugh score/Child-Turcotte-Pugh score is used to assess the prognosis of chronic liver disease, mainly cirrhosis. The score employs five clinical measures of liver disease such as Total bilirubin in mg/dl (<2:1; 2–3:2; >3:3), serum albumin in g/dl (>3.5:1; 3.5–2.8:2; <2.8:3), PT-INR (<1.7:1; 1.7–2.3:2; >2.3:3), ascites (none: 1; mild: 2; moderate to severe: 3), and hepatic encephalopathy (none: 1; supressed with medication: 2; refractory to treatment: 3). Each measure is scored 1–3, with 3 indicating most severe derangement.[16,17] Interpretation classifies chronic liver disease into Child-Pugh Class A (5–6), B (7–9) and C (10–15), employing the added score from above. Survival after 1 year for Class A is 100%, Class B is 81%, and for Class C is 41% whereas survival after 2 years for Class A is 85%, Class B is 57%, and for Class C is 35%. MELD is a scoring system for assessing the severity of chronic liver disease using the patient's values for serum bilirubin, serum creatinine and the INR for PT to predict survival. It is calculated according to the following formula:[18]

MELD = 3.78 × ln (serum bilirubin [mg/dL]) + 11.2 × ln (INR) + 9.57 × ln (serum creatinine [mg/dL]) + 6.43 × etiology (0: Cholestatic or alcoholic, 1: Otherwise)

United Network for Organ Sharing has made the following modifications to the score:[19] If the patient has been dialyzed twice within the last 7 days, then the value for serum creatinine used should be 4.0. Any value < 1 is given a value of 1 (i.e., if bilirubin is 0.8, a value of 1.0 is used) to prevent the occurrence of scores below 0 (the natural logarithm of 1 is 0, and any value below 1 would yield a negative result). In interpreting the MELD score in hospitalized patients,[20] the 3 months mortality is: >40: 71.3% mortality, 30–39: 52.6% mortality, 20–29: 19.6% mortality, 10–19: 6.0% mortality and <9:1.9% mortality.

Statistical analysis

Data collected under each group was summarized as mean ± standard deviation. Statistical analysis was carried out using paired *t*-test for within the group comparisons and unpaired *t*-test for between the group comparisons. A $P < 0.001$ is considered statistically highly significant whereas a $P < 0.05$ is considered statistically significant and a $P > 0.05$ was considered statistically not significant. The IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp (International Business Machines Corporation-Statistical Package for Social Science program version 21.0) software was used for statistical analysis of data.

RESULTS

A total of 41 patients with ALD were assessed for eligibility, of which thirty patients were selected based on inclusion and exclusion criteria, and also based on their willingness to participate in the study. Three patients did not meet the inclusion or exclusion criteria, whereas eight patients declined to participate in the study. The remaining thirty patients were randomized into two groups of fifteen each and were designated as Group A (received standard treatment) and Group B (received standard treatment and Vitamin E). One patient was lost during the follow-up in Group B and 29 patients - 15 in Group A and 14 in Group B were followed up after the treatment duration of 8 weeks.

Table 2: Baseline demographic data and other parameters of the patients

Parameters	Baseline parameters (before the treatment) mean±SD		*P*
	Group A (standard treatment)	Group B (Vitamin E with standard treatment)	
Age	43.93±6.78	40.47±6.71	0.170*
Years of alcohol consumption	17.20±8.14	18.13±6.80	0.736*
Total protein	5.65±0.93	6.41±0.64	0.015†
Albumin	3.15±0.91	2.69±0.63	0.114*
Globulin	2.5±0.82	3.72±0.63	<0.001‡
Albumin:globulin ratio	1.50±0.91	0.77±0.29	0.009†
Total bilirubin	2.55±2.80	7.50±7.64	0.030†
Conjugated bilirubin	1.51±2.15	4.54±4.92	0.042†
Unconjugated bilirubin	1.04±0.80	2.97±3.13	0.034†
ALP[a]	129.91±59.77	155.59±122.30	0.473*
AST[b] (SGOT)	43.75±54.87	61.95±50.51	0.353*
ALT[c] (SGPT)	40.96±34.65	53.99±43.31	0.371*
De-Ritis ratio (AST/ALT)	1.21±0.74	1.19±0.51	0.955*
PT[d]	18.68±2.94	16.74±3.60	0.118*
PT-INR[e]	1.46±0.32	1.61±0.26	0.157*
Child-Pugh score	8.60±1.96	9.53±2.00	0.206*
Blood urea	31.77±19.78	29.07±22.82	0.731*
Serum creatinine	1.22±0.74	1.46±1.69	0.620*
MELD[f] score	14.87±5.79	19.47±6.83	0.057*
Hemoglobin	8.66±3.00	8.97±2.00	0.739*
Total leukocyte count	10386.67±4857.96	11543.33±7182.34	0.610*

**P value is statistically not significant (≥0.05); †P value is statistically significant (<0.05); ‡P value is statistically highly significant (<0.001). All the values are given as mean±SD. [a]ALP: Alkaline phosphatase; [b]AST: Aspartate transaminase; [c]ALT: Alanine transaminase; [d]PT: Prothrombin time; [e]PT-INR: Prothrombin time-international normalized ratio; [f]MELD: Model for end-stage liver disease; SD: Standard deviation; SGPT: Serum glutamate pyruvate transaminase; SGOT: Serum glutamate oxaloacetate transaminase*

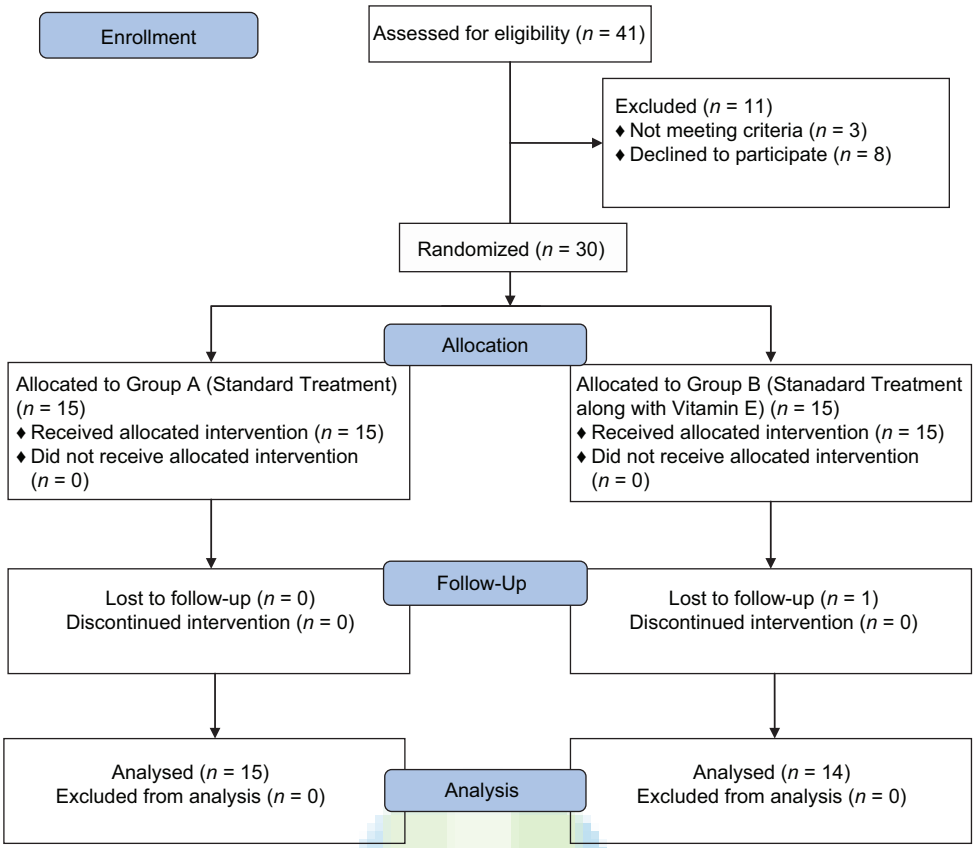

Figure 1: Consort flow diagram of patients of the study through its various phases

Figure 1 shows the flow of participants of this study through its various phases.

The two groups were homogenous with respect to most of the baseline demographic data, including patient's age, duration of alcohol consumption and all the biochemical parameters except for total protein, globulin, A:G ratio, and bilirubin levels [Table 2]. All the patients in our study belonged to the low socioeconomic status.

In Group A, when the changes in the LFT parameters were compared before and after the treatment, only the total protein value was significantly ($P < 0.05$) increased [Table 3]. There is an improvement in the other LFT parameters indicated by an increase in the levels of albumin and globulin, a decrease in the levels of total bilirubin, conjugated and unconjugated bilirubin values. There is also a decrease in the serum levels of ALT and ALP, as well as A:G ratio and De Ritis ratio, but these changes were not statistically significant.

There was a decrease of PT value observed in Group A after the treatment which was statistically highly significant ($P < 0.001$). Even though, there was a decrease in the PT-INR in the same group after the treatment, the change was not statistically significant. There was a statistically significant decrease in the Child-Pugh score after the treatment. A decrease in the -MELD score was also observed after the treatment in Group A, but the change was not statistically significant. As far as renal parameters such as serum creatinine and blood urea are concerned, there was an improvement in both the parameters, but it was not statistically significant. Even hematological parameters such as Hb and TLC showed improvement but not significant [Table 3].

In Group B, to whom Vitamin E was given in addition to standard treatment, the improvement in albumin was highly significant ($P < 0.001$) and most of the other LFT parameters such as total protein, A:G ratio, total bilirubin, conjugated and unconjugated bilirubin showed a statistically significant ($P < 0.05$) improvement. There was an improvement in the globulin, ALP, ALT, AST and De Ritis ratio, but these changes were not statistically significant [Table 4].

In contrast to Group A, where the change in the PT-INR was not statistically significant, the change in the PT-INR value in Group B was highly significant

Table 3: Student paired *t*-test results for within the group comparison of Group A

Parameters	Mean±SD		*P* (before the treatment vs. after the treatment)
	Group A (standard treatment)		
	Before the treatment	After the treatment	
Total protein	5.65±0.93	6.38±0.80	0.030†
Albumin	3.15±0.91	3.26±0.82	0.746*
Globulin	2.5±0.82	3.12±0.81	0.051*
Albumin:globulin ratio	1.50±0.91	1.15±0.48	0.207*
Total bilirubin	2.55±2.80	1.54±1.06	0.146*
Conjugated bilirubin	1.51±2.15	0.77±0.65	0.187*
Unconjugated bilirubin	1.04±0.80	0.77±0.68	0.196*
ALP[a]	129.91±59.77	124.27±46.50	0.604*
AST[b] (SGOT)	43.75±54.87	37.51±26.61	0.705*
ALT[c] (SGPT)	40.96±34.65	38.20±17.57	0.781*
De-Ritis ratio (AST/ALT)	1.21±0.74	1.07±0.62	0.571*
PT[d]	18.68±2.94	13.37±2.38	<0.001‡
PT-INR[e]	1.46±0.32	1.22±0.35	0.071*
Child-Pugh score	8.60±1.96	6.47±1.36	0.005†
Blood urea	31.77±19.78	27.87±8.98	0.484*
Serum creatinine	1.22±0.74	1.05±0.31	0.405*
MELD[f] score	14.87±5.79	11.27±3.79	0.050*
Hemoglobin	8.66±3.00	10.31±1.71	0.054*
Total leukocyte count	10,386.67± 4857.96	8460.00± 1827.88	0.133*

**P value is statistically not significant (≥0.05); †P value is statistically significant (<0.05); ‡P value is statistically highly significant (<0.001). All the values are given as mean±SD. [a]ALP: Alkaline phosphatase; [b]AST: Aspartate transaminase; [c]ALT: Alanine transaminase; [d]PT: Prothrombin time; [e]PT-INR: Prothrombin time-international normalized ratio; [f]MELD: Model for end-stage liver disease; SD: Standard deviation; SGPT: Serum glutamate pyruvate transaminase; SGOT: Serum glutamate oxaloacetate transaminase*

Table 4: Student paired *t*-test results for within the group comparison of Group B

Parameters	Mean±SD		*P* (before the treatment vs. after the treatment)
	Group B (Vitamin E with standard treatment)		
	Before the treatment	After the treatment	
Total protein	6.36±0.64	7.16±0.47	0.004†
Albumin	2.68±0.66	3.75±0.37	<0.001‡
Globulin	3.68±0.64	3.41±0.49	0.291*
Albumin:globulin ratio	0.78±0.31	1.13±0.23	0.008†
Total bilirubin	7.88±7.79	2.93±3.15	0.007†
Conjugated bilirubin	4.77±5.03	1.38±1.56	0.007†
Unconjugated bilirubin	3.11±3.19	1.55±1.99	0.008†
ALP[a]	160.63±125.29	112.91±27.09	0.138*
AST[b] (SGOT)	62.37±52.38	51.71±37.68	0.054*
ALT[c] (SGPT)	55.49±44.54	48.81±36.15	0.466*
De-Ritis ratio (AST/ALT)	1.16±0.51	1.23±0.71	0.688*
PT[d]	16.87±3.70	14.25±2.20	0.008†
PT-INR[e]	1.63±0.26	1.40±0.28	<0.001‡
Child-Pugh score	9.57±2.07	5.93±1.07	<0.001‡
Blood urea	29.07±23.69	20.41±4.78	0.210*
Serum creatinine	1.50±1.75	0.81±0.23	0.187*
MELD[f] score	19.93±6.84	12.93±4.14	0.001†
Hemoglobin	8.94±2.07	11.01±2.11	0.034†
Total leukocyte count	11,889.29± 7322.63	7678.57± 2788.21	0.027†

**P value is statistically not significant (≥0.05); †P value is statistically significant (<0.05); ‡P value is statistically highly significant (<0.001). All the values are given as mean±SD. [a]ALP: Alkaline phosphatase; [b]AST: Aspartate transaminase; [c]ALT: Alanine transaminase; [d]PT: Prothrombin time; [e]PT-INR: Prothrombin time-international normalized ratio; [f]MELD: Model for end-stage liver disease; SD: Standard deviation; SGPT: Serum glutamate pyruvate transaminase; SGOT: Serum glutamate oxaloacetate transaminase*

and that of PT was statistically significant. Even the decrease in the Child-Pugh score was highly significant. In relation to MELD score, the decrease in the score after treatment in Group B was statistically significant which contrasts with that in Group A, where the decrease in the score after the treatment was not statistically significant. There was a statistically significant ($P < 0.05$) improvement in the hematological parameters like TLC. The renal parameters such as serum creatinine and blood urea showed improvement but were not statistically significant [Table 4].

The mean percentage changes of various parameters in Group A and Group B are shown in [Table 5]. On the whole, the mean percentage changes observed were more in Group B compared to Group A, except in the cases of total protein, globulin, conjugated bilirubin, AST, ALT, PT and Hb where we observe a higher change in mean percentage in Group A compared to Group B. In Group A, the highest change was observed in conjugated bilirubin (49.82%) followed by change in ALT (45.83%), whereas in Group B,

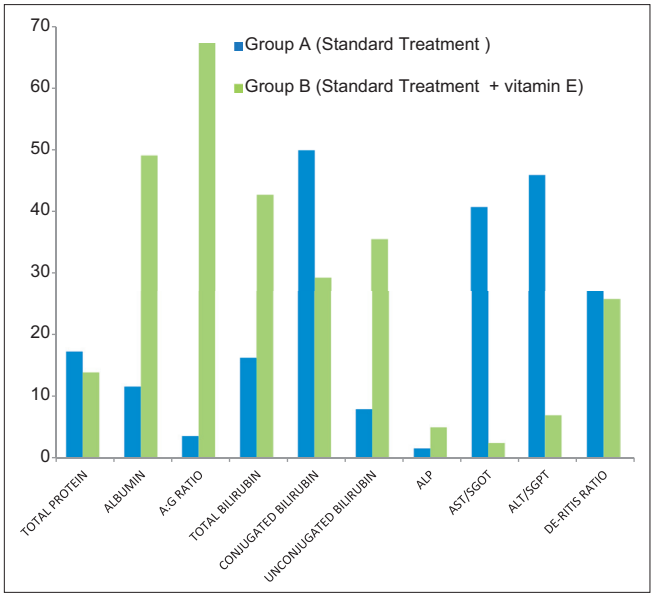

Figure 2: Mean percentage change observed in both the groups for biochemical parameters. A:G ratio = Albumin to globulin ratio; ALP = Alkaline phosphatase; AST/SGOT = Aspartate aminotransferase also known as serum glutamate oxaloacetate transaminase; ALP/SGPT = Alanine aminotransferase also known as serum glutamate pyruvate transaminase; De-ritis ratio = Ratio of AST to ALT

Table 5: Mean percentage change in Group A and Group B

Parameters	Group A (standard treatment)	Group B (Vitamin E with standard treatment)
Total protein	17.25	13.86
Albumin	11.58	49.03
Globulin	43.00	3.62
Albumin:globulin ratio	3.60	67.2
Total bilirubin	16.27	42.74
Conjugated bilirubin	49.82	29.40
Unconjugated bilirubin	7.97	35.59
ALP[a]	1.55	4.97
AST[b] (SGOT)	40.72	2.49
ALT[c] (SGPT)	45.83	6.91
De-Ritis ratio (AST/ALT)	27.05	25.72
PT[d]	27.07	11.60
PT-INR[e]	13.14	13.86
Child-Pugh score	20.29	36.57
Blood urea	13.69	10.28
Serum creatinine	4.49	16.18
MELD[f] score	16.79	31.36
Hemoglobin	35.28	32.26
Total leukocyte count	3.68	23.67

[a]ALP: Alkaline phosphatase; [b]AST: Aspartate transaminase; [c]ALT: Alanine transaminase; [d]PT: Prothrombin time; [e]PT-INR: Prothrombin time-international normalized ratio; [f]MELD: Model for end-stage liver disease; SGPT: Serum glutamate pyruvate transaminase; SGOT: Serum glutamate oxaloacetate transaminase

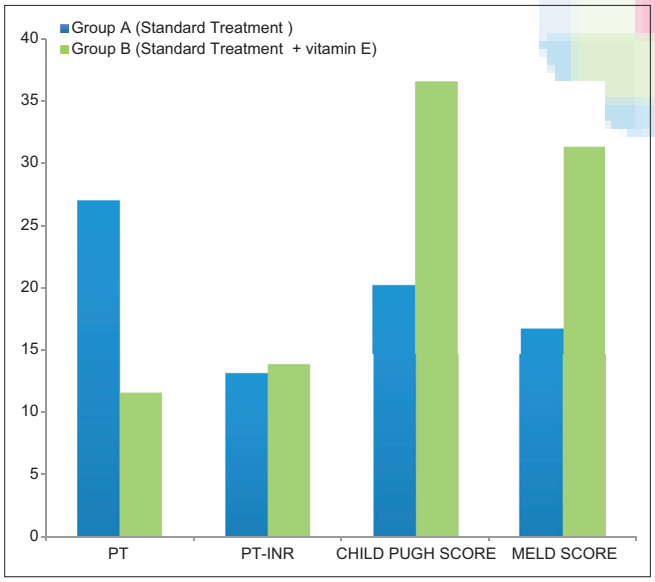

Figure 3: Mean percentage changes observed in both the groups for prothrombin time, prothrombin time-international normalized ratio, Child-Pugh score and MELD score. PT = Prothrombin time; PT-INR = Prothrombin time-international normalized ratio; MELD = Model for end-stage liver disease

highest change was observed in A:G ratio (67.2%) followed by change in albumin (49.03%) [Figure 2]. The mean percentage changes in PT, PT-INR, Child-Pugh score and MELD score are shown in Figure 3. In relation to Child-Pugh score, the mean percentage change in Group B was 36.57% which is more than that in Group A of 20.29%. Similarly in MELD score, the mean percentage change in Group B was 31.36% which is more than that in Group A of 16.79%.

DISCUSSION

To the best of our knowledge, there is no study evaluating clinical efficacy of Vitamin E on Indian patients with ALD. This study for the 1st time assessed the effect of Vitamin E supplementation to the standard treatment in patients with ALD.

The two groups were homogenous with respect to most of the baseline demographic data, including patient's age, duration of alcohol consumption and all the biochemical parameters except for total protein, globulin, A:G ratio and bilirubin levels [Table 2]. Nonsignificance of the demographic variables between Group A and B indicates that the two groups were properly randomized [Table 2]. Even though, there are several studies demonstrating that women develop liver disease after exposure to lower quantities of alcohol and over shorter time periods,[21,22] in our study, all the thirty patients who were included in the study were males. This may be due to sociocultural aspects of this country, where almost exclusively males are involved in alcohol intake. Furthermore, we observe that, at a very early age only, people of this region are suffering from ALD. This may be ascribed to the habit of consuming the alcohol from a very early age compared to other parts of the country. Furthermore, as our hospital is located in a union territory, the reduced cost of alcohol beverages due to the reduced tax compared to other parts of the country, also is contributing here to the increased prevalence of alcoholism and subsequently ALD in this region.

The normal range of various parameters that were analyzed as a part of this study are as follows: Total protein is 6.7–8.6 g/dl; albumin is 3.5–5.5 g/dl; globulin is 2.0–3.5 g/dl; A:G ratio is 1.2–1.5:1; total bilirubin is 0.3–1.3 mg/dl; conjugated bilirubin is 0.1–0.4 mg/dl; unconjugated bilirubin is 0.2–0.9 mg/dl; ALP is 45–115 U/L; AST is 12–38 U/L; ALT is 7–41 U/L; PT is 12.7–15.4 s; De Ritis ratio: 1.1:1; PT-INR is 0.8–1.2; TLC is 3.54–9.06 × 10^3/mm^3; blood urea is 7–20 mg/dl; serum creatinine is 0.6–1.2 mg/dl; Hb for adult males is 13.3–16.2 g/dl and for adult females is 12.0–15.8 g/dl.[23]

In patients with ALD, the albumin levels will be deceased because of decrease in the synthetic function of the liver, globulin levels will be increased in response to antigenic stimulation reflecting the immune changes associated with ALD, A:G ratio reversed obviously. Total bilirubin and especially the conjugated bilirubin levels will rise, levels of ALP, AST, and ALT will rise and because AST will be much more raised compared to ALT, the De Ritis ratio will also be increased. PT and PT-INR are increased as there is reduced synthesis of clotting factors. Hb is decreased due both to loss of synthetic function and to hemolysis and TLC is increased because of the inflammation.

In relation to the LFT parameters, in Group A, only the total protein value was significantly ($P < 0.05$) improved [Table 3], whereas in Group B, to whom Vitamin E was given in addition to standard treatment, in addition to total protein, A:G ratio, total bilirubin, conjugated and unconjugated bilirubin showed a statistically significant ($P < 0.05$) improvement and also the improvement in albumin and PT-INR values were highly significant ($P < 0.001$) indicating that addition of Vitamin E has actually improved the outcome of many more LFT parameters compared to standard treatment given alone [Table 4]. This was in accordance with a previous study carried out by Mahmood *et al*., where Vitamin E supplementation (500 mg/d) for 3 months in 17 chronic hepatitis C patients resulted in modest reduction of serum ALT levels to 63 IU/l from baseline levels of 73 IU/l.[24] Similar results were reported in another study with a prospective randomized double-blind cross over design in 23 chronic hepatitis C patients who were refractory to interferon. Vitamin E supplementation (800 IU/day) for 12 weeks reduced serum ALT from 90 to 68 IU/l at the end of treatment.[25] However, both the above studies evaluated the effect of Vitamin E in patients of chronic hepatitis C and not in those having ALD.

In relation prognostic scores, the decrease in the Child-Pugh score was highly significant in Group B, whereas it was just significant in Group A [Tables 3 and 4]. The decrease in the MELD score Group B was statistically significant, whereas in Group A, it was not statistically significant [Tables 5 and 6]. Both these results indicate an improved chance of survival with addition of Vitamin E to the treatment.

In relation to hematological parameters, in Group B, there was a statistically significant ($P < 0.05$) reduction of TLC and increase in Hb which were not significant in Group A [Tables 3 and 4] which indicate that supplementing Vitamin E has caused a reduction in the hepatic inflammation and an improvement in the synthetic function of the liver, which resulted in an increase in the synthesis of the protein part-globin of Hb.

Table 6: Independent *t*-test results for comparison of differences in both the groups

Parameters	Mean percentage change (mean±SD)		*P* (Δ in Group A vs. Δ in Group B)
	Group A (standard treatment)	Group B (Vitamin E with standard treatment)	
Total protein	17.25±31.15	13.86±15.43	0.712*
Albumin	11.58±42.14	49.03±43.95	0.027†
Globulin	43.00±72.66	3.62±27.22	0.032†
Albumin:globulin ratio	3.60±49.88	67.2±73.46	0.006†
Total bilirubin	16.27±54.13	42.74±36.07	0.132*
Conjugated bilirubin	49.82±191.51	29.40±89.73	0.165*
Unconjugated bilirubin	7.97±127.62	35.59±35.37	0.222*
ALP[a]	1.55±31.75	4.97±69.89	0.753*
AST[b] (SGOT)	40.72±137.88	2.49±45.15	0.266*
ALT[c] (SGPT)	45.83±125.79	6.91±54.00	0.287*
De-Ritis ratio (AST/ALT)	27.05±100.44	25.72±124.69	0.975*
PT[d]	27.07±15.72	11.60±27.20	0.077*
PT-INR[e]	13.14±29.35	13.86±11.20	0.931*
Child-Pugh score	20.29±27.97	36.57±11.19	0.051*
Blood urea	13.69±63.23	10.28±38.78	0.227*
Serum creatinine	4.49±45.06	16.18±34.34	0.175*
MELD[f] score	16.79±35.51	31.36±22.09	0.194*
Hemoglobin	35.28±61.39	32.26±54.97	0.890*
Total leukocyte count	3.68±52.94	23.67±34.85	0.238*

**P value is statistically not significant (≥0.05); †P value is statistically significant (<0.05). All the values are given as mean±SD. Δ: Difference; [a]ALP: Alkaline phosphatase; [b]AST: Aspartate transaminase; [c]ALT: Alanine transaminase; [d]PT: Prothrombin time; [e]PT-INR: Prothrombin time-international normalized ratio; [f]MELD: Model for end-stage liver disease; SD: Standard deviation; SGPT: Serum glutamate pyruvate transaminase; SGOT: Serum glutamate oxaloacetate transaminase*

When the differences observed in various parameters in Group A were compared with those seen in Group B using independent Student's *t*-test, the changes observed in albumin, globulin and A:G ratio in Group B were statistically significant ($P < 0.05$) when compared to the respective changes in Group A [Table 6]. Even though, the remaining parameters have improved better in Group B compared to Group A, these changes were not statistically significant [Table 6]. Increase in albumin indicates the improved synthetic function of the liver. Globulin levels increased following treatment in Group A but decreased in Group B. As mentioned earlier, the globulin levels will increase during liver disease in response to antigenic stimulation reflecting the immune changes associated with ALD. The decrease in globulin levels in Group B signifies the improvement

because of addition of Vitamin E which being an antioxidant decreases the antigenic stimulation and thus reducing the immunological damage. These results indicate that addition of Vitamin E improved the liver function in comparison to the standard treatment given alone. This is in contrast to the results seen in a previous study of Vitamin E in ALD patients, in which some normalization of serum hyaluronic acid levels was observed, but without significant changes in indices of liver function.[14] This may be due to the use of Vitamin E as monotherapy only, in that study. However in our study, we used it only as a supplementation to standard treatment for ALD patients.

In a previous study which was carried out with Vitamin E alone in ALD patients, has showed no benefit which might be due to an inadequate dose (500 mg/day)[15] used in that study. In this study, we have used a recommended and an adequate dose of Vitamin E of 800 mg/day.

As mentioned earlier, oxidative stress plays a key role in the pathogenesis of ALD. Metabolism of alcohol leads to increased liver oxidative stress via formation of acetaldehyde which can form hybrid-adducts with reactive residues mediating lipid peroxidation and nucleic acid oxidation,[26] excessive reduction of NAD leading to the transfer of an electron to molecular oxygen to generate reactive species such as superoxide anion[27] and induction of CYP2E1 which interacts with cytochrome reductase leading to electron leaks in the respiratory chain and reactive oxygen species production.[28] Even urinary levels of 8-isoprostanes, a marker of oxidative stress and lipid peroxidation are elevated in subjects after alcohol intake compared to normal.[29]

Alcohol intake also increases the intestinal permeability to a variety of substances that include bacterial endotoxins, such as lipopolysaccharide[30] that activates the NF-κB in Kupffer cells causing exaggerated transcription of pro-inflammatory cytokines such as TNF-α, interleukin-6 (IL-6), and transforming growth factor-beta (TGF-β).[31,32] Whereas TNF-α and IL-6 are mainly involved in cholestasis and synthesis of acute-phase proteins, TGF-β may be critically involved in fibrogenesis through the activation of hepatic stellate cells.

Vitamin E, being an antioxidant has caused a statistically significant improvement in some of the liver function parameters and prognostic indicators in our study which can be explained by its proven hepatoprotective mechanisms mentioned previously which include membrane stabilization, reduced NF-κB activation, reduced TNF production, and inhibition of hepatic stellate cell activation.[7,11-13]

CONCLUSION

This study shows that in patients with ALD, Vitamin E supplementation to standard treatment has shown better improvement in liver function parameters and prognostic indicators than standard treatment given alone, although it was not statistically significant in most of them except in albumin, globulin, and A:G ratio. Among the prognostic indicators, even though both the Child-Pugh score and MELD score showed significant improvement, it was the MELD score that showed a very high improvement indicating that short-term mortality is better improved compared to long-term mortality with Vitamin E supplementation. These findings suggest that Vitamin E, given in adequate dose will be a useful addition for treating ALD, although larger studies involving more number of patients should be done.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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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

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^{o}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^{o}C before use in test procedure
- Recap the reagent vial and replace immediately to 2-8^{o}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^{o}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':

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

Or as International Normalized Ratio (INR), $\textbf{INR} = (\textbf{R})^{ISI}$, 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, ɣ-1-globulin, and α-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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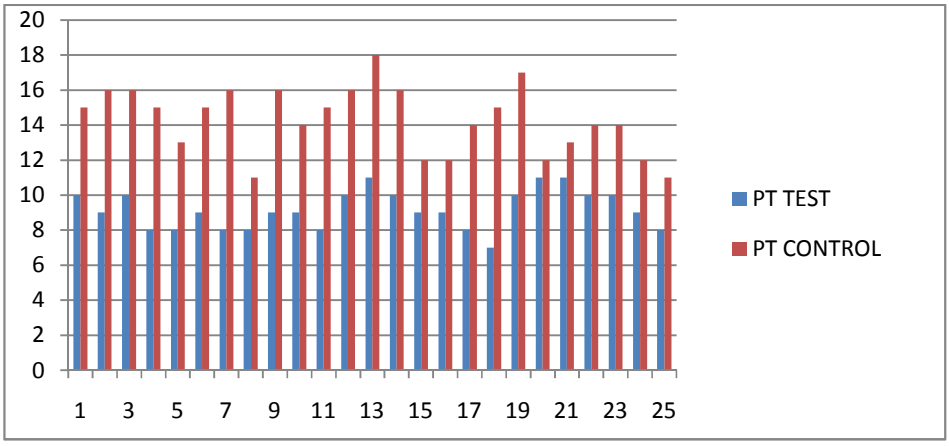
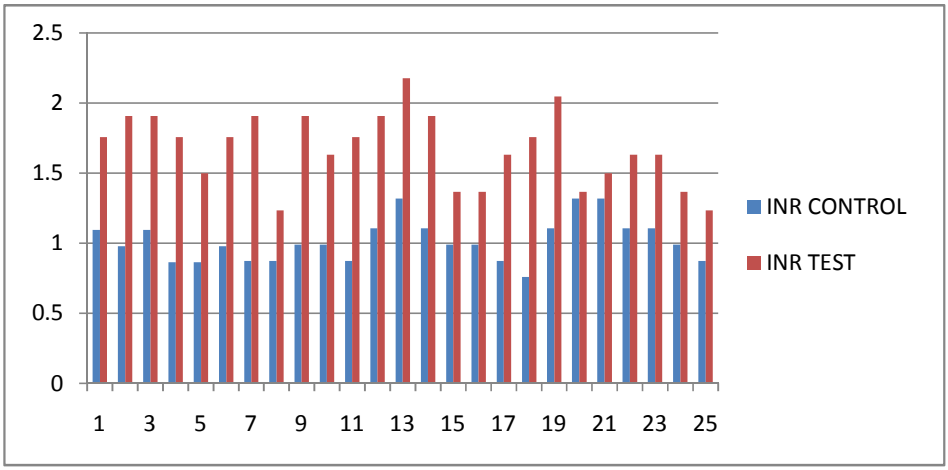
RESULT PT:

	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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COMPARATIVE ACTIVATED PARTIAL THROMBOPLASTIN TIME (APTT) AND PROTHROMBIN TIME (PT) PROFILE OF INDIAN SNAKES *Naja naja, Echis carinatus, Vipera russelli* HELPFUL IN ESTABLISHING THEIR SUPERIOR THERAPEUTIC PROCOAGULANT EFFICACY

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ABSTRACT
Comparative profile studies on the Activated Partial Thromboplastin Time (APTT) and the Prothrombin Time (PT) of Naja naja (Indian cobra), Vipera russelli (Indian Russell's viper) Echis carinatus (Indian saw scaled viper) proving their superior Procoagulant efficacy than the normal platelet poor plasma.

KEYWORDS: Naja naja, Vipera russelli, Echis carinatus, Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT).

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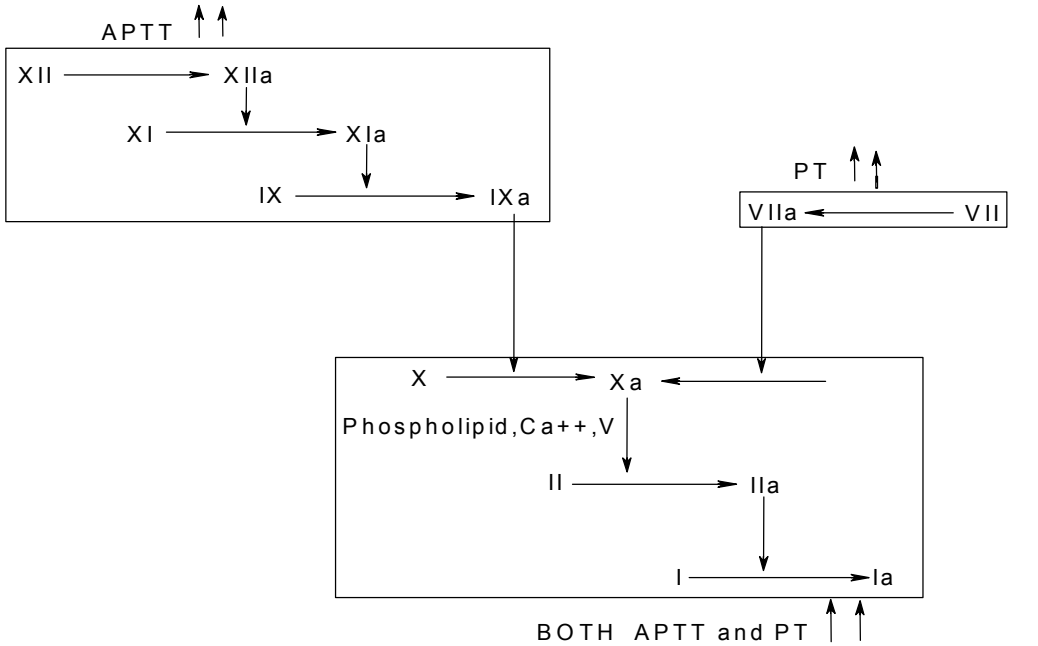
INTRODUCTION

Demonstrative methods for the measurement of blood coagulation time conventionally employed, include those measuring the Activated APTT, PT and fibrinogen levels. Alternatively, a thrombotic event may also be confirmed by measuring the levels of soluble fibrin or fibrin degradation products in circulation[1].The venoms of several species of snakes contain enzymes that convert the Zymogen Prothrombin into the enzyme thrombin and /or its catalytically active precursor meizothrombin. Both the activated products convert fibrinogen into fibrin, thereby resulting in plasma coagulation[2].Activated partial Thromboplastin time's (APTT) normal range is approximately 25-35 seconds, the time taken by citrated platelet poor plasma to clot in the presence of optimum concentration of contact activator, phospholipid (platelet substitute /partial Thromboplastin) and calcium. It screens for all coagulation factors deficiencies; except for factor VII.[3] The Activated Partial Thromboplastin (APTT) tests the integrity of the intrinsic and the final coagulation pathways. Thromboplastin is a tissue extract containing tissue factor and a phospholipid. As the test uses only the phospholipid part as a substitute for the platelet membrane in activating factor XII, it is known as partial Thromboplastin. Deficiency of factors XII, XI, IX, VII, High molecular weight kininogen (HMWK) or kallikerin will prolong the APTT to accelerate the PTT reaction. An activator (Celite-diatomaceous earth, Kaolin) is added and hence the term Activated[4]. An abnormal APTT is associated with quantitative or qualitative deficiencies in factor XII, XI, IX, VIII, and V or X[5]. Abnormal APTT values are obtained in the following conditions like i) Factor deficiencies, which may be corrected by the addition of normal plasma. ii) Presence of inhibitors like heparin, lupus anticoagulant (LA), specific factor inhibitors, fibrinogen degraded products (FDP), which cannot be corrected by normal plasma addition.

Prothrombin time (PT); The Prothrombin time (PT) measures the integrity of the extrinsic pathway and the common coagulation pathway. Deficiencies of factor VII or vitamin K and warfarin therapy cause an elevation in the PT .Inactivation of factor II by large doses of heparin also prolong the PT. PT measures the time taken by citrated platelet poor plasma to clot in presence of optimum concentration of tissue Thromboplastin and calcium. The results are expressed as PR Prothrombin ratio (Patients/control). PR greater than 1.2 is considered as abnormal**.** It is very important that in the event of an injury or accident or a blood disorder, blood has to clot in particular time range (The normal clotting time for a healthy individual weighing 70kgs is (5-15minutes) and therefore, if the clotting time is prolonged, there would be copious loss of blood resulting in the drop of systolic blood pressure, followed by circulatory collapse, ultimately resulting in a state of shock and heart failure .Hence, such severe loss of blood should be prevented and if coagulation cannot be brought about due to reasons like genetic disorders like hemophilic and hemorrhagic syndromes, circulating anticoagulants, thrombocytopenic purpuras, dental extractions, prostractomy, Ophthalmological surgeries, gastroenterology, cosmetic surgeries and post delivery bleedings, one must make use of therapeutic external procoagulant support to hasten the blood coagulation process[6-7].



Implication of raised PT & APTT in the Coagulation system

In the present investigation we have compared the APTT and PT of crude venoms Indian venomous snakes with the normal APTT and PT of human platelet poor plasma.

MATERIALS AND METHODS

Crude venoms of Naja naja (Indian cobra), Vipera russelli (Indian Russell's viper), Echis carinatus (Indian saw scaled viper) were procured from The Irula snake catcher's society, Chennai, India. Human citrated platelet poor plasma was procured from the Karnatak Cancer Research Institute Navnagar Hubli.,Test kits for APTT and PT determination were procured from Tulip Diagnostics (P) LTD. Unit II first floor, Plot nos. 92/96,Phase II C, Verna IND. EST. Verna, Goa -403 722,India

Activated Partial Thromboplastin Time (APTT) Determination

Reagents attained room temperature before prewarming to 37^0C for testing purposes. The kit reagents were mixed well by gentle swirling. To a 12x15mm test tube, add 0.1ml crude venom sample and 0.1ml liquiceline-E, shake the tube gently to mix. Next place the tube in an incubator for 3minutes at 37^0C. Following incubation, add 0.1ml of prewarmed calcium chloride and simultaneously start the stop watch to measure the time of clot formation[8].

Prothrombin Time (PT) Determination

Reagents were brought to room temperature before prewarming to 37^0C for testing purposes. Kit reagents were mixed well. To a 12x15mm tube add0.1ml of crude snake venom which was incubated for 3minutes at 37^0C.Next add 0.2ml of liquiplastin reagent (prewarmed at 37^0C for at least 3minutes) and simultaneously start the stop watch and note the time of appearance of first fibrin strand[9-10].

RESULT

Comparative APTT and PT of the crude venoms of Naja naja, Vipera russelli and Echis carinatus proving their superior procoagulant efficacy is shown in the **table 1**.

DISCUSSION

Indian venomous snakes, Echis carinatus and Naja naja take about 13 times less time for the clot formation, where as Vipera russelli crude venom takes about 2.23 times less time for clot formation, as their APTT and PT times are far less than the normal platelet poor human plasma. Hence, one could

make use of these venoms in the treatment of coagulation disorders, thus proving their superior Procoagulant efficacy over the existing commercial pharmaceutical preparations[11].

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Table 1: The time taken for the APTT and PT by the different snake venoms

Samples	APTT (Time in seconds)	PT (Time in seconds)
Normal platelet poor human plasma	35	5.62
Vipera russelli crude venom	15.66	4.32
Echis carinatus crude venom	2.65	2.57
Naja naja crude venom	2.65	2.53

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The antioxidant, anticancer and anticoagulant activities of *Acanthus ilicifolius* L. roots and *Lumnitzera racemosa* Willd. leaves, from southeast coast of India

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ABSTRACT

The present study was designed to evaluate and determine the phytochemical composition, antioxidant, anticancer and anticoagulant activities of the aqueous extracts of *Acanthus ilicifolius* roots and *Lumnitzera racemosa* leaves from Pichavaram mangrove forest, Tamil Nadu, India based on folklore knowledge. The preliminary phytochemical screening revealed the presence of the following classes of bioactive compounds: phenols, flavonoids, alkaloids, terpenoids, sterols, tannins, carbohydrates, cardiac glycosides, saponins and quinones. The total phenolic content was reported much higher as compared to the total flavonoid content mainly in the leaf extract of *L.racemosa*. This mostly contributes to the antioxidant power of the extracts, which is affirmed by the IC_{50} values of the crude extracts in the DPPH assay, which was lower than the ABTS assay. The FRAP assay also exhibited a consistent increase in reducing ability with increase in the concentration which is indicative of the extract's antioxidant potential. The extracts were also reported to exhibit *in vitro* cytotoxicity and apoptosis inducing ability in Hep G2 cancer cells. And the anticoagulant study conducted provided a first hand report for the plants exhibiting the property. However, further studies must be conducted for secondary metabolite profiling to decipher and clarify the compound(s) responsible for the reported activities of the plant crude extracts.

INTRODUCTION

Mangroves are a category of plants, the use of which for medicinal purposes dates back to the year 1230 (Bandaranayake, 1998). The current study aims at documenting a maiden report on the antioxidant, anti-cancerous and anti-coagulant properties of specific parts of the mangrove species: *Acanthus ilicifolius* L. (Acanthaceae) and *Lumnitzera racemosa* Willd. (Combretaceae), based on folklore knowledge, from the Pichavaram mangrove forest situated in the state of Tamil Nadu, India. Prominent survey reports from India suggests the use of roots of *Acanthus ilicifolius* and leaves of *Lumnitzera racemosa* for treatment of snake bites, rheumatism, skin allergies, blood purifier, asthma, diabetics etc. (Bandaranayake, 1998; Pattanaik *et al*., 2008) And as such, the specific plant parts were collected to primarily investigate their phytochemical & antioxidant properties and their role as anti-cancer agents. They were also tested for blood anti-coagulating property, which in sum would scientifically validate their folklore functionality.

MATERIALS AND METHODS

Sample collection and authentication

Fresh samples of mangroves (*Acanthus ilicifolius* and *Lumnitzera racemosa*) were collected from Pichavaram mangrove forest located along the southeastern coastline, in the state of Tamil Nadu, India. Guided by the knowledge of folklore use of plants for different medicinal purposes, specifically, the roots of *Acanthus ilicifolius* and the leaves of *Lumnitzera racemosa* were collected from this region during the month of April-May 2013. The plant sample specimens were identified and preserved in the herbarium under the collection number AUCASMBTR01 and AUCASMBTR 09.

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Sample preparation and extraction

The plant samples collected were then thoroughly washed with distilled water for the removal of contaminants, mud and dirt. After shade drying at room temperature for 3-4 days, the plant samples were pulverized for use in the extraction purpose.

For the preparation of extracts (root and leaf), 5g of the powdered plant parts (root and leaf) were soaked in 50ml of water in a beaker and was kept on a magnetic stirrer for 24h at room temperature. The extracts were then filtered using Whatman No. 1 filter paper and the filtrates were concentrated using rotary vacuum evaporator. The dried extracts were finally stored at -20 °C until further use (Solomon Charles Ugochukwu *et al*., 2013).

Chemicals, reagents and cell lines

All the chemicals and reagents used in the phytochemical and antioxidant assays were obtained from the certified suppliers and were of the highest analytical grade. The Hep G2 (human liver hepatocellular carcinoma) cell line was procured from the cell repository of National Center for Cell Science (NCCS), Pune, India. Liquicelin-E and Liquiplastin reagents, for the anticoagulation studies were purchased from Tulip Diagnostics Pvt. Ltd., India.

Phytochemical screening of the extracts

Using standard protocol (Solomon Charles Ugochukwu *et al*., 2013), the freshly prepared aqueous extracts were subjected to qualitative phytochemical screening for detecting the presence of the following bioactive chemical constituents: phenols, flavonoids, alkaloids, terpenoids, sterols, tannins, proteins and amino acids, carbohydrates, cardiac glycosides, saponins and quinones.

Determination of total phenolic content (TPC*)*

The standard protocol (Ainsworth and Gillespie, 2007; Barku *et al*., 2013) for estimating the total phenol content of the extracts using Folin-Ciocalteu reagent was adapted with little modifications. Gallic acid (20-100 µg/ml) was used as a standard. To a volume of 1 ml (100 µg/ml) of aqueous plant extract, 5 ml of Folin-Ciocalteu reagent (diluted to 10 folds) and 4ml of sodium carbonate solution (7.5%) were added. The reaction mix was then allowed to stand in dark at room temperature for 30 min and absorbance of the blue color developed was recorded at a wavelength of 765 nm using a Shimadzu UV-1800 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). The TPC of the extracts were determined using the linear regression equation acquired by plotting gallic acid standard curve. The results were calculated as mean ± SD (n=3) and expressed as µg/ml of gallic acid equivalent (GAE) of dry extract.

Determination of total flavonoid content (TFC)

The aluminium chloride colorimetric method (Barku *et al*., 2013) was used for determining the total flavonoid content of the plant aqueous extracts. Quercetin (20-100 µg/ml) was used as the standard. Each plant extract (1 mg/ml, 0.25 ml) was added to 1.25 ml of distilled water and then 0.075 ml of sodium nitrite solution (5%) was added. The reaction mix was incubated at room temperature for 5 min, following which 0.15 ml of 10% aluminum chloride solution was added and the mix was again allowed to stand for another 6 min at room temperature before adding 0.5 ml of 1M sodium hydroxide solution and finally diluting the reaction mix with 0.275 ml of distilled water. The absorbance was recorded at 510 nm in a Shimadzu UV-1800 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). The TFC of the plant extracts were calculated as mean ± SD (n=3) using the linear regression equation obtained by plotting quercetin standard curve and expressed as µg/ml of quercetin equivalent (QE) of dry extract.

In vitro antioxidant assays

DPPH radical scavenging activity

The scavenging activity of the plant aqueous extracts against DPPH (2-diphenyl-2-picrylhydrazyl) radical was determined by the standard method (Banerjee *et al*., 2008; Zheleva-Dimitrova *et al*., 2010), with few modifications. An aliquot of 200 µl of different concentrations (20-100 µg/ml) of each of the extracts was mixed with 3.9 ml of freshly prepared DPPH solution (25 mg/L) in methanol. The reaction mixture was mixed and incubated for 30 min at room temperature in dark and its absorbance was recorded at 517 nm. Ascorbic acid was used as the reference standard. The DPPH scavenging capability was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [(Abs_{(control)} - Abs_{(sample)})/Abs_{(control)}] \times 100$$

where $Abs_{(control)}$ is the absorbance of the DPPH radical in methanol; $Abs_{(sample)}$ is the absorbance of the DPPH solution in presence of the aqueous extract or standard. The antioxidant value was expressed as IC_{50}, which is defined as the concentration in µg of the dry extract per ml that inhibits the formation of DPPH radical by 50%. Each value was determined from the slope of the linear regression equation (y = mx + c), obtained by plotting the ascorbic acid standard curve. All results were calculated as mean ± SD (n=3).

ABTS radical scavenging assay

For ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid**)]** assay the standard protocol (Floegel *et al*., 2011; Shalaby and Shanab, 2013) was followed with slight modifications. Initially, two stock solutions of 7 mM ABTS and 2.4 mM potassium persulfate were prepared. The working stock solution was then prepared by mixing the two solutions in equal volume and was allowed to react for 16 h at room temperature in the dark. After incubation, the solution was diluted by mixing 1 ml of ABTS·+ with 60 ml methanol to obtain an absorbance of 0.706 ± 0.01 units at 734nm. For each assay the ABTS·+ solution was freshly prepared. Then, 1 ml of aqueous extract was added to 1 ml of ABTS·+ solution and was allowed to react for 7 min at room temperature. After the incubation, absorbance of the reaction mix

was recorded at 734 nm. Ascorbic acid was used as the experimental standard with which the ABTS radical scavenging capacities of the extracts were compared. The percentage inhibition value was calculated as:

ABTS radical scavenging activity (%)= [($Abs_{(control)} - Abs_{(sample)}$)/$Abs_{(control)}$] × 100

where $Abs_{(control)}$ is the absorbance of the ABTS radical in methanol; $Abs_{(sample)}$ is the absorbance of the ABTS in presence of the aqueous extract or standard. The antioxidant value was expressed as IC_{50}, which is defined as the concentration in µg of the dry extract per ml that inhibits the formation of ABTS radical by 50%. Each value was determined from the slope of the linear regression equation (y = mx + c), obtained by plotting the ascorbic acid standard curve. All results were calculated as mean ± SD (n=3).

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing power method (Oyaizu, 1986; Chen *et al*., 2015) was followed for deducing the reducing power of the aqueous extracts. 200 µl of extract was added to 500 µl of phosphate buffer (0.2 M, pH 6.6) and 500 µl of 1 % potassium ferricyanide. The reaction mix was then incubated at 50 °C for 20 min, after which 10 % trichloroacetic acid was added and was subjected to centrifugation at 3000 rpm for 10 min. 700 µl of the supernatant was then taken into a fresh tube and to it 700 µl of distilled water and 140 µl of freshly prepared 1 % ferric chloride solution were added. The absorbance was then recorded at 700 nm. Ascorbic acid was used as the reference standard.

Cytotoxicity and apoptosis studies on Hep G2 cancer cell line

For the MTT assay (Nguyen *et al*., 2015), the Hep G2 (human liver hepatocellular carcinoma) cells were harvested during the logarithmic growth phase and seeded in 96-well plates when the cell density in the culture flask had attained 70-80 % confluency. In each well, a cell density of 3×10^3 cells in a volume of 100 µl was maintained and the plate was incubated for 24 h in a CO_2 incubator. The crude extracts were prepared as a stock of 1 mg/ml in the Dulbecco's Modified Eagle's Medium (DMEM). The cells were then treated with increasing concentrations of the crude extracts (10, 25, 50, 100 µg/ml) for 48 h in a CO_2 incubator. 10 µl of MTT [3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] solution (5 mg/ml in PBS, pH 7.2) was then added to each well and the plate was further incubated for 4 h in an incubator. After incubation, the medium was carefully decanted to air-dry the formazan crystals in dark. Later, 100 µl of DMSO was added to the wells followed by gentle shaking to solubilize the formazan dye for 15 min at room temperature. Absorbance was then measured using a Synergy H4 microplate reader at 570 nm and the percent cell viability was calculated.

In order to determine the level of apoptosis in Hep G2 cells, DAPI (4',6-diamidino-2-phenylindole) staining method was performed (Lian *et al*., 1998). The Hep G2 cells were treated with respective crude extracts at its IC_{50} concentration and incubated for 24 hours. The cells were then harvested and pelleted by centrifugation at 1100 rpm for 10 min. The medium was aspirated and cells were re-suspended in 1 ml PBS. Again the cells were pelleted and 1 ml of permeabilization buffer (PBS/0.01 M glycine/0.1% Triton X-100) was added followed by incubation for 10 min with gentle rocking. The cells were re-washed with 1 ml PBS and fixed with 1 ml of 2% paraformaldehyde in PBS buffer containing 10 µg of DAPI [2µl from stock: 5 mg/ml DAPI solution in N,N-Dimethyl formamide (DMF)] for 10 min in the dark. The stained cells were then washed in 1 ml PBS for 5 min. Finally an aliquot was put onto a clean slide, mount with a coverslip and apoptotic cells were observed using a fluorescent microscope at an excitation wavelength of 350nm. The Image J (Version 2.1) software was used for calculating the pixel value statistics of the stained cells that helps quantify the score of DNA damage in the form of a density histogram which represents normalized values of the treated cells against control.

Determination of the anticoagulant activity

The crude extracts for the assays were prepared by dissolving the dried extract in normal saline (0.9% sodium chloride) at different concentrations. Blood samples were collected from 4 healthy volunteers using disposable polypropylene syringe and 3.8% tri-sodium citrate (9 parts of blood to 1 part of tri-sodium citrate solution) as the anticoagulant. The blood was immediately centrifuged at 4000 x g for 15 min. The freshly prepared plasma was separated, pooled and was stored at 4 °C for use in the assay. The steps followed in this assay had been adapted from (Kathiresan *et al*., 2006), with slight modifications.

In this study, the action of the crude extracts in the intrinsic and common blood coagulation pathway is evaluated by the APTT assay and that of the extrinsic pathway is determined by the PT assay. In the APTT assay, 0.1 ml of the blood plasma was added to 0.1ml of Liquicelin-E (a phospholipid preparation derived from the rabbit brain with ellagic acid as an activator) and shaken briefly to mix them. The reaction tube was incubated at 37 °C for 20 min. After incubation, 0.1 ml of pre-warmed calcium chloride solution was forcibly added into the reaction tube. Finally, 0.1 ml of each of the already prepared saline plant extracts of different concentrations (100, 500, 1000 µg/ml) were added to separate reaction tubes and kept at 37 °C. The tubes were shaken gently and tilted back and forth to allow the mixing of the reaction and the time taken to coagulate was recorded in seconds using a stopwatch. As soon as the clot formation begins the stopwatch is stopped. The assay was carried out in triplicates for each sample and the average value was noted and the activity was expressed as clotting time ratio in relation to the control taken, which is heparin.

In the PT assay, 0.1 ml of the blood plasma was taken in a tube and was placed on a water bath at 37 °C for 3-5 min. To this 0.2 ml of pre-warmed (37 °C) Liquiplastin reagent (liquid calcified Thromboplastin reagent, derived from rabbit brain) was added. And then 0.1 ml of the already prepared saline plant extracts of different concentrations (100, 500, 1000 µg/ml) were added to

separate tubes and kept at 37 °C. The time required for the formation of clot was recorded similar to that in the APTT assay.

Statistical analysis

The results of all series of experiments were performed in triplicates and were expressed as mean ± SD (standard deviation). Quantitative and graphical data were analyzed using GraphPad Prism 5 software and Microsoft Excel 2011 Package. The data in apoptosis study was analyzed using ImageJ (Version 2.1) package, a public domain, JAVA based image processing software developed at the National Institutes of Health (NIH).

RESULTS AND DISCUSSION

Phytochemical screening of aqueous extracts

The aqueous extracts of roots of *Acanthus ilicifolius* and leaves of *Lumnitzera racemosa* showed the presence of several classes of bioactive compounds (Table 1). These are the chemical compounds produced in plant tissues as a result of their defense mechanism and are known to have several health benefits (Gavamukulya *et al*., 2014). The presence of the above class of compounds in the crude extracts validates the presence of molecules that are extensively used in the field of medicine (traditional and pharmaceutical industry) and also weighs on the importance of traditional knowledge for use of plants in drug discovery process.

Table 1: Results of preliminary phytochemical screening of the aqueous plant extracts.

Phytochemicals	Name of Tests	*Acanthus ilicifolius*	*Lumnitzera racemosa*
Phenols	Ferric chloride test	+	+
	Gelatin test	+	+
Flavonoids	Ferric chloride test	+	+
	Alkaline reagent test	+	+
Alkaloids	Wagner's test	+	+
Terpenoids	Salkowski's test	+	+
Sterols	Libermann-Burchard test	+	+
Tannins	Braymer's test	+	+
Proteins and Amino acids	Biuret test and Ninhydrin test	-	-
Carbohydrates	Molisch's test	+	+
Cardiac glycosides	Keller-Kiliani test	+	+
Saponins	Foam test	+	+
Quinones	-	+	+

+: Presence and -: Absence

Total phenolic content (TPC) & Total flavonoid content (TFC)

The total phenolic content and total flavonoid content of the extracts were expressed as µg/ml of gallic acid equilavent (GAE) and quercetin equivalent (QE), respectively (Table 2). The TPC in the extracts were determined from the gallic acid calibration curve with a regression equation of y=0.0046x+0.0891 and R^2=0.995 (Figure 1). And the TFC in the extracts were determined from the quercetin calibration curve with a regression equation of y=0.0096x+0.0521 and R^2=0.993 (Figure 2). The values clearly indicate and confirm the presence of phenolic compounds in the leaf extract of *Lumnitzera racemosa* as well as in the root extracts of *Acanthus ilicifolius* and literature data abounds in reports that supports the fact that higher phenolic content directly contributes to the overall antioxidant activity of the extract (Luximon-Ramma *et al*., 2002). The antioxidant activity of the phenolic compounds is due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Saha *et al*., 2009).

Table 2: TPC and TFC of the aqueous plant extracts (n=3). Values are mean ± SD.

Crude Extract	*Acanthus ilicifolius*	*Lumnitzera racemosa*
Total phenol content (µg/ml of GAE)	200.98±0.004	476.37±0.007
Total flavonoid content (µg/ml of QE)	17.28±0.005	24.96±0.004

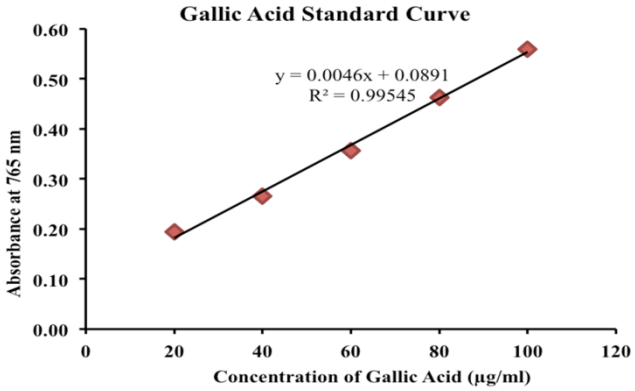

Fig. 1: Gallic acid standard calibration curve.

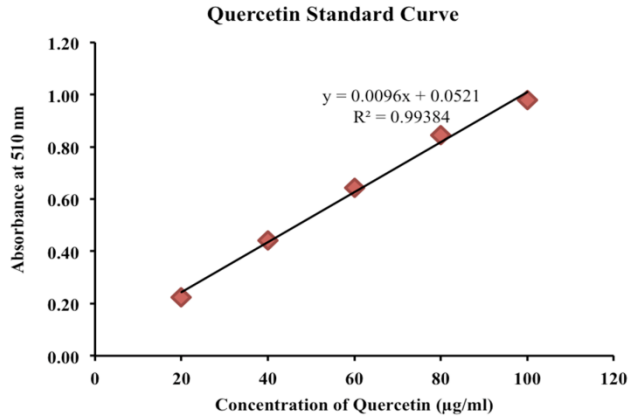

Fig. 2: Quercetin standard calibration curve.

In vitro antioxidant assays

DPPH radical scavenging activity and ABTS radical scavenging activity

Figure 3 clearly shows a decrease in the concentration of DPPH due to the scavenging capability of the plant aqueous extracts. The percentage scavenging effect increases with the simultaneous increase in the concentration of extracts. This concludes that the leaf extracts of *Lumnitzera racemosa* is an efficient DPPH-free radical scavenger, exhibiting a lower IC_{50}

value of 38.89 µg/ml which is in fair proximity to the IC_{50} value of the experimental standard, ascorbic acid (21.71 µg/ml), thus proving to have good antioxidant power.

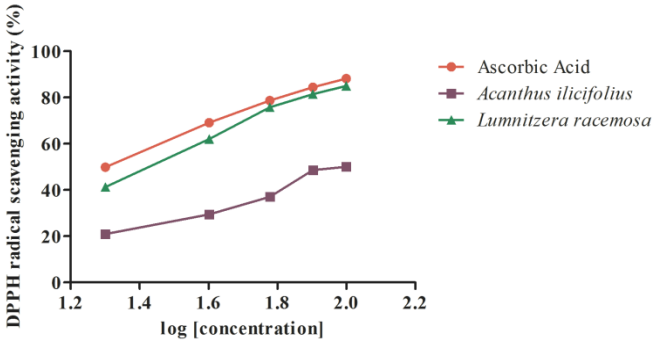

Fig. 3: Antioxidant activity of the crude extracts on DPPH.

Similarly in the ABTS radical scavenging activity assay too, the scavenging effect increases with the increase in the concentration of the plant extracts (Figure 4) and the IC_{50} values of the leaf extracts of *Lumnitzera racemosa* and the root extracts of *Acanthus ilicifolius* were calculated as 44.38 µg/ml and 60.89 µg/ml respectively. The IC_{50} value of the experimental standard ascorbic acid was only 19.93 µg/ml.

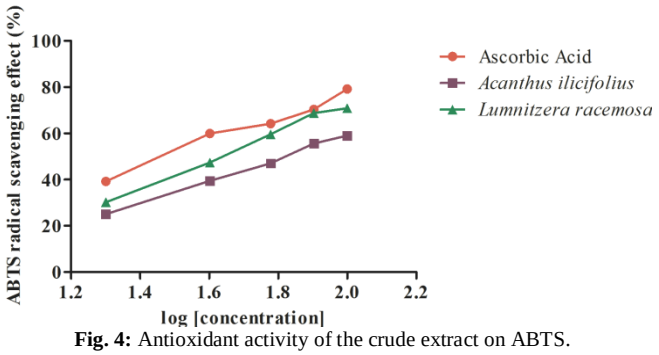

Fig. 4: Antioxidant activity of the crude extract on ABTS.

Table 3: IC_{50} values of Ascorbic Acid, root extracts of *Acanthus ilicifolius* and leaf extracts of *Lumnitzera racemosa* with DPPH and ABTS antioxidant assays (µg/ml).

Parameters	Ascorbic Acid IC_{50}	*Acanthus ilicifolius* root extract IC_{50}	*Lumnitzera racemosa* leaf extract IC_{50}
DPPH radical scavenging assay	21.71	59.85	38.89
ABTS radical scavenging assay	19.93	60.89	44.38

The present antioxidant study thus reflects that the plant aqueous extracts, particularly the leaf extract of *Lumnitzera racemosa* was a potent antioxidant. It showed 85 % DPPH-free radical scavenging activity at 100 µg/ml. Also the IC_{50} values of DPPH assay was lower than the ABTS assay, which might be due to the reason that the aqueous extracts had compounds that are better capable of scavenging the DPPH radicals than the ABTS radicals. According to Mukherjee *et al*., 2011, various factors like (i) stereo-selectivity of the radicals, (ii) solubility of the extract in different testing systems, (iii) polarity of the solvent, (iv) functional groups present in the bioactive compounds, have been reported to affect the capacity of extracts to react and quench different radicals. In sum, the results of the current antioxidant study comprehends and establishes the possibility of the use of these plant extracts for treatment of free radical induced pathological damages (Aiyegoro and Okoh, 2010).

Ferric reducing antioxidant power (FRAP) assay

In the FRAP assay, Figure 5 clearly indicates a steady increase in absorbance with the increase in the concentration of plant aqueous extracts which signifies that the ferric reducing ability of the extract increases with increasing concentration. And this reducing capacity is the indication of the extracts having potential antioxidant activity (Babu *et al*., 2001; Ravikumar and Gnanadesigan, 2011; Firdaus *et al*., 2013). However, in comparison to the experimental standard ascorbic acid, the plant extracts exhibited moderately lower ferric reducing capability.

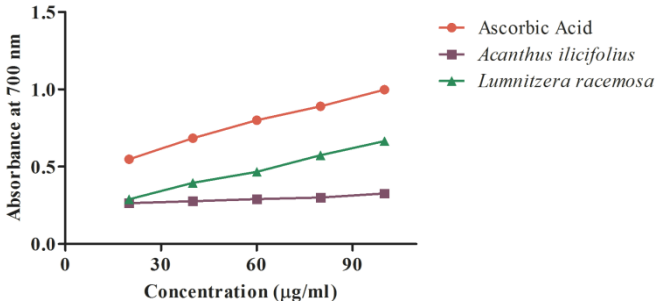

Fig. 5: Reducing power of the crude extracts as compared to ascorbic acid.

Cytotoxicity and apoptosis studies on Hep G2 cancer cell line

The present study in addition to previously reported studies (Bandaranayake, 2002; Huo *et al*., 2005; Raut and Khan, 2012; Singh and Aeri, 2013; Huang *et al*., 2014; Nguyen *et al*., 2015) suggest that mangroves are a rich source of secondary metabolites probably because they grow and survive under very hostile environmental conditions (high salinity, low nutrition, low oxygen conditions of waterlogged mud and high solar radiation during low tide) (Kathiresan and Bingham, 2001). These naturally occurring bioactive constituents (eg- phenolic compounds) are majorly known to play an important role in the mechanism of anticancer (Babu *et al*., 2002; Bunyapraphatsara *et al*., 2003; Van Kiem *et al*., 2008; Ravikumar and Gnanadesigan, 2011; Firdaus *et al*., 2013). This is a maiden report of the mangrove plant samples from Pichavaram mangrove forest, Tamil Nadu, India that states the cytotoxicity of the aqueous root extract of *Acanthus ilicifolius* and leaf extract of *Lumnitzera racemosa*. In this study the cytotoxic effect of the crude plant extracts were tested against Hep G2 cancer cell line using MTT assay. The IC_{50} values for root extract of *Acanthus ilicifolius* and leaf extract of *Lumnitzera racemosa* were reported as 39.76 µg/ml and 26.05 µg/ml respectively. Also, the percent cell viability (Figure 6) of the leaf extract of *Lumnitzera racemosa* exhibited more potent cytotoxicity on Hep G2 cell lines at different concentrations. The higher

cytotoxicity of the crude extracts may be due to their higher antioxidant potentials or due to the synergistic effect of the multiple bioactive components present in the extract. The antioxidants are capable of scavenging the free radicals that are known to damage healthy cells and is a prime reason for the occurrence of cancer and other free radical induced conditions.

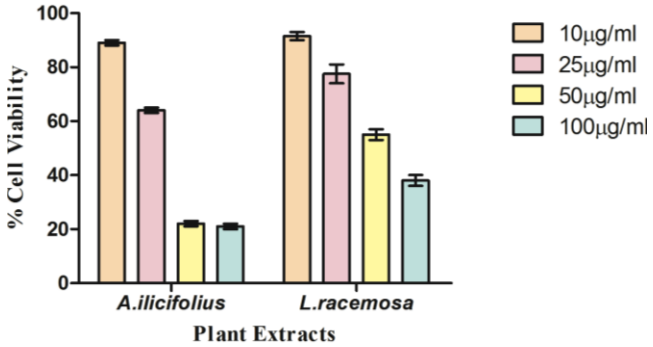

Fig. 6: Effect of the aqueous plant extracts at different concentrations on Hep G2 cancer cells.

Resistance of programmed cell death or apoptosis is an integrated part of the cancer cell development and re-inducing the apoptosis mechanism would be a good indicator of anticancer agent. The apoptosis study was conducted using DAPI staining protocol to examine the apoptosis inducing capability of the crude extracts by determining the score of DNA damage using the ImageJ software. The output is based on pixel value comparison of the treated cells against the control cells. The score for DNA damage (*A.ilicifolius*=2.193 a.u. and *L.racemosa*=2.683 a.u.) in Figure 7, clearly supports our claim of the crude extracts being suitable anticancer agent owing to their apoptosis inducing ability by causing DNA damage. This is at par with a study which proved that any extract had anticancer and cytotoxic activity if it had an IC_{50} value less than 1000 µg/mL after 24 h contact time, and that the smaller the IC_{50} value of a test compound, the more toxic the compound was (Gavamukulya *et al*., 2014). However, further detailed studies should be conducted to decode the anti cancer mechanism by the crude extracts.

Fig. 7: Effect of aqueous plant extracts on inducing cell apoptosis.

Determination of anticoagulant activity

Heparin is majorly used for limiting blood coagulation in the field of medicine. But owing to some of its side effects like hemorrhage there has been an urge to look for an alternate to it and hence researchers have been trying to find anticoagulant from natural sources (Edemeka and Ogwu, 2000; Félix-Silva *et al*., 2014; Khouya *et al*., 2015). The anticoagulant study involving the APTT and PT assays using normal citrated plasma is an attempt to check the anticoagulation effect of these mangrove plant extracts. Studies however suggest that the anticoagulant property of mangrove plant extract varies over a great range depending on the species and also on the part of the plant (Kathiresan *et al*., 2006). This assay was performed just to check and report if the plants exhibited anticoagulant property. This is the maiden report for the plants used in this study.

The results show that both the assays vary slightly in the anticoagulant activity and the prolongation of the APTT is slightly higher than the PT in case of both the plant extracts. Table 4 indicates that aqueous extracts of *A.ilicifolius* and *L.racemosa* had only slightly prolonged the coagulation times compared with the control sample treated with PBS, suggesting that extracts inhibited the common pathways but isn't too effective compared to Heparin. A previous research however showed that certain other mangrove species like *Aviccenia marina*, *Aegiceras corniculatum* exhibited a higher anticoagulant property (Kathiresan *et al*., 2006), which further proves that the property is totally independent of the plant genera and varies greatly with regional location.

Table 4: Clotting time ratio in mangrove extracts assayed by using APTT and PT methods. The clotting ratio is the time taken for blood clotting in the mangrove extracts treated to the control. The clotting time for control was 80 s and 32 s for APTT and PT methods respectively.

Mangrove Species	Clotting Time Ratio					
	APTT Assay (µg/ml)			PT Assay (µg/ml)		
	100	500	1000	100	500	1000
Acanthus ilicifolius	1.3	1.35	1.5	1	1.25	1.31
Lumnitzera racemosa	1.2	1.4	1.6	1.25	1.31	1.34
Heparin (Positive Control)	315	-	-	120	-	-

CONCLUSION

We have investigated the aqueous extracts of the mangroves for their phytochemical constituents, *in vitro* antioxidant, anti-cancer and anticoagulant activities to scientifically validate their folklore use in treatment of diseases. This is a first hand report that provides sufficient evidence for carrying out further research on the selected plants to decipher the exact mechanism involved in anticancer and anticoagulant activity. Thereby suggesting *in vitro*, *in vivo* and secondary metabolite profiling studies to unravel and identify the bioactive compound(s) responsible, and ultimately provide alternative treatment strategies.

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Original Article

CLOT PROMOTING AND DISSOLVING PROPERTIES OF CUCUMBER (*CUCUMIS SATIVUS*) SAP, VALIDATING ITS USE IN TRADITIONAL MEDICINE

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ABSTRACT

Objective: To investigate the biochemical events that are associated with the skin softening, cleansing and wound healing properties of the cucumber (C*ucumis sativus* L) sap extract.

Methods: Preparation of cucumber sap extract (CSE). Assay of CSE for proteolytic activity, plasma re-calcification time, APTT, PT, thrombin-like activity, plasmin-like activity, and effect on platelet aggregation and wound healing property by physical, biochemical and histological examinations. Appropriate positive and negative controls were maintained wherever necessary.

Results: CSE decreased the plasma re-calcification time and prothrombin time (PT) and showed factor VII (pro-convertin) like activity. EGTA or EDTA pre-treated CSE did not alter the plasma recalcification time and PT. CSE readily hydrolyzed the plasma clot and azocasein; while, IAA pre-treated CSE did not hydrolyze the plasma clot and azocasein. CSE inhibited the agonists collagen, ADP and epinephrine induced platelet aggregation in PRP in the order epinephrine>collagen>ADP with the respective IC_{50} of 22 ± 2.5, 20 ± 3 and 11 ± 2 µg/ml. PMSF pre-treated but not IAA and EDTA pre-treated CSE lost the platelet aggregation inhibition property. Further, CSE augmented wound healing process including the scar removal in a mouse model. The SOD, CAT, GSH activities and hydroxyproline, hexosamine and hexuronic acid contents were increased while, NO, LPO and MPO activities were decreased compared to control values. Histological study revealed accelerated wound healing involving epithelialisation and re-formation of skin following CSE treatment compared to Neosporin.

Conclusion: CSE contain metallo-, serine and cysteine proteases, and interfere in clot formation, dissolution and wound healing process, which validates the use of cucumber as cosmetics and to treat wounds by traditional healers.

Keywords: Cucumber sap extract (CSE), Hemostasis, fibrin clot dissolution, Ethylene diamine tetra acetic acid (EDTA), Phenyl methyl sulphonyl fluoride (PMSF), Iodoacetic acid (IAA), Metalloprotease, Serine protease, Cysteine protease, Wound healing.

INTRODUCTION

Wounds are physical injuries that result in an opening or breaking of the tissue. The wound may be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissues. Healing involves a complex biological process initiated in response to an injury that restores the function and integrity of damaged tissues. Clotting and removal of debris through inflammation, replacement of damaged tissue through proliferation of epithelial cells and maturation of scar are the essential events [1]. Untreated wounds are the potential place for the infection and chronic wounds may even lead to multiple organ failure. For example, management of diabetic wounds and eczema are still remained challenging and the lack of knowledge on the molecular mechanism of wound healing is the major limitation in identification of the precise target for the better management. Since time immemorial, herbal extracts are being tried extensively to achieve the better cure. Several plant species have been used in the Indian traditional systems of medicine such as Ayurveda, Siddha and Unani to treat wounds. Plants such as *Azardica indica* (Meliaceae), *Lantana camara* (Verbenaceae*), Tridax procumbens* (Asteraceae), *Hydnocarpus wightianus* (Achariaceae), *Ginkgo biloba* (Ginkgoaceae) and *Centella asiatica* (Apiaceae) with promising wound healing efficacy have been evaluated scientifically [2].

Cucumber from *Cucumis sativus*. L (Cucurbitaceae) commonly known as melon or gourd or cucurbit has been extensively used for external applications to treat various skin disorders such as wrinkles, chink, skin mold, freckles, sunburn, hyper-pigmentation, burning sensation, acne, dark circles, skin rashes, burns, wounds and bedsores. It is also used to treat tuberculosis, fever, insomnia, headache, jaundice, haemorrhage, kidney diseases, calculi, dyspepsia, diabetes, gout, obesity, arthritis, bronchitis, stomach pains, rheumatic problems, constipation, tonsillitis, cancer, inflammation and heart problems [3-5]. Thus, from ancient to modern world, the cucumber is being used extensively as an external applicant in the cosmetic industry and as well as to treat wounds, but without having any scientific validation. Therefore in the present study cucumber sap extract in tissue remodelling and wound healing process have been studied in a mouse model and the results are presented.

MATERIALS AND METHODS

Cucumbers (*Cucumis sativus* L.) were purchased from Krishna Raja Market in month March 2013 and authenticated by expertise/botanist, Botany department, University of Mysore Mysore, India (voucher specimen facility is not available). Thrombin, urokinase, ADP, collagen type I and epinephrine were purchased from Sigma Chemicals Company (St. Louis, USA). UNIPLASTIN, LIQUICELIN-E and factor-X and VII deficient plasma were purchased from Tulip Diagnostics Pvt. Limited. Blood was collected from healthy donors (Sanction order-IHEC-UOM No.71). Adult Swiss Albino mice (30-35 g; either gender) were obtained from the Central Animal House Facility, DOS in Zoology, University of Mysore (Sanction order-UOM/IAEC/6/2012). The animal care and experimental procedures performed were in compliance with the Regulations for Animal Research and Animal Ethical Committee of the UOM. Ketamine and xylocaine were purchased from the University Medical facility with a prescription from the University authorized medical practitioner. Ethylene diamine tetra acetic acid (EDTA), phenyl methyl sulphonyl fluoride (PMSF), and iodoacetic acid (IAA), was purchased from Sigma Chemicals Company (St. Louis, USA) and PDMAB (para-dimethyl amino benzaldehyde) from Sisco Research Laboratory (Mumbai, India). Gelatin and antibiotic Neosporin skin ointment (Neomycine+polymyxin B sulphate+bacitracin zinc) were obtained from Qualigens Fine Chemicals and GlaxoSmithKline Pharmaceuticals (Mumbai, India). All other chemicals used in this study were of analytical grade.

Preparation of cucumber sap

The outer green layer of cucumber was peeled and crushed in saline (0.9%), stirred overnight using magnetic stirrer. The extract was centrifuged for 10 min at 8000 rpm, the supernatant was collected and the pellet was discarded. Chilled acetone was added to the supernatant in the ratio 2:1 and kept overnight at 4°C and centrifuged for 10 min at 8000 rpm. The pellet was collected and the supernatant was discarded. The pellet was dissolved in minimum distilled water and dialyzed using dialysing bag (10 KDa cut off) in double distilled water. The dialyzed sample was centrifuged for 10 min at 8000 rpm and the supernatant was collected and designated as cucumber sap extract (CSE) and stored at-20 °C for further use [6].

Plasma re-calcification time

The plasma re-calcification time was determined according to the method of Quick [7]. Briefly, the CSE (2.5 to 100 µg) was pre-incubated with 0.2 ml of citrated human plasma in presence of 10 mM Tris-HCl (20 µl) buffer pH 7.4 for 5 min at 37°C. For the inhibition of proteolytic activity, the CSE (25 µg) was pre-incubated independently with known protease inhibitors such as EDTA, PMSF and IAA (10 mM) for 30 min at 37 °C and then 20 µl of 0.25 M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded in seconds.

Activated partial thromboplastin time (APTT) and prothrombin time (PT)

Briefly, 100 µl of normal citrated human plasma was pre incubated for 1 min with CSE (2.5 to 100 µg). For APTT, 100 µl reagent (LIQUICELIN–E Phospholipids preparation derived from rabbit brain with ellagic acid) which was activated for 3 min at 37 °C was added and the clotting was initiated by adding 100 µl 0.25 M $CaCl_2$. For PT activity, the clotting was initiated directly by adding 200 µl PT reagent (UNIPLASTIN/liQUIPLASTIN-rabbit brain thromboplastin). In both the cases, the clotting time was recorded in seconds by observing the formation of visible clot against a light source. Plasma sample treated with no CSE served as controls in both the cases. CSE (25 µg) was preincubated independently for 30 min with 10 mM each of EDTA, PMSF and IAA at 37 °C for inhibition of proteolytic activity,

Thrombin-like activity

Thrombin-like activity was determined according to the method of Denson [8]. The assay volume of 0.4 ml with 0.5% human fibrinogen in the presence of 10 mM Tris-HCl (40 µl) buffer pH 7.4 was treated with the CSE (2.5 to 100 µg) and the mixture was agitated gently against a light source to record the formation of the visible clot in seconds at room temperature. For control experiments, fibrinogen was treated with the thrombin. The clotting time was then determined after the addition of 100 µl of thrombin (2.5 NIH units/ml) to 100 µl of the incubation sample. For inhibition of proteolytic activity, CSE (100 µg) was preincubated independently for 30 min with 10 mM each of EDTA, EGTA, PMSF and IAA at 37 °C.

Factor VIIa-like activity

Factor VIIa-like activity was assayed using the congenital factor VII deficient human plasma. The different concentration of CSE (2.5 to 100 µg) was incubated with 100 µl of congenital factor VII deficient plasma for 2 min at 37 °C. The clotting time was recorded in seconds after adding 30 µl of 0.25 M $CaCl_2$ against a light source. Normal plasma, 200 µl treated with 30 µl 0.25 M $CaCl_2$ served as control experiments. For inhibition of proteolytic activity, CSE (100 µg) was pre incubated independently for 30 min with 10 mM each of EDTA, PMSF and IAA at 37 °C.

Fibrinolytic activity

The citrated human plasma (100 µl) was mixed with 30 µl of 0.25 M $CaCl_2$ and kept at 37 °C for 3 h to form the soft fibrin clot. The clot was washed thoroughly for 5-6 times with phosphate buffered saline (PBS), suspended and incubated with the CSE (5 to 150 µg) independently in a final volume of 500 µl of 0.2 M Tris-HCl buffer pH 8.5 for 2 h at 37 °C. The undigested clot was precipitated by adding 750 µl of 0.44 M of TCA and allowed to stand for 30 min at room temperature; it was then centrifuged for 15 min at 1500x*g*. About 0.5 ml of the supernatant was transferred to a clean glass tube followed by the addition of 1.25 ml of 0.44 M Na_2CO_3, 0.25 ml of 1:3 diluted Folin ciocalteus reagent and the colour developed was read at 660 nm. For inhibition of proteolytic activity, CSE (100 µg) was pre incubated independently for 30 min with 10 mM each of EDTA, PMSF and IAA at 37 °C. The semi-quantitative assay was also done using the plate method. Fresh human blood was mixed with anticoagulant (0.11 M trisodium citrate, 0.2% EDTA) in the ratio 9:1 and centrifuged for 15 min at 500g to separate platelet poor plasma. A mixture consisting of 2 ml of platelet poor plasma, 3 ml of 1.2% agarose in 10 mM Tris–HCl, 0.15 M NaCl, 0.05% sodium azide and 0.25 M $CaCl_2$ was poured into 10 mm x 99 cm flat Petri dish and left for 2 h at 25 °C. CSE (25 to 100 µg) in 10 mM Tris-HCl buffer (pH 7.4), protease inhibitors such as EDTA, 1,10-Phenanthroline, PMSF, Benzydamine hydrochloride and IAA (10 mM) were pre-incubated independently with CSE (25 µg) for 30 min at 37 °C and placed on the surface and incubated overnight at room temperature. Then 0.01% TCA was added over the surface and the diameter of the trans lucent clear zones due to lyses of the fibrin clot (plaque) were measured in millimetre (mm). Urokinase, 2.5 units served as control.

Plasminogen activation assay

The plasminogen activation assay was done according to the method described by Chakrabarty *et al*., [9]. (a) Human citrated plasma (20 µl), (b) Human citrated plasma (20 µl) with 1 mg (10 µl) urokinase, (c) Human citrated plasma (20 µl) with 50 µg CSE, (d) 50 µg CSE alone and (e) 1 mg urokinase alone were independently incubated for 1 h at 37 °C in 100 µl of 100 mM potassium phosphate buffer and the reaction was initiated by adding 500 µl of azocasein (0.25% in 100 mM potassium phosphate buffer pH 7.4). Addition of 400 µl of 25% trichloroacetic acid abolished the activity and it was centrifuged at 1000x*g* for 15 min. The supernatant (600 µl) was diluted with an equal volume of 0.5 N NaOH and absorbance was read at 440 nm. For inhibition of proteolytic activity, CSE (50 µg) was pre incubated independently for 30 min with 10 mM each of EDTA, PMSF and IAA at 37 °C.

Preparation of platelet-rich plasma and platelet-poor plasma

The freshly collected blood from healthy donors, who were non-smokers and non-medicated at least for the previous 15 days, was mixed with 0.11 M trisodium citrate (9:1 ratio) and centrifuged at 90x*g* for 15 min to obtain platelet-rich plasma (PRP). The remaining blood was again centrifuged at 500 x*g* for 15 min to obtain the platelet poor plasma (PPP). The platelet number in the PRP was adjusted to 3.8×10^8 platelets/ml by diluting with PPP and used within 2 h. All the above preparations were carried out using plastic wares or siliconized glass wares.

Platelet aggregation

The turbidometric method of Born [10] was followed using a Chronolog dual channel aggregometer connected to an omniscribe dual pen recorder to record the light transmission as a function of time. The reaction volume 500 µl contain 0.45 ml PRP was pre-incubated with CSE (5 to 50 µg) for 3 min in a cylindrical glass cuvette under constant stirring. The aggregation was initiated by the addition of agonists such as collagen (2 µg/ml), ADP (5 mM) and epinephrine (5 mM) respectively and the aggregation was followed for 6 min. Further, CSE was also checked for its effect on platelet aggregation in the absence of any agonists. As platelets aggregate, light transmission increases progressively producing an aggregation trace on the recorder. The aggregation trace was the plot of light transmission between platelet rich plasma (PRP) and platelet poor plasma (PPP) base line, which represent 0% and 100% aggregation respectively. For inhibition of proteolytic activity, CSE (50 µg) was pre incubated independently for 30 min with 10 mM each of EDTA, PMSF and IAA at 37 °C.

Wound healing activity

The wound healing activity of CSE was determined using the excision wound model [11]. About 10 cm diameter area was shaved and sterilized with 70% alcohol on the dorsal side of the mouse. Further, a full thickness excision wound of 1 cm diameter was made

using sharp scissor and samples were applied over the entire wound twice a day up to 21 days starting from day of wounding. Four groups (n = 3) of mice were used for the study. Group I_a-I_d: wounds were treated with saline (negative control), Group II_a-II_d: wounds were treated with CSE (10 mg/kg body wt), Group III_a-III_d: wounds were treated with heat denatured CSE and Group IV_a-IV_d: wounds were treated with Neosporin (positive control) respectively for 3, 7, 14 and 21 days. Wound contraction (diameter of the wound calculated by tracing margins of the wound on a graph sheet) was monitored daily and the percentage of wound closure was calculated using the formula, % wound contraction = [Healed area/Total wound area] × 100 (Healed area= original wound areapresent wound area). Mice were anesthetized by intraperitoneal injection of ketamine-xylocaine mixture [12] on the respective days. The healing skin from each group was taken and fixed in Bouin's solution for a period of three days for histopathology.

Histopathology studies

Skin samples were stored in Bouin`s solution was subjected to dehydration process through different grades of alcohol and chloroform mixture. Finally, the samples were placed in chloroform. The tissues were embedded in molten paraffin wax and 5 µm thick sections were prepared using Spencer 800 microtome. The sections were stained with hematoxylin-eosin stain for microscopic observations according to the standard procedure. The sections were observed under Leitz wetzlar Germany type (307-148.002) microscope and photographed using Photomet-rics colorsnap CF camera (Leitz Diaplan Germany, Roper Scientific Photometrics type-A014872002) attached to the microscope.

Estimation of antioxidants and free radicals in granulation tissue

Wet granulation tissues of mice from each group were homogenized in a glass teflon homogenizer (10% w/v) at 4 °C in phosphate buffered saline (PBS, pH 7.2) and used for the assays. Antioxidant markers such as superoxide dismutase (SOD) [13], catalase (CAT) [14] and reduced glutathione (GSH) [15] were estimated. Free radical markers such as nitric oxide (NO) [16], lipid peroxidation (LPO) [17], acute inflammatory marker myeloperoxidase (MPO) [18] were estimated. In addition the protein content [19] was also estimated. The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitro blue tetrazolium formazan. One unit of enzyme activity was defined as the amount of enzyme that gave 50% inhibition of nitro blue tetrazolium reduction in one minute. CAT measurement was done based on the ability to oxidize hydrogen peroxide to water and molecular oxygen. One unit of enzyme activity was defined as the enzyme which decomposes one mM of H_2O_2/min at 25 °C. GSH activity was estimated by the ability of GSH to reduce DTNB (5, 5'-dithiobis nitro benzoic acid) to 2-nitro-5-thiobenzoate (TNB) within 5 min against blank and the amount of reduced GSH was expressed as nanomoles of GSH/mg. LPO levels were estimated in terms of malondialdehyde (MDA) released during lipid peroxidation and expressed in nanomoles of MDA/mg. Nitrites and nitrates formed during NO formation were measured by using Griess reagent and expressed as units/mg. For myeloperoxidase (MPO) estimation, granulation tissue (5% w/v) was homogenized in 0.5% hexa decyl trimethyl ammonium bromide (HTAB, Sigma-Aldrich, Co., St. Louis, MO, USA) with 50 mM potassium phosphate buffer (pH 6). The previous homogenate was freeze-thawed three times, sonicated for 10 seconds and then centrifuged at 14000×g for 45 minutes at 4 °C and the resulting supernatant was used for estimation of MPO. One unit of MPO activity was defined as that converting 1 µmol of H_2O_2 to water in 1 min at 25 °C.

Estimation of connective tissue parameters

The wet granulation tissue samples from each group were dried at 50 °C for 24 h and 40 mg was weighed from each group and transferred to clean and dry test tubes and suspended in 1 ml of 6 N HCl and boiled in the water bath for 24 h. The hydrolysate was then cooled and excess of acid was neutralized by adding 10 N NaOH using phenolphthalein as an indicator. The volume of neutral hydrolysate was diluted to a concentration of 20 mg/ml with distilled water and used for the estimation of hydroxyproline [20], hexosamine [21] and hexuronic acid [22]. The respective standards such as 4-Hydroxy-L-proline (75 to 900µg/0.3 ml), D (+) glucosamine hydrochloride (5 to 50µg/0.5 ml) and D (+) Glucurono-6, 3-lactone (5 to 40 µg/0.5 ml) were used to prepare standard curves.

Protein estimation

Protein content was determined by Biuret method [23].

Statistical analysis

The experiments were repeated for three independent observations. Results were expressed as mean±SEM values. Data were compared by analysis of variance (ANOVA) followed by Bonferroni post hoc test for multiple compari-sons; significance was accepted at $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***). Data were analyzed using the statistical package GraphPad Prism (GraphPad Soft-ware, Inc., USA).

RESULTS

Initial screening for the proteolytic activity revealed that the cucumber pulp showed insignificant activity while the activity was significant in the green skin peel and thus the cucumber sap was prepared from the later. The crude saline extract of cucumber sap was subjected to chilled acetone precipitation in the ratio 1: 2 (extract: acetone). The precipitate was re-suspended and dialyzed in distilled water and centrifuged, the supernatant was called (CSE) and used for further studies as a source of protease activity. CSE showed proteolytic activity on casein with the specific activity of 2.27±0.6 units/mg/min [6].

CSE showed procoagulant activity as it reduced the re-calcification time of citrated human plasma from 266±3.5 sec to 141±0.7 sec (fig. 1A) and the effect was found to be dose dependent. EDTA and EGTA abolished, while PMSF and IAA did not affect the plasma recalcification time of CSE (table 1). CSE also reduced the prothrombin time (PT) of citrated human plasma dose dependently from 16±2 sec to 5±2 sec (fig. 1B). Further, preincubation of CSE with EDTA abolished the PT activity while it was insensitive to PMSF and IAA pre-treatment (table 1). CSE also showed the thrombin time (TT) dose dependently, but the effect observed was very feeble as compared to the control where thrombin showed the clotting time of 26±2 sec. Further, the thrombin time was also abolished by EDTA while PMSF and IAA did not affect (data not shown).

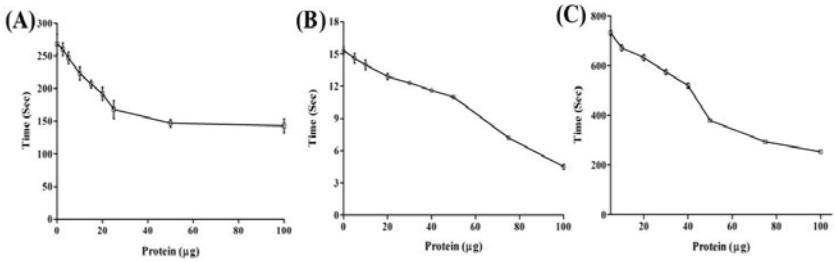

Fig. 1: Effect of CSE on plasma recalcification time, Prothrombin time and congenital factor VII deficient human plasma
(A) Dose dependent effect of CSE (5-100 µg) on human citrated plasma. (B) Dose dependent effect of CSE (5-100 µg) on citrate human plasma with prothrombin time reagent. (C) Dose dependent effect of CSE (5-100 µg) on congenital factor VII deficient human plasma and the assay was carried out as explained in 'materials and methods' section. Results are presented as the mean±SEM

When tested on the factor VII deficient congenital human plasma, CSE induced the clotting in a dose dependent manner, and at 100 µg, the clotting time observed was 254±13 sec (fig. 1C), and this effect was abolished by EDTA but not by PMSF and IAA (table 1). The factor VII deficient plasma was insensitive to the added $CaCl_2$, while the normal citrated human plasma showed the recalcification time of 267±4 sec which served as control.

Table 1: Effect of protease inhibitors on recalcification time, prothrombin time and factor VII eficient plasma clotting property of CSE

Samples	Clotting time
Recalcification time (Sec)	
Control (Normal plasma)	262±04
CSE (25µg)	182±07 [a]***
CSE (25µg)+PMSF (10 mM)	179±14
CSE (25µg)+IAA (10 mM)	180±13
CSE (25µg)+EDTA (10 mM)	No clot formation [b]***
CSE (25µg)+EGTA (10 mM)	No clot formation [b]***
Prothrombin time (Sec)	
Control (Normal plasma)	16±2
CSE (25µg)	13±2 [a]***
CSE (25µg)+PMSF (10 mM)	12±2
CSE (25µg)+IAA (10 mM)	13±3
CSE (25µg)+EDTA (10 mM)	No clot formation [b]***
CSE (25µg)+EGTA (10 mM)	No clot formation [b]***
Factor VII deficient plasma clotting time (Sec)	
Control (Normal plasma)	262±04
CSE (50µg)	382±20 [a]***
CSE (50µg)+PMSF (10 mM)	379±21
CSE (50µg)+IAA (10 mM)	381±23
CSE (50µg)+EDTA (10 mM)	No clot formation [b]***
CSE (50µg)+EGTA (10 mM)	No clot formation [b]***

Results are presented as the mean±SEM and analyzed using one-way ANOVA followed by 'Bonferroni's multiple comparison test (***P<0.0001, [a] significant when compared to clotting time of control, [b] significant when compared to CSE treated samples).

CSE hydrolysed the washed fibrin clot dose dependently with the specific activity of 0.475±0.026 units/mg/min. PMSF and IAA inhibited the clot hydrolysing activity and the inhibition achieved was (95±2%) and (11±4%) respectively while, EDTA didn't inhibit (fig. 2A and 2B). The degradation of fibrin was further confirmed by the semi-quantitative fibrinolytic assay on agarose plate method in which the CSE (100 µg) revealed the clear zone of fibrin hydrolysis of 7.5±0.04 mm diameter. PMSF, benzidine hydrochloride and IAA inhibited, while EDTA and EGTA did not inhibit the fibrinolytic activity of CSE (fig. 2C and 2D). Urokinase (2.5 units) revealed the zone of hydrolysis of 12±0.02 mm which served as positive control. In plasminogen activation assay, the urokinase didn't hydrolyze azocasein while CSE hydrolyzed the azocasein dose dependently when incubated independently. The azocasein hydrolysing activity of CSE was inhibited to the extent of 86% by IAA and 12% by PMSF while EDTA did not inhibit (data not shown).

CSE inhibited the epinephrine, collagen and ADP induced platelet aggregation dose dependently and the order of inhibition was found to be epinephrine>collagen>ADP with the respective IC_{50} values of 22±2.5, 20±3, and 11±2 µg/ml (fig. 3A, B and C). The platelet aggregation property of CSE was abolished by PMSF while it was insensitive to EDTA and IAA (fig. 3D, E and F).

CSE was tested for wound healing property and it was followed from day 3rd to 21st days. CSE readily promoted wound healing in a Swiss albino mouse model. The rate of wound contraction achieved in CSE, positive control (PC), negative control (NC), and B-CSE (boiled CSE) treated wounds were found to be 25 to 99%, 27 to 96%, 16 to 75%, 17 to 78% respectively (fig. 4 A and B). In case of CSE treated wound, complete scar free healing was observed on 21st day of treatment and this was highly comparable to the healing rate of Neosporin treated control (PC) wound, while the healing was achieved at 25th day of treatment in case of NC and BSE treated wounds.

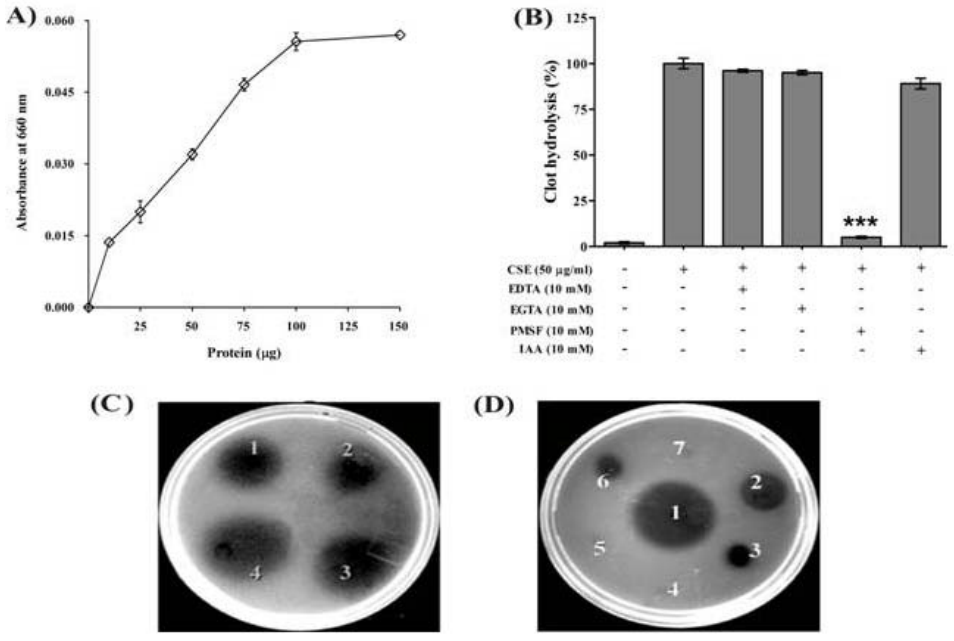

Fig. 2: Effect of CSE on fibrin clot hydrolysis and inhibition by protease inhibitors. A represents the effect of CSE on fibrin clot hydrolysis. B represents the effect of protease inhibitors on CSE towards hydrolysis of fibrin clot. Protease inhibitors (10 mM) were preincubated independently with CSE (100 µg) for 30 min at 37 °C. C represents the effect of CSE on fibrin clot hydrolysis by plate method. 1 (2.5 units urokinase), 2, 3, 4 (25, 50, and 100 µg CSE respectively). D represents the effect of protease inhibitors on fibrinolytic activity of CSE. 1 (2.5 units urokinase), 2 (CSE 25 µg), 3 (CSE 25 µg+EDTA), 4 (CSE 25 µg+PMSF), 5 (CSE 25 µg+benzydamine hydrochloride), 6 (CSE 25 µg+EGTA) and 7 (CSE 25 µg+IAA) respectively and the assay was carried out as explained in the Methods section. Results are presented as the mean±SEM (*P<0.0001, significant when compared to CSE.)**

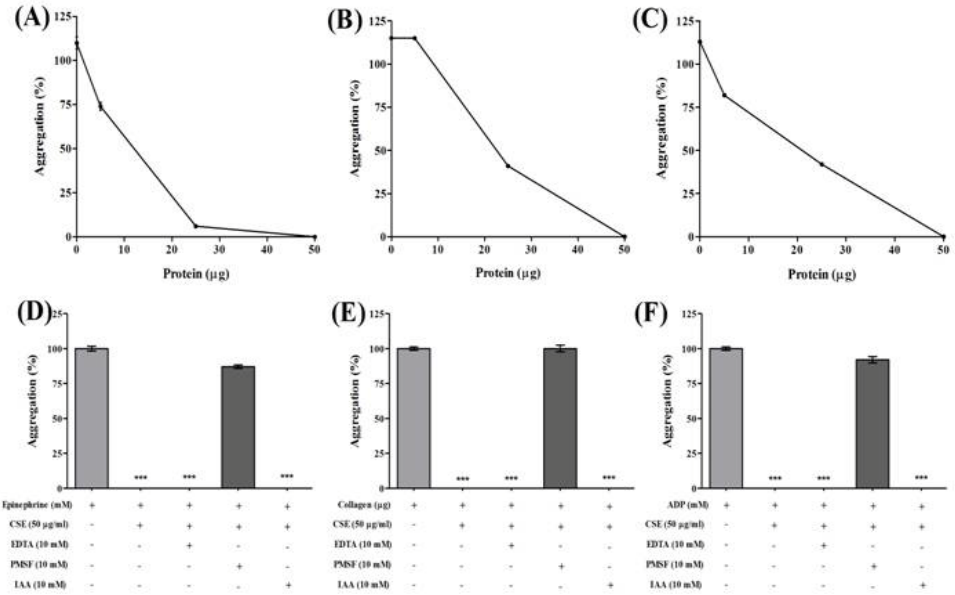

Fig. 3: Effect of CSE and protease inhibitors on platelet aggregation of human platelet rich plasma: A, B and C represents the dose dependent effect of CSE (5-50 µg) on platelet aggregation induced by epinephrine, collagen and ADP respectively. D, E and F represents the effect of EDTA, PMSF and IAA (10 mM) on platelet aggregation activity of CSE (50 µg) induced by ADP, collagen and epinephrine respectively. For inhibition studies the protease inhibitors were pre-incubated independently with CSE for 30 min at 37 °C and platelet aggregation was performed as described in the methods section. Results are presented as the mean±SEM and analyzed using one-way ANOVA followed by Bonferroni's multiple comparison Test (*P<0.0001, significant when compared to aggregation induced by epinephrine, collagen and ADP)**

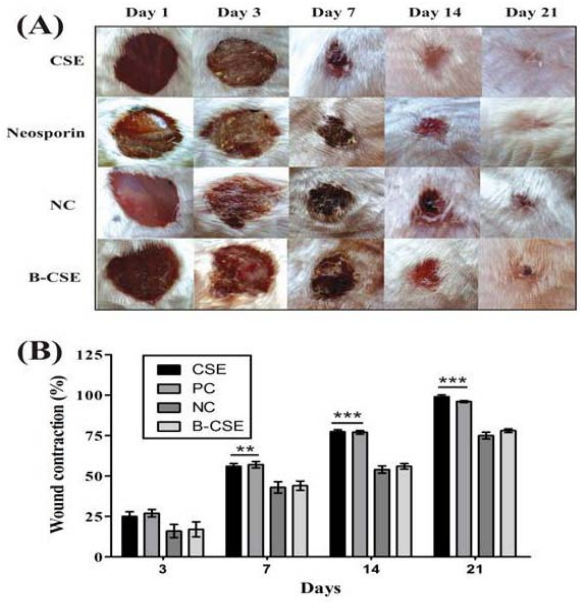

Fig. 4: Effect of CSE on wound healing activity using mouse model: 10 mm diameter full thickness excision wounds were surgically made on the dorsal portion of mice. An amount of 250 µg of CSE was applied twice daily on the wound (10 mg/kg/day). A represents the photographic representation of percentage of wound contraction from day 3 to 21 of cucumber sap extract (CSE) Neosporin, negative control (NC), and boiled extract of CSE (B-CSE). B represents the wound contraction which was monitored by measuring the diameter of the wound using a graph sheet. Results are expressed as a percentage of wound closure and are presented as the mean±SEM and analyzed using two-way ANOVA followed by Bonferroni's multiple comparison Test (* P<0.0001, signi ficant when compared to negative control (NC) group of mice)**

On respective days, such as 3rd, 7th, 14th, and 21st days of treatment, the animals from all the groups were sacrificed to remove skin tissue at the site of wound healing and subjected for histopathology. The CSE and Neosporin treated wound tissue sections revealed the accumulation of large number of inflammatory cells at 3rd day, as compared to NC and B-CSE.

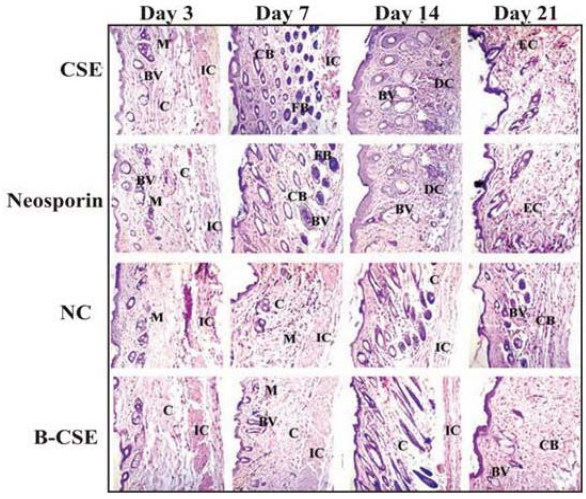

Fig. 5: Histopathology of skin granulation tissue of mice from day 3 to 21 stained with Hematoxylin-Eosin. Tropical application of CSE (250 µg), standard cream Neosporin and boiled CSE extract twice a day for wounded skin of albino mice till 21 days. After 3, 7, 14 and 21 days the mice were scarified in each group. The skin was dissected and processed for hematoxylin-eosin staining. Images were under microscope and photographed (10X). [Note: Cucumber sap extracts (CSE), Neosporin, Negative control (NC), Boiled extract of CSE (B-CSE) M-Macrophages, IC-inflammatory cells, C-collagen, BV-blood vessel, CB-collagen bundles, FB-fibroblast, DC-dense collagen and EC-intact extracellular matrix]

At 7th day, there observed an increased formation and accumulation of collagen bundles in CSE and Neosporin treated tissue sections as

compared to NC and B-CSE. On day 14, an increased collagen network and less numbers of inflammatory cells with a relatively intact extracellular matrix in CSE and Neosporin treated sections were prominently noticed while contrasting features were associated with NC and B-CSE treated wound tissue sections. On day 21st, the normal collagen, intact extracellular matrix and with no significant inflammatory cells characteristic of normal tissue has been restored in CSE and Neosporin treated wound tissue sections. However, the normal histological features did not restore even after day 25th in NC and B-CSE treated wound tissue sections (fig. 5).

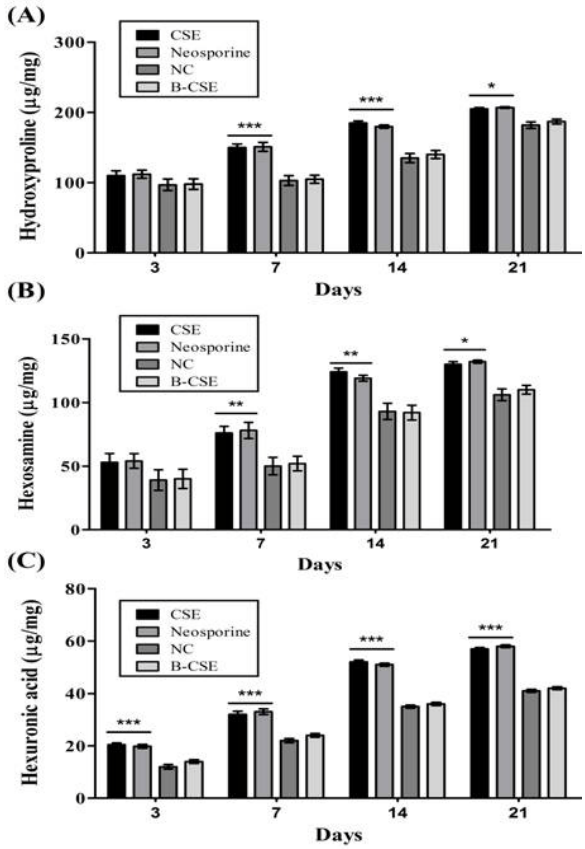

Fig. 6: Estimation of biochemical parameters of granulation tissue. A, B, and C represents the content of hydroxyproline, hexosamine and hexuronic acid of 3 to 21 days granulation tissue of mice treated with CSE (cucumber sap extract), Neosporin, NC (negative control, without any treatment) and B-CSE (boiled extract of CSE). Respective assay was performed as described in the Methods section. Results are presented as the mean±SEM and then analyzed using two-way ANOVA followed by Bonferroni post-tests (* P<0.001, significant when compared to the negative control group of mice)**

Further, the tissues at the site of wound from each group of mice were studied for antioxidant markers, free radical scavenging activity and biochemical parameters. The antioxidant markers such as SOD and CAT activities, and GSH levels were increased significantly from day 3rd to 14th day, however their levels decreased drastically in CSE and Neosporin treated wound tissues at 21st day, as compared to NC and B-CSE (table **2**). Free radicals such as NO and LPOs levels, and acute inflammatory marker MPO activity were decreased gradually from day 3rd to 21st day in CSE and Neosporin treated wound tissues, while their levels remained high in NC and B-CSE treated wound tissues (table 2). The biochemical parameters such as hydroxyproline, hexosamine and hexuronic acid contents were significantly increased from day 3rd to 21st day of CSE and Neosporin treated tissue sections as compared to NC and B-CSE which showed marked decreased levels (fig. 6A, B and C).

DISCUSSION

The wound is generally characterized by the damage of tissues and vascular endothelium resulting in the onset of complex acute phase events such as primary and secondary hemostasis and inflammatory response which not only offer protection to the wound but also prevent spreading of wound due to the possible infection. However, removal of the fibrin clot and debris, and replacement of damaged tissue through the proliferation of epithelial cells and maturation of scar are the essential events of wound healing. It is a highly complex and tightly regulated process involving the symphony of variety of factors such as platelets, collagen, blood clotting factors, cytokines, chemokines, hormones, proteases etc. At the time of wound formation, the clotting of blood is critical to prevent the fatal haemorrhage while the dissolution of clot is equally essential during wound healing. Proteases, which are the key factors known to participate in both of these processes. The results of this study systematically evaluated the beneficial effects of the CSE where the proteolytic activity play a critical role during hemostasis and tissue repair as it promoted clot formation at the time of tissue injury while it dissolved the fibrin clot and promoted tissue regeneration during wound healing. CSE readily reduced the plasma recalcification time and thus it showed strong procoagulant activity and the procoagulant activity was abolished by both EDTA and EGTA. EDTA is a non specific divalent metal ion chelator while EGTA is specific for calcium ions. Therefore, it appears that the procoagulant activity was due to the metalloprotease/s which is most likely calcium ion dependent.

Generally the metallo-proteases are predominantly the zinc ion dependent enzymes, however there are metalloprotease which are dependent on both calcium and zinc ions for their activity, for example, NN-PF3, the metalloprotease isolated from the Indian cobra (Naja naja) venom was known to contain both zinc and calcium ions and the activity was abolished by both EDTA and EGTA [24]. Normally the metal ions in metalloprotease are known to play both structural and catalytic role, and affecting any of the metal ion will affect the activity, thus CSE protease/s needs thorough investigation for its metal ion composition and biological activity. CSE did not alter the APTT while it reduced PT suggesting that it is affecting the extrinsic/tissue factor pathway of coagulation. Both EDTA and EGTA abolished the PT of CSE. Further, CSE readily caused the clotting of factor VII deficient plasma; therefore, it is highly likely that the CSE interfered in the plasma coagulation process and work in place of factor VII of the extrinsic pathway. This activity was also abolished by both EDTA and EGTA. In addition, CSE also hydrolyzed the fibrinogen and caused the formation of fibrin clot but at a very slower rate as clot formation seen only after 30 min of incubation and it was also abolished by EDTA and EGTA. Thus, during tissue damage, CSE appear to promote the clot formation through metalloprotease/s as both EDTA and EGTA abolished the plasma recalcification time, PT and coagulation of factor VII deficient plasma. Although not studied extensively, only few studies reported the procoagulant activity of plant products that are working through the extrinsic pathway of coagulation, for example, the methanolic leaf extract of Ageratum conyzoides and the cysteine protease from Ficus carica were found to show procoagulant activity through extrinsic pathway of coagulation [25-26]. However, the procoagulant activity has been reported in different extracts of plant latex, fruits like kiwi, pineapple and raspberries [27-28], while the activity has been extensively studied in animal venoms [29-31]. As the injury enter in to the healing phase, this will further trigger a complex event including clot dissolution, scavenging of the debris and tissue regeneration through controlled proliferation of cells including angiogenesis. Plasmin digests the fibrin net and the clot breaks down which will eventually be engulfed and removed by phagocytic system. This step appears crucial, as little alteration of this step would result in prominent scar and keloid formation. Further, excess accumulation of collagen and other connective tissue materials would lead to fibrosis and has been commonly seen in many surgical wounds.

Table 2: Estimation of antioxidant and free radical of granulation tissue

Groups	Day 3	Day 7	Day 14	Day 21
Superoxide dismutase (Units/mg)				
CSE treated	4.7±0.1***	5.7±0.2***	6.3±0.4***	3.2±0.1***
Neosporin	4.8±0.2***	5.5±0.2***	6.0±0.3***	3.4±0.3***
NC	2.9±0.4	4.5±0.3	5.0±0.4	5.5±0.1
B-CSE treated	2.8±0.3	4.6±0.3	5.2±0.2	5.4±0.3
Catalase (Units/mg)				
CSE treated	1.8±0.3**	2.4±0.4	3.0±0.4*	1.6±0.5***
Neosporin	1.9±0.3**	2.2±0.3	2.8±0.4	1.8±0.4**
NC	0.7±0.2	1.6±0.3	2.1±0.4	2.9±0.4
B-CSE treated	0.8±0.2	1.8±0.3	2.2±0.2	2.7±0.3
Reduced glutathione (nano moles/mg)				
CSE treated	98±6***	119±5 ***	173±4***	202±4***
Neosporin	94±4***	115±4***	168±4***	198±3***
NC	60±6	84±5	105±5	112±4
B-CSE treated	62±5	86±5	106±3	121±4
Scavenging of nitric oxide (Units/mg)				
CSE treated	0.56±0.03*	0.50±0.02*	0.15±0.02***	0.062±0.01
Neosporin	0.54±0.02*	0.50±0.01*	0.12±0.01***	0.060±0.01*
NC	0.60±0.03	0.57±0.02	0.52±0.03	0.110±0.03
B-CSE treated	0.59±0.03	0.55±0.03	0.52±0.02	0.100±0.03
Lipid peroxidation (nano moles/mg)				
CSE treated	32±6**	26±5	20±4	12±4*
Neosporin	32±4 **	25±4	20±3	12±3*
NC	45±6	34±5	24±4	22±4
B-CSE treated	42±5	32±5	25±3	22±4
Myeloperoxidase (Units/mg)				
CSE treated	1.2±0.02***	0.8±0.02***	0.5±0.02***	0.3±0.01***
Neosporin	1.3±0.02***	0.9±0.01***	0.5±0.01***	0.4±0.01***
NC	1.9±0.02	1.3±0.02	0.9±0.02	0.8±0.01
B-CSE treated	1.8±0.03	1.3±0.20	1.0±0.02	0.8±0.01

Data are presented as the mean±SEM and analyzed using two-way ANOVA followed by Bonferroni post-tests (*** P<0.001, significant when compared to negative control group of mice).

CSE hydrolyzed azocasein suggesting the plasminogen activation property and this was abolished by IAA and thus the role of Cysteine protease in the process. This is the first study reporting plasminogen activation by the cysteine protease; however cysteine proteases are abundantly present in lysosomes. Further, CSE readily hydrolyzed plasminogen free washed human fibrin clot suggesting the fibrinolytic or plasmin-like activity and the activity was abolished by PMSF, suggesting the role of serine protease. Several plants serine proteases degrade fibrin clot such as from *Euphorbia hirta* [32], *Clausena suffruticosa, Leea indica* and *Leucas aspera* [33]. Interestingly CSE inhibited platelet aggregation induced by collagen, ADP and epinephrine significantly and this activity was abolished by PMSF suggesting the role of serine proteases. Similarly the antiplatelet activity was reported from several fruits and vegetables, including black grapes (*Vitis vinifera L.*), pineapple (*Ananas comosus L. Merr.*), strawberry (*Fragaria x ananassa L. Duch.*), kiwi (*Actinidia chinensis Planchon*), vegetables such as garlic (*Allium sativum L.*), onion (*Allium cepa L.*), scallion (*Allium schoenoprasum L.*), tomato (*Solanum lycopersicon Mill.*), melon (*Cucumis melo L. var. inodorus*) and green beans [28]. Many serine proteases isolated from plants source such as bromelain from *Ananas comosus* [34] and Crinumin from *Crinum asiaticum* [35] showed the inhibition of platelet aggregation.

Clot formation and clot dissolution properties are the crucial steps during wound healing and the wound contraction is the process of mobilizing healthy skin surrounding the wound to cover the denuded(without epidermis) area and this involves complex and orchestrate interaction of cells, extracellular matrix, and cytokines. Since CSE enhanced wound contraction, it would either have enhanced contractile property of myofibroblasts or increased the number of myofibroblasts through controlled prolifereation. An increased hydroxyproline, hexosamine and hexuronic acid content of granulation tissue reflects the increased rate of wound healing. In addition, the histology of granulation tissue sections of CSE and Neosporin treated mice showed abundance of collagen tissue and neovascularisation with few inflammatory cells on 14th day of treatment compared to NC and B-CSE which suggests augmented the wound healing process. During wound healing ROS and as well as non radical oxidants play vital role in healing and serve as cellular messengers that drive numerous biological pathways. For example, at optimum micro molar concentration, hydrogen peroxide can promote vascular endothelial growth factor (VEGF) expression in keratinocytes [36]. It has been reported earlier that topical application of compounds with free radical scavenging properties in patients has shown to improve wound healing significantly and protect tissues from oxidative damage [37]. Over production of ROS results in oxidative stress, thereby causing cytotoxicity and delayed wound healing. Therefore, elimination of ROS could be an important strategy in healing of chronic wounds [38]. The initial increased SOD, CAT, GSH levels and their decreased pattern after 14th day and the decreased levels of NO, LPO and MPO at 21st day in CSE treated granulation tissue suggests the significant antioxidant activity. Reduction of free radicals and MPO levels could prevent oxidative damage and promote the healing processes. Thus, cucumber sap is a depot of a spectrum of proteases which readily interfere in hemostasis. Hemostasis is an acute phase response to vascular injury and involves platelet activation, clot formation and clot dissolution. Metalloproteases (factor VIIa like) promote blood coagulation that is required to stop bleeding while cysteine (plasminogen activation) and serine (fibrinolytic and antiplatelet) proteases are found to play essential role in clot dissolution and platelet aggregation inhibition during wound healing.

CONCLUSION

In conclusion this systematic study not only uncover the beneficial properties of the proteases of cucumber sap but also provide the scientific basis for the wide use of cucumber in cosmetic industry and as well as in traditional medicine as a skin conditioner and cleansing agent during wound healing and in the treatment of conditions such as acne and other skin disorders.

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CONFLICT OF INTERESTS

The authors have declared that there is no conflict of interest

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Research Article

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Antithrombotic and anticoagulant activities of *Desmodium gyrans (DC)*

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ABSTRACT

Antithrombotic, anticoagulant properties of medicinally used Desmodium gyrans (DC)was studied using 70% methanolic extract. Clotting time, prothrombin time, plasma recalcification time and platelet aggregation studies were performed in plasma collected from Desmodium gyrans extract treated male Sprague Dawley rats. Inhibition of lipid peroxidation was assessed in vitro using platelet rich plasma sample. The treatment with extract gave a delay in coagulation by 14.6% in prothrombin time assay. Plasma recalcification time also was enhanced with treatment. Collagen induced platelet aggregation of platelet rich plasma was brought down significantly in dose dependent manner. On treatment lipid peroxidation was brought down by 29.94% compared to the control.

INTRODUCTION

The use of herbal drugs against various diseases is attracting considerable prominence these days. Various plants have been employed in Indian traditional clinical practice and as ingredients in Ayurveda and Unani preparations. There has been a recent upsurge in research in this area after scientific authentication that they are effective for the therapeutic conditions to which they have originally been employed[1].

Thrombosis is the pathological development of blood clots and these clots subsequently may break free and become mobile. Thrombosis typically occurs during myocardial infarction as a result of atherosclerotic plaque rupture[2]. Antithrombotic agent is any medication that prevents clot formation or dissolves already formed clots. They can be used therapeutically for primary and secondary prevention or treatment of acute thrombus [3]. Anticoagulant agents also prevent formation of internal clotin the vessels by affecting either the availability or activation of blood coagulation factors and platelets. Anticoagulants and antiplatelet agents are amongst the most commonly used medications that inhibit activation of clotting factors that prevent venous thrombosis [4].

Desmodium gyrans DC, found in Kerala forests belong to the family Fabaceae. *D. gyrans* is popularly used in traditional and folk medicine since its leaves have diuretic, febrifugal and tonic properties and roots are used in Indian medicine as a remedy for asthma, coughs, as antidysenteric and as emollient. It has got a remarkable wound healing effect also. But the main focus of the current study is based on the traditionally assumed cardioprotective effect of the plant [5].*D. gyrans* has a long history of use in Chinese traditional medicine to treat various ailments[6]. Closely related species *Desmodium gangeticum* has proven cardio protective properties and give protection against cardiac reperfusion injury [7]. The presence of safe and bioactive natural antioxidants raises a substantial interest in deploying these medicinal plants in cardiovascular and other diseases[8].

A number of medicinal plants have been evaluated for their antithrombotic and anticoagulant properties and there have been some significant findings also, *Careyaarborea, Bauhinia forficata, Gloriosa superba* and *Jatropha curcas* being a few among them [9,10]. The objective of the present study was to evaluate the antithrombotic and anticoagulant potential of methanolic extract of traditionally used medicinal plant *Desmodium gangeticum*.

EXPERIMENTAL SECTION

2.1 Plant material

Leaves of plant *Desmodium gyrans* were collected from Ayurvedic Garden maintained in the campus of Amala Institute of Medical Sciences, Thrissur, Kerala, India and were authenticated by Dr. N Sasidharan, scientist, NWFP Division, Kerala Forest Research Centre, Peechi, Kerala, India. A voucher specimen was deposited in the herbarium of Amala Cancer Research Centre (ACRH No.036).

2.2 Preparation of Plant Extract

The dried leaves were powdered and subjected to extraction with 70% methanol using a soxhlet apparatus. The methanol extract of *Desmodium gyrans* (MDG) was filtered, concentrated and evaporated to dryness and the dried extract was dissolved again in distilled water was used for the study.

2.3 Animals

Male Sprague Dawley rats (200 gm) were obtained from the Small Animal Breeding Station (SABS), Veterinary and Agriculture University, Mannuthy, Kerala, India. The animals were kept under standardized environmental condition (22-30^{0}C, 60-70% relative humidity, 12 hr dark/light cycle) and fed with standard rat feed (Kerala Feeds, Thrissur, India) and water *ad libitum.* Animal experiments conducted during the study had prior permission from Institutional Animal Ethics Committee (IAEC) and followed the guideline of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India.

2.4 Determination of total flavonoid content

Colorimetric aluminum chloride method was used for determination of total flavonoid content [11]. Exactly 0.5 ml solution of plant extract was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water, and kept at room temperature for 30minutes. The absorbance of the reaction mixture was measured at 415 nm using spectrophotometer. Total flavonoids contents were calculated from a calibration curve using quercetin as standard. Quercetin solutions at serial concentrations 12.5, 25, 37.5, 50,62.5, 75, 87.5 and100 mg/ml in methanol were prepared. These standards were run using the same procedure and standard curve was prepared. Total flavonoid values are expressed in terms of quercetin equivalent.

2.5 Determination of total phenol content

Total phenolic compound contents were determined by Folin-Ciocalteau method [11]. Exactly 0.5 ml of extract was added to5 ml pre diluted Folin-Ciocalteu reagent (1:10 diluted with distilled water), mixed for 5 min and then 4 ml aqueous Na_2CO_3 (1M) was added. The mixture was allowed to stand for 15 min and the phenols were determined colourimetrically at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200mg/ml solutions of gallic acid in methanol and water solvent (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent.

2.6 Clotting Time

Clotting time is used as a screening test to monitor all phases of intrinsic coagulation system and to monitor heparin therapy[12].The coagulation time of whole blood is the time required for blood to clot under normal standard conditions. Blood was collected from normal rat through the tail vein and was transferred directly to capillary tube. The end of the capillary tube was broken at regular intervals and clotting time was noted when a fibrin thread appears between broken pieces of capillary tubes. Time taken for clot to appear is noted. Clotting time was also assayed on rats injected with standard dose heparin injected through tail vein as well as on rats fed with MDG.

2.7 Study groups and sampling

The rats were divided into three groups, normal, standard and extract treated group consisting of 6 male Sprague Dawley rats in each group. The standard group received heparin standard drug injected to tail vein at a dose of 250 U/kg b.wt of the animal for one day. The extract treated group received MDG extract at a dose of 250 mg/kg b.wt of the animal for a continuous period of seven days.

2.8 Prothrombin Time (PTT)

The arrest of bleeding depends upon formation of primary platelet plug formed on site of injury followed by the formation of a stable fibrin network. Formation of this clot involves the sequential interaction of a series of plasma proteins and the interaction of these complexes with blood platelet and components released from tissues. Tissue thromboplastin, in presence of calcium initiates the extrinsic pathway of coagulation, which comprises plasma coagulation factors VII, X, V, Prothrombin and Fibrinogen[13]. During oral anticoagulant therapy specific blood coagulation factors are depressed.

Prothrombin time determination is the preferred method for screening and diagnosis of congenital deficiency of factors II, V, VII and X. PTT is also used for monitoring of patients on oral anticoagulant therapy and as a liver function test.

Plasma collected from normal male Sprague Dawley rat 0.1 ml is taken in a glass test tube and incubated at 37^0C for 5 minutes. Liquiplastin 0.2 ml is mixed with plasma and time is noted instantly. Formation of a visually detectable solid plasma clot occurs inside glass tube within a specified period of time. The time required for clot formation is noted in seconds. The experiment is repeated using heparin treated standard plasma and plasma from extract treated group of animals. Tissue thromboplastin in the presence of calcium initiates the extrinsic pathway of coagulation mechanism. When liquiplastin reagent is added to normal plasma, the clotting mechanism is initiated. PTT would be prolonged if there is a deficiency or delay of blood coagulation factor activity in the extrinsic pathway of the coagulation mechanism.

2.9Plasma Recalcification Time

Plasma recalcification time was calculated by the addition of M/100 $CaCl_2$ solution to the previously warmed plasma at 37^0C**.** Platelet rich plasma (PRP) was prepared by centrifugation (1000rpm × 5 min) of blood collected from normal aspirin free blood bank donors. 400 µl of PRP was taken in tubes marked control, standard and extract treated, and were incubated for 1 minute at 37^0C. To one of these tubes added 20 µl of saline followed by 200 µl of M/100 $CaCl_2$ and a stopwatch was started immediately. The time taken for formation of a firm plasma clot was noted. The experiment was repeated using 20 µl of heparin in standard tube and 20 µl of MDG in two concentrations of 100 µg and 150 µg in place of saline used in normal control. The procedure was continued to determine the respective plasma recalcification time. The values were noted in each of the tubes and were compared with the value of the normal control.

2.10Platelet Aggregation Study- Collagen induced

Platelet rich plasma (PRP) was prepared by centrifugation (1000rpm × 5 min) of blood collected from normal aspirin free blood bank donors. Platelet aggregation can be done using specific agents to induce platelet aggregation or cause platelets to release endogenous ADP, or both. Platelet aggregation can be induced in vitro using thrombin, ADP, arachidonic acid, epinephrine or collagen and different mediators can be studied for their inhibition of platelet aggregation [14]**.**

In glass cuvettes 0.45 ml of PRP was taken and incubated with 50 µl of saline. The cuvette was incubated at 37^0C for 5 minutes without disturbing the content. Platelet aggregation was initiated by adding 1µg/ml of collagen. Aggregation was recorded for every minute continuously for 5 minutes using spectrophotometer at 340nm. Decreases in optical density were recorded and a graph was plotted against time taken in minutes. The procedure was repeated substituting 50 µl of MDG in place of saline, in two concentrations of 100 µg and 150 µg. The graph obtained using normal saline and plant extract as test material were compared.

2.11 Lipid Peroxidation

Malondialdehyde (MDA) produced during peroxidation can react with thiobarbituric acid (TBA) reagent to form a pink coloured product which has an absorption maximum at 532nm. The assay is calibrated with 1,1,3,3, tetramethoxypropane, which on hydrolysis produces malondialdehyde. The results are expressed in terms of the amount of malondialdehyde produced during the reaction.

PRP sample 0.1ml in Tris buffer was added to a reaction mixture containing KCl (0.1 ml), ascorbic acid (0.1ml), ferrous ammonium sulphate (0.1 ml) and Tris buffer (0.1 ml).Final volume is 0.5 ml. The reaction mixture was incubated for 1hr at 37°C. To 400µl of this reaction mixture added 0.2ml SDS, 1.5 ml of acetic acid and 1.5 ml TBA and incubated for 1 hour at 95^0C. After incubation, the reaction mixture was cooled and added 1 ml distilled water. To this mixture 5 ml of butanol-pyridine mixture (15:1, v/v) was added, mixed thoroughly and centrifuged at 3000 rpm for 10 minutes. Absorbance of upper layer containing the chromophore was measured at 532 nm against pyridine butanol mixture. In control sample, 0.1ml of PRP sample (25%) in Tris buffer was added to a reaction mixture containing KCl (0.1 ml), ascorbic acid (0.1ml), ferrous ammonium sulphate (0.1 ml) and Tris buffer (0.05 ml). In addition 0.05 ml collagen is also added. In test solution 0.05 ml of drug extract was added in place of Tris buffer used in control protocol. All the procedures were done in triplicate. The amount of MDA formed was expressed as n mol/mg protein.

RESULTS AND DISCUSSION

3.1Total flavonoid and total phenolic contents

The phytochemical analysis showed a total flavonoid 70.5±2.0 mg quercetin equivalent /g of extract and total phenol 41.5±2.29 mg gallic acid equivalent /g of extract

3.2Clotting Time

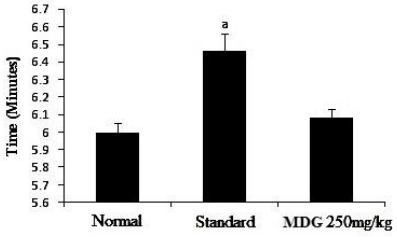
Heparin treated standard group of rats gave a significant delay in blood clotting in clotting experiment with an average increase of clotting time by 0.46 minutes when compared to the untreated group of rats (Figure 1). But clotting time of MDG treated rats showed only a mild delay in clotting time of 0.08 minutes which is only 1.35% increase and statistically insignificant.

Table.1: Effect of MDG on clotting time

Group	Clotting time (Minutes)
Normal	6.0 ± 0.05
Standard	6.47 ± 0.1
MDG	6.09 ± 0.05

Fig. 1: Effect of MDG on Clotting Time

Values are expressed as mean ± SD for 5 animals: a = p<0.01, b = p<0.05 when compared to normal.



3.3 Prothrombin Time

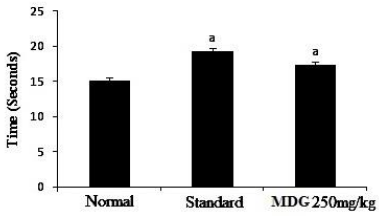
The standard group of animals treated with heparin showed a delay in coagulation by 28% when compared to the normal group of animals (Figure 2).The treatment with MDG gave a delay in coagulation by 14.6% which is a significant elevation in comparison to the normal group.

Table.2: Effect of MDG on prothrombin time

Group	Prothrombin time(seconds)
Normal	15 ± 0.5
Standard	19.2 ± 0.5
MDG	17.6 ± 0.5

Fig. 2: Effect of MDG on Prothrombin Time

Values are expressed as mean ± SD for 5 animals: a = p<0.01, b = p<0.05 when compared to normal.



3.4 Plasma recalcification time

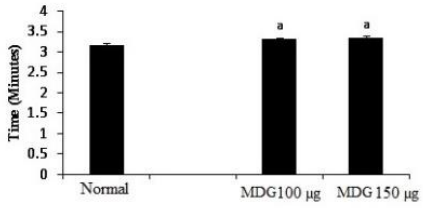
Normal plasma recalcification time of PRP was 3.15 minutes. The standard tubes in which heparin were used as the standard substance showed no clotting of plasma even after a prolonged time period. The tubes in which different concentrations of MDG(100µg and 150µg) were added showed a delay of 0.15 minutes and 0.18 minutes respectively when compared to the normal (Figure 3).

Table.3: Effect of MDG on plasma recalcification time

Group	Plasma recalcification time (Minutes)
Normal	3.15±0.05
Standard Heparin	No coagulation
MDG (100µg)	3.29 ±0.05
MDG (150µg)	3.33 ±0.05

Fig. 3: Effect of MDG on Plasma recalcification Time

Values are expressed as mean ± SD for 5 samples: a = p<0.01, b = p<0.05 when compared to normal



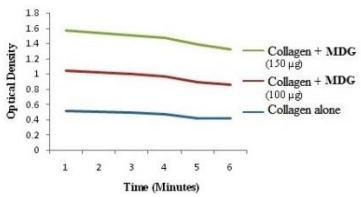
3.5 Platelet aggregation study

From the graph of platelet aggregation study, it can be seen that there was marked aggregation of PRP with usage of collagen, which was ameliorated in dose dependent manner up on treatment with MDG in two different concentrations of 100µg and 150µg (Figure 4). Collagen induced aggregation of PRP was brought down significantly as is indicated by the change in the optical densities.

Table.4:Effect of MDG on platelet aggregation study

Time (Minutes)	Optical density		
	Collagen	Collagen + MDG (100 µg)	Collagen + MDG (150 µg)
0	0.514	0.525	0.535
1	0.501	0.512	0.524
2	0.486	0.505	0.513
3	0.471	0.494	0.509
4	0.416	0.477	0.489
5	0.411	0.446	0.463

Fig. 4: Effect of MDG on Platelet aggregation study



3.6 Lipid peroxidation

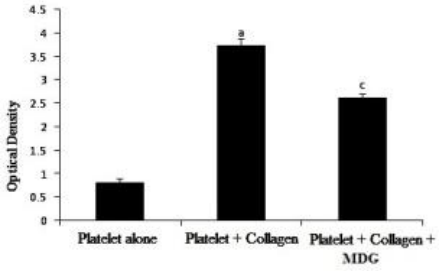
The value of lipid peroxidation was increased by 78.19% in the control, which is highly significant in comparison to the normal in the experiment (Figure 5).On treatment with MDGlipid peroxidation was brought down by 29.94% when compared to the control.

Table.5: Effect of MDG on lipid peroxidation

Group	Optical density
Normal: Platelet alone	0.8155 ± 0 .08
Control: Platelet + collagen	3.7393 ± 0.15
Test: Platelet + MDG + collagen	2.6207± 0.08

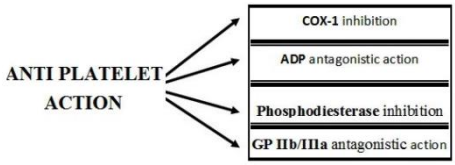
Fig. 5: Effect of MDG on Lipid Peroxidation

Values are expressed as mean ± SD for 5 samples: a = p<0.01, b = p<0.05 when compared to normal; c= p<0.01, d= p<0.05 compared to control.



The results of *in vitro* antiplatelet aggregation study revealed the property of MDG to reduce clumping of platelets in platelet rich plasma. Some of the proven antiplatelet aggregation agents like aspirin are having an effect of even irreversibly reducing platelet count in the blood. But unlike aspirin, MDG is not having such a property as assessed from the platelet count analysis done during the study. The property to act as antiplatelet aggregation agent may be due anyone of the mode of action of antiplatelet aggregation pathways as described in Figure 6. The mode of action can be classified into four types, namely COX-1 inhibition, ADP antagonistic action, phosphodiesterase inhibition or GP IIb/IIIa antagonistic action[15].

Fig. 6: Showing mode of action of antiplatelet agents



One of the popularly used drugs namely Aspirin in low dose induces reversible inactivation of a key enzyme in platelet arachidonate metabolism through acetylation of a critical serine residue near its catalytic site. This key enzyme, cyclooxygenase (COX)-1, is responsible for the formation of prostaglandin (PG) H_2, the precursor of thromboxane (TX)A_2. Thromboxane (TX) A_2 is directly involved in platelet activation and aggregation functions.

Inhibition of adenosine diphosphate (ADP)-dependent platelet function by irreversible modification of the platelet $P2Y_{12}$ receptor through short-lived active metabolites, generated by liver cytochrome P-450 (CYP) isozymes is another mechanism under examination. These metabolites are found to form covalent bonds with critical cysteine residues within the receptor which inhibit ADP recognition of the receptor thus effectively preventing platelet aggregation.

Phosphodiesterase inhibitors can acts as vasodilators and antiplatelet agents. It inhibits adenosine uptake and cyclic GMP phosphodiesterase activity, which decreases platelet aggregation process.

Glycoprotein IIb/IIIa (also known as integrin $\alpha_{IIb}\beta_3$) is an integrin complex found on platelets. It is a receptor for fibrinogen and von Willebr and factor and assists in platelet activation. The complex is formed through calcium-dependent association of GPIIb and GPIIIa, a required step in normal platelet aggregation and endothelial adherence. GPIIb/IIIa antagonists prevent fibrinogen attachment to activated GPIIb/IIIa receptors and, thus,

formation of fibrinogen bridges between platelets. Activation of GPIIb/IIIa constitutes the final common pathway of platelet aggregation. Studies are going on regarding GPIIb/IIIa blockers.

Phytochemical screening of extract of *Desmodium gyrans* revealed the presence of phenolic compounds and flavonoids. These secondary metabolites are known to have various biological activities of which can be credited to the medicinal properties of *Desmodium gyrans*. Although a study on the exact mode of action is still has to be done, various mechanisms as illustrated can be attributed as the possible mechanism by which MDG brings about inhibition of platelet aggregation as revealed in the present study.

Anticoagulant action of *Desmodium gyrans* was verified through clotting time and plasma recalcification experiments. Clotting of blood is delayed by a number of agents in blood which are having different mode of actions. Most of the agents depend on the inhibition of coagulation factors by one method or the other. Activation of antithrombin III is the mechanism of action of heparin and other anticoagulant drugs like Dalteparin, Lepirudin, Enoxaparin and Fondaparinux[16,17]. Antithrombin III (ATIII) is a 432 amino acid glycoprotein produced by the liverthat inactivates several enzymes of the coagulation system. It contains three disulfide bonds and a total of four potential glycosylation sites. α-Antithrombin is the principal form of antithrombin found in blood plasma and has an oligosaccharide occupying each of its four glycosylation sites. A single glycosylation site remains consistently free in the minor form of antithrombin namely β-antithrombin. Its activity is amplified many times by heparin, which enhances the binding of antithrombin to factor II and factor X[18].

Another regularly used anticoagulant agent namely Warfarin inhibits vitamin K reductase, resulting in exhaustion of the reduced form of vitamin K (vitamin KH2). Since vitamin K is a cofactor for carboxylation of glutamate residues on the N-terminal regions of vitamin K-dependent proteins, this limits the gamma-carboxylation and subsequent activation of the vitamin K-dependent coagulant proteins [19,20]. The synthesis of vitamin K-dependent coagulation factors II, VII, IX, and X and anticoagulant proteins C and S are inhibited. Depression of three of the four vitamin K-dependent coagulation factors (factors II, VII, and X) results in decreased prothrombin levels and a reduced amount of generated thrombin. This reduces the thrombogenicity of clots [21]. Anticoagulant action of *Desmodium gyrans* can be anyone of these modes and opens up scope for further study on its mechanism of action.

CONCLUSION

Despite recent advances, there is still scope for safe, oral anticoagulants for both short term and long term therapeutic purposes. As observed, the normal coagulation function of the different blood coagulation factors can be influenced through diverse routes which have to be probed for demonstrating the role of *Desmodium gyrans* as an effective anticoagulant and antithrombotic agent.

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Original Article

Hematological Changes in Pregnancy-induced Hypertension

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Abstract

Background: Pregnancy-induced hypertension (PIH) is the most common medical disorder of pregnancy contributing significantly to maternal/fetal morbidity and mortality. Hemostatic abnormalities that range from thrombocytopenia, consumption coagulopathy to hemolysis, elevated liver enzymes, and low platelets (HELLP) are the most ominous complications seen. This study was taken to evaluate the nature of these special hematological abnormalities in PIH.

Materials and Methods: A total 200 patients with clinical diagnosis of PIH referred to the department of pathology for hematologic evaluation over a period of 1½ year were included in the study. Complete hemogram, routine urine examination, and aspartate aminotransferase/alanine aminotransferase were done in all patients. Coagulation tests such as prothrombin time activated partial thromboplastin time, thrombin time, and D-dimer were carried out only in patients with thrombocytopenia (platelet count <1.5 lakhs), i.e., on 42 patients.

Results: Around 112 patients were grouped as severe PIH and 88 patients were grouped as mild PIH. Five of the severe PIH patients and only one of the mild PIH patient progressed toward HELLP syndrome. Useful parameters in the hemogram were blood picture to indicate microangiopathic hemolytic anemia, consumption coagulopathy, reticulocytosis, and leukocytosis which helped to identify the need for early initiation of specific therapy. The D-dimer test along with the platelet count was useful in predicting impending disseminated intravascular coagulation. HELLP syndrome with its grave prognosis was identified in 6 patients using blood picture and elevated liver enzymes.

Conclusion: This study shows that repeated hemogram and study of blood smear can go a long way toward identifying patients who are likely to go in for one or the other complications of PIH and identify those requiring specific component therapy by undertaking coagulation studies in a certain percentage of these patients.

Key words: Coagulation, Hemolysis; elevated liver enzymes and low platelets, Pregnancy-induced hypertension, Thrombocytopenia

INTRODUCTION

Pregnancy-induced hypertension (PIH) is the most common disorder of pregnancy affecting approximately 5-7% of pregnancies and is a significant cause of maternal and fetal morbidity and mortality.[1] The incidence of PIH in India ranges from 5% to 15%.[2] The majority of patients remains in mild to moderate group and does not have any major obstetric problems. However, in a certain proportion of patients, the risk to the mother can be significant and includes the possible development of disseminated intravascular coagulation (DIC), intracranial hemorrhage, renal failure, retinal detachment, pulmonary edema, liver rupture, abruptio placentae, and death. However, in a certain percentage of patients, the disease can progress to a more severe form with maternal risk of convulsions, cerebrovascular accidents, or increasing morbidity. For the fetus, it is also associated with placental insufficiency, intrauterine growth retardation, and rarely even intrauterine device.[3]

Hemostatic abnormalities ranging from thrombocytopenia, consumption coagulopathy to the triad of hemolysis, elevated liver enzymes, and low platelets (HELLP) are

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the more ominous complications seen in severe PIH. Thrombocytopenia is the most common hemostatic abnormality and its detection is important as it is one of the preventable factors contributing to some cases of life threatening cerebral and hepatic hemorrhage.[4] In view of the magnitude of coagulation changes that occurs in normal pregnancy, it i not surprising that pregnant or puerperal patient develops overt thromboembolic or coagulation abnormalities. It is equally reasonable to explore whether more subtle coagulation fibrinolytic changes develop into patterns of pathologic significance in diseases unique to pregnancy like toxemia.[5]

Superimposed HELLP syndrome develops in 4-12% of women with pre-eclampsia or eclampsia.[6] HELLP syndrome is severe form of pre-eclampsia, which poses a significant threat to both mother and fetus. This acronym HELLP was first coined by Weinstein, in 1982, to emphasize the triad of hemolysis, elevated liver, and low platelets. Based on the lowest observed maternal platelet count, HELLP syndrome is classified into three classes: Class 1 - If platelet count <50,000/cumm, Class 2 - If platelet count is >50,000 and <100,000/cumm, and Class 3 - If platelet count >100,000 and <150,000/cumm.[7]

Pathophysiologially, it is characterized by microangiopathic hemolytic anemia associated with liver and kidney damage that can progress to DIC having fatal termination.[6]

This study was taken up to evaluate the nature of these hematological abnormalities in PIH. Evaluation of peripheral smear with a special reference to red blood cell morphology, platelet morphology, aggregation and number has been the important focus of the study. Abnormal and premature forms of erythrocytes can identify microangiopathic hemolytic anemia cases which can progress to levels which require aggressive therapy. Cases having platelet counts below 1.5 lakhs were selected for performing battery of coagulation tests. Special emphasis was laid on D-dimer testing which can be used as a sensitive screening and follow-up tool for pre-eclamptic coagulopathy helping to define a subset of patients with severe disease. The D-dimer testing has been preferred over the test for fibrin degradation products (FDPs) as it has been established as a more sensitive tool for fibrinolysis.

MATERIALS AND METHODS

This was a prospective study carried out over a period of 1½ years at the Department of Pathology, Karnataka Institute of Medical Sciences, Hubli, a major tertiary health center for Karnataka. The total cases attending the outpatient department (OPD) per year are 3.5 lakhs of which around 15,000 cases attend antenatal OPD. Average number of antenatal cases admitted for delivery is 7000 per year of which about 450 cases are diagnosed to be having PIH.

About 200 patients diagnosed with PIH admitted to antenatal ward of KIMS and referred to the pathology department for hematological studies over a period of 1½ year were evaluated. Patients with essential hypertension, malnutrition, sepsis, neoplastic diseases, chronic diseases, valvular heart diseases, and those on anticoagulants were excluded. Clinical examination, complete hemogram, bleeding time, urine examination, and liver function tests were done on all the patients (200 patients).

Coagulation tests of prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), and D-dimer were done only on patients with platelet count below 1.5 lakhs (42 patients).

After obtaining informed consent from all the patients, venous blood was collected using 21G disposable needle and disposable plastic syringe. 4 cc of blood was collected for complete hemogram, of which, 2 cc of blood was collected in ethylenediaminetetra-acetic acid bulb for determination of hemoglobin (Hb), red cell indices, packed cell volume, total count, and platelet count. This was determined using Sysmex K-1000 automated blood cell counter. The remaining 2 cc of blood was collected in a citrate bulb for estimation of erythrocyte sedimentation rate (ESR) by Westergren's method. One drop of blood was obtained by finger prick for preparing peripheral smear and stained by Wrights stain. Bleeding time was estimated by Ivy's method. In those cases, where coagulation tests were done an additional 1.8 cc of venous blood was collected in citrate bulb mixed with 0.2 ml of citrate and used for coagulation studies.

The reagent used for PT was liquiplastin (Tulip Diagnostics). Normal values using liquiplastin are between 10 and 14 s. For each lot of liquiplastin, the mean normal PT was established by taking plasma from 20 normal healthy individuals and obtaining the average of their PT values.

The reagent used for APTT was liquicelin (Tulip Diagnostics), and the normal value is 21-29 s. Controls were run simultaneously with each test using plasma from healthy individuals.

The reagent used for TT was Fibroscreen (Tulip Diagnostics). Normal values using this reagent are formation of solid gel clot in 5-15 s.

D-dimer was estimated using Tulip XL FDP. Quantification was done by preparing serial dilutions of plasma sample

using phosphate-buffered saline buffer solution − 1:2, 1:4, 1:8, 1:16, 1:32, and so on. Positive result was indicated by agglutination indicating a D-dimer level above 200 ng/ml. The absence of agglutination indicates a negative result. D-dimer levels in ng/ml were calculated using the formula:

$200 \times d$

Where, d = Highest dilution of plasma showing agglutination during quantitative test of the sample.

Statistical Analysis

Students *t*-test was used.

RESULTS

Of the 200 cases, 112 (56%) had severe PIH (diastolic blood pressure [BP] ≥110 mmHg) and 88 cases (44%) had mild PIH (diastolic BP ≤100 mmHg). Table 1 shows age distribution of PIH cases.

The mean age in mild PIH was 23.57 ± 3.76 and that in severe PIH was 23.49 ± 4.1. Of the 200 cases, 109 (54.5%) were primigravidas and 91 (45.5%) were multigravidas. Cases with mild PIH were asymptomatic, whereas all cases of severe PIH were symptomatic with headache being the predominant symptom present in 52 cases (46.43%) followed by epigastric pain in 21 (18.75%), blurring of vision in 8 (7.14%), reduced urine output in 6 (5.35%), vomiting in 8 (7.14%), and giddiness in 3 (2.68%) cases. Biochemical examination revealed 1+ proteinuria in 43 cases (48.86%) of mild PIH while majority (55 cases, 49.10%) of those with severe PIH had 3+ proteinuria. Serum bilirubin levels were raised in 1 case (1.14%) of mild PIH and 9 (8.04%) of severe PIH and was normal in the remaining cases. The mean serum bilirubin in the cases with HELLP was as shown in Table 2. The highest level of serum bilirubin of 13.5 mg/dl was seen in a case of HELLP, the range being 1.3-13.5 mg/dl.

Aspartate aminotransferase (AST) levels were slightly elevated (41-70 IU/L) in 3 cases (3.40%) of mild PIH and 28 cases (25%) of severe PIH. Mean AST in cases with HELLP was high as shown in Table 3. Alanine aminotransferase levels were markedly elevated (>70 IU/L) in 9 cases (8.04%) of severe PIH. Hb levels of all these 200 patients varied from 2.3 to 14.8 g%, the mean being 9.05 g%. ESR of these patients ranged from 5 to 170 mm/h. The mean corpuscular volume in mild PIH was 82.91 ± 10.35 and, in severe PIH, it was 81.21 ± 10.26. The mean corpuscular hemoglobin (MCH) in mild PIH was 23.83 ± 6.11 and, in severe PIH, it was 24.13 ± 4.63. The mean MCH concentration in mild PIH was 28.35 ± 5.21 and, in severe PIH, it was 29.30 ± 3.88. Total leukocyte count varied from 4000 to 43,000 cells/cumm. The reticulocyte count varied between 0.5% and 10%. It was raised (>2.5%) in 18 cases (20.45%) of mild PIH and 40 (35.71%) of severe PIH.

Platelet count was normal (>1.5 lakhs) in 81 cases (92.05%) of mild PIH and 77 (68.75%) of severe PIH. Platelet count was between 1 and 1.5 lakhs in 3 cases (3.40%) of mild PIH and in 10 (8.93%) of severe PIH. The count was between 50,000 and 1 lakh in 4 cases (4.55%) of mild PIH and 20 (17.86%) of severe PIH. Platelet count was below 50,000 in 5 cases (4.46%) of severe PIH, whereas in none of those with mild PIH. Bleeding time was prolonged (>6 min) in one case (1.14%) of mild PIH and 10 (8.93%) of severe PIH. 13 cases (11.60%) of severe PIH showed giant platelets in the peripheral smear. Table 4 shows the red cell morphology in mild and severe PIH.

Coagulation tests were done in only those who had platelet count below 1.5 lakhs/cumm, i.e. in 42 cases. 2 cases (28.57%) of mild PIH and 29 (82.86%) of severe PIH had prolonged PT (>14 s). The PT in severe PIH was significantly prolonged ($P < 0.05$). APTT was normal in all 7 cases of mild PIH and 27 cases of severe PIH but prolonged (>29 s) in 8 cases (22.86%) of severe PIH.

Table 1: Age distribution of PIH cases

Age group (years)	Number of cases (%)	
	Mild PIH	Severe PIH
15-20	13 (14.77)	16 (14.29)
20-25	34 (38.64)	51 (45.53)
25-30	35 (39.77)	35 (31.25)
30-35	04 (4.55)	06 (5.36)
35-40	02 (2.27)	04 (3.57)
Total	88 (100)	112 (100)

PIH: Pregnancy-induced hypertension

Table 2: Mean serum bilirubin levels in HELLP and non-HELLP cases

Patients groups	Mean±SD
HELLP	3.53±4.88
Non-HELLP	0.72±0.18

SD: Standard deviation, HELLP: Hemolysis, elevated liver enzymes, and low platelets

Table 3: Mean AST levels in HELLP and non-HELLP cases

Patients groups	Mean±SD
HELLP	253.83±196.33
Non-HELLP	34.35±16.09

SD: Standard deviation, HELLP: Hemolysis, elevated liver enzymes, and low platelets, AST: Aspartate aminotransferase

APTT was not significantly prolonged in severe PIH ($P > 0.05$). The TT was normal in 6 cases (85.7%) of mild PIH and 28 (80%) of severe PIH. TT was raised (>15 s) in 1 case (14.29%) of mild PIH and 7 (20%) of severe PIH. The TT in severe PIH was significantly prolonged ($P < 0.05$).

D-dimer levels in cases of mild and severe PIH are shown in Table 5. In the present study, HELLP syndrome was diagnosed in six patients based on the hematological parameters, blood smear examination, and liver function tests.

Table 4: Red cell morphology in mild and severe PIH cases

RBC morphology	Mild PIH	Severe PIH
	N (%)	*N* (%)
Normocytic normochromic	38 (43.18)	39 (34.82)
Dimorphic	16 (18.18)	24 (21.43)
Normocytic hypochromic	15 (17.05)	18 (16.07)
Microcytic hypochromic	13 (14.77)	17 (15.18)
Macrocytic	03 (3.40)	08 (7.15)
Microangiopathic hemolytic	01 (1.14)	05 (4.46)
Leukoerythroblastic	01 (1.14)	01 (0.89)
Megaloblastic	01 (1.14)	00 (0.0)
Total	88 (100)	112 (100)

RBC: Red blood cell, PIH: Pregnancy-induced hypertension

Table 5: D-dimer levels in mild and severe PIH

D-dimer (ng/ml)	Number of cases (%)	
	Mild PIH	Severe PIH
Undetectable	4 (57.14)	9 (25.71)
200	2 (28.50)	16 (45.71)
>200	1 (14.2)	10 (28.57)
Total	7 (100)	35 (100)

PIH: Pregnancy-induced hypertension

Table 6 shows hematological parameters, coagulation profile, liver enzymes, and outcome in 6 cases of HELLP syndrome. In this study, 2 cases belonged to Class 1 HELLP syndrome and they had prolonged PT and APTT with TT on the higher side and marked increase in D-dimer levels indicating a state of DIC. Remaining 4 cases belonged to Class 2 HELLP and none of them belonged to Class 3. Four cases with HELLP syndrome underwent cesarean section. One patient died after cesarean and all the 6 patients delivered low birth weight babies.

DISCUSSION

Women with severe pre-eclampsia develop a variety of hematologic aberrations which have an impact on the outcome of these patients so that aggressive therapy can be initiated to prevent maternal and neonatal morbidity and mortality. Simple investigations such as complete hemogram, urine examination, and liver enzymes were done on all the cases that can detect platelet abnormalities, red cell abnormality, and detect patients likely to progress to HELLP syndrome. Coagulation profile was done only on patients with thrombocytopenia which is an important parameter for detecting DIC.

Women at any age are said to be at a greater risk for PIH.[3] In the present study, the mean age in mild PIH was 23.57 ± 3.76 and, in severe PIH, it was 23.49 ± 4.1. Similar observation was made by O'Brien *et al.*, who reported a mean age of 21.5 ± 0.9 in mild PIH and 21.3 ± 1.4 in severe PIH.[8] PIH is mainly a disease of young primigravidas. This observation was made by various authors and also in the present study.[9] Epigastric pain was the predominant symptom seen in 50% of severe PIH patients with HELLP in this study, similar observation was made by Weinstein.[10]

Table 6: Hematological parameters, coagulation profile, liver enzymes, and outcome in 6 cases of HELLP syndrome

Tests	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Hb (g%)	3.4	4.3	3.6	6.1	3.9	3.5
Total leukocyte count	8200	20.000	15,000	26,000	9900	4000
ESR (mm/h)	110	105	120	85	66	112
Reticulocyte count (%)	2.5	6	8	6	8	8
Platelet count (lakhs/cumm)	0.80	0.92	0.90	0.88	0.28	0.40
Bleeding time (min)	6'48"	6'40"	3'40"	3'50"	7'45"	7'40"
PT (s)	13.6	14.6	16	16.8	16	16
APTT (s)	28	25	30	26	32	24
TT (s)	10	13.6	16.2	16	14.8	16
D-dimer (ng/ml)	200	200	400	400	600	800
Serum bilirubin (md/dl)	1.3	1.8	1.9	13.5	1.4	1.3
AST (IU/L)	90	518	380	265	120	90
ALT (IU/L)	48	488	246	376	98	48
Mode of delivery	Vaginal	Cesarean	Cesarean	Cesarean (patient died)	Cesarean	Vaginal
Birth weight (kg)	2.2	2.1	2.2	2.3	2.3	2.1

HELLP: Hemolysis, elevated liver enzymes, and low platelets, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, PT: Prothrombin time, APTT: Activated partial thromboplastin time, TT: Thrombin time, ESR: Erythrocyte sedimentation rate, Hb: Hemoglobin

Proteinuria is an important sign of pre-eclampsia and diagnosis of pre-eclampsia is doubtful in its absence. In our study, proteinuria was present in all the cases (100%) of mild and severe PIH. However, Jambhulkar *et al.* observed proteinuria in only 68% cases of mild PIH and 92% cases of severe PIH.[11]

Estimation of serum bilirubin is important as it not only forms important criteria for diagnosis of HELLP syndrome but also its rise signifies the severity of the condition. Entman *et al.* in their study reported mean bilirubin concentration in severe PIH to be significantly higher than that in mild PIH.[12] However, in our study, there was no significant difference in mean bilirubin concentration between the mild and severe PIH groups. Serum AST appears to be the dominant transaminase released into the peripheral circulation with severe pre-eclampsia and HELLP syndrome. In the present study, the mean AST was higher in HELLP cases compared to non-HELLP cases which was similar to the observation made by de Boer *et al.*[13]

The present study showed raised ESR in most of the patients (85%). This is explained by the fact that pregnancy is one of the physiological causes of raised ESR and infection was ruled out in all these patients. Patients with severe pre-eclampsia have a microangiopathic hemolytic anemia, but it is not known whether increased red cell turnover occurs with milder form of this syndrome. Although hemolytic peripheral blood picture was present in only six cases, 18 (20.45%) of mild PIH and 40 (35.71%) of severe PIH showed raised reticulocyte counts (>2.5%). The highest value recorded was 10% in severe PIH with microangiopathic hemolytic anemia. Thrombocytopenia is the most common hemostatic abnormality of pre-eclampsia seen in approximately 50% of patients with pre-eclampsia according to a recent study done by Donimath *et al.*[14] In the present study, 22.5% patients had thrombocytopenia. Thomas *et al.* in their study observed that 16% of their patients had thrombocytopenia. Kelton *et al.* in his study concluded that there is evidence of both *in vivo* and *in vitro* platelet functional defect as the patients had disproportionate prolongation of bleeding time. Thus, patients with pre-eclampsia can have a significant defect in platelet function as well as number. The bleeding time may be important for evaluation of pre-eclamptic patients and provide information about the risk of any surgical procedures. In the present study, only 5.5% of patients had prolonged bleeding time, whereas in the study by Kelton *et al.* 34.6% patients with PIH had prolonged bleeding time.[15]

Microangiopathic hemolytic anemia is present to some degree in all patients with HELLP syndrome. This diagnosis is confirmed by finding of burr cells, schistocytes, and polychromasia on peripheral smear.[6] In the present study, six cases revealed these findings. All these six cases had thrombocytopenia and elevated liver enzymes. Documentation of HELLP syndrome is essential as aggressive therapy is initiated to prevent neonatal morbidity and mortality.

It is impossible to say which part of the maternal pathology reflects the HELLP syndrome. The associated DIC is an important aggravating factor often leading to deterioration of maternal status. The diffuse organ system damage particularly in liver, lungs, kidney, and brain may be a direct consequence of DIC causing vessel wall damage and increased vascular permeability. Arterial and venous macro and microthrombosis producing tissue hypoxia and ischemic necrosis. Hence, coagulation test is important in these patients and can reduce maternal morbidity and mortality if delivery is expedited as soon as diagnosis of suspected DIC is made.[6,16]

The reported hematologic findings in toxemia of thrombocytopenia, hemolysis, increased platelet adhesiveness, and increased FDPs are indicators compatible with intravascular coagulation. In the present study, an attempt was made to determine if the clinical categories of toxemia of pregnancy could be related to the syndromes of DIC on the basis of plasma assays of PT, APTT, and D-dimer estimation. Leduc*et al.* in their study concluded that DIC occurs once severe thrombocytopenia is present. Hence, one needs to obtain a complete blood count with platelet count at admission followed by serial platelet counts. Evaluation of PT, APTT, and fibrinogen should be added only if platelet count is <1 lakh in pre-eclampsia. This also saves the cost.[17] In this study, coagulation studies were done in patients with platelet count below 1.5 lakhs and showed that the mean PT was significantly prolonged in cases with severe PIH. Similar observation was made by Thomas *et al.* The prolongation of PT reflects picture of utilization of clotting factors due to mild intravascular coagulation. There were no significant differences between the mean APTT of mild and severe PIH patients in this study. However, the mean APTT in severe PIH was significantly prolonged in the study by Thomas *et al.* and Jambhulkar *et al.*[5,11] Significant prolongation of PTT in severe PIH indicates consumption of coagulation factors, especially factor VIII. In the present study, absence of prolongation of APTT could not be explained. TT was significantly prolonged in our study and similar observation was made by Jambhulkar *et al.*[11] Prolonged TT is ascribed to low concentration of substrate for thrombin, i.e., hypofibrinogenemia.

Coagulation abnormalities are considered one of the more ominous maternal complications in pre-eclampsia.

Unfortunately, there is no sensitive, reliable cost-effective screening tool to detect this and usually a battery of tests such as platelet count, PT, APTT, fibrinogen, and FDP are performed. None of these consistently reflect coagulation abnormalities such as D-dimer test. Although detection of degradation products traditionally has been used to assess fibrin formation, most of these assays cannot actually distinguish whether the products origin is fibrin or fibrinogen and, therefore, not specific for coagulation. The dimeric fragments on the other hand being unique to the process of fibrin polymerization specifically reflects its formation and breakdown.[18] Hence, in the present study, D-dimer was done rather than FDP. D-dimer was detectable, and it was above 200 ng/ml in 1 case of mild PIH and 10 of severe PIH in our study. We observed that D-dimer positive women had greater risk of cesarean section, premature delivery, and low birth weight. Similar observation was made by Trofatter *et al.* in their study.

Testing for D-dimer may be useful in early screening and follow-up for coagulopathy in PIH and may also help to define the subset of patients with severe disease.[19] D-dimer was also a better indicator of DIC compared to all other tests and correlated well with the outcome of pregnancy in the present study.

CONCLUSION

This study gives an outline of the investigation to be done in cases of PIH which can alert the physician of the severity of the disease so that appropriate and timely management can be initiated. Further, it proves the importance of peripheral blood smear examination which is a very simple and cost-effective tool and can detect the red cell abnormalities and qualitative and quantitative abnormalities of platelets frequently seen in PIH. Coagulation tests can be added only once there is thrombocytopenia, as increased platelet consumption is an early feature of this disorder. This also reduces the expenses of investigations. The importance of liver enzymes is furthermore emphasized, especially in patients with thrombocytopenia to detect HELLP syndrome.

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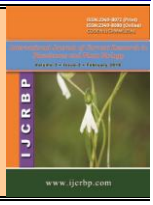

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Supplementation of Virgin Coconut Oil Compared with Copra Oil, Olive Oil and Sunflower Oil on Thrombotic Factors in Rats and *In Vitro* Platelet Aggregation

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Abstract

Virgin coconut oil (VCO) extracted from fresh coconut kernel is becoming very valuable because of its numerous beneficial properties. In the present study, comparative effect of VCO with copra oil (CO), olive oil (OO) and sunflower oil (SFO) on thrombotic factors and platelet aggregation were investigated. Male Sprague-Dawley rats were fed test oils at 8% level for 45 days along with the synthetic diet. Results demonstrated that compared to CO, a prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT) were observed in VCO fed rats and was comparable with OO and SFO. Supplementation of VCO reduced the coagulation factors namely factor V, fibrin, fibrinogen and thromboxane B2 levels in plasma compared to those fed CO, OO and SFO. Compared to other test oils, platelet aggregating tendency was also reduced in VCO fed rats. The polyphenolic fraction (PF) isolated from VCO inhibited *in vitro* platelet aggregation induced by ADP compared to PF from other oils. These results indicated that supplementation of VCO has significant antithrombotic effect by inhibiting the activation of platelets and coagulation factors compared to rats fed other test oils.

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Introduction

Coronary thrombosis is a major cause of sudden cardiac death, acute myocardial infarction, unstable angina pectoris and silent myocardial ischaemia (Davies and Thomas, 1984; De Wood et al., 1980; Fuster and Chesebro, 1986; Gurfinkel et al., 1994). The thrombogenic state arises when an imbalance exists between procoagulant and profibrinolytic activity (Astrup, 1958; Nossel, 1998). A significant component of thrombogenesis is contributed by platelet and its reactivity (Huo and Ley, 2004). Dietary fat appears to influence both the atherosclerotic and thrombogenic components of coronary heart disease (CHD) (Miller, 1997; Hornstra, 1980). Previous studies have shown that fatty acids affect coagulation of blood (Leray, 2001; Tholstrup, 2003; Nelson et al., 1997), but the effects of individual fatty acids on haemostasis are still controversial (Hoak, 1997; Knapp, 1997), which depends on both the fatty acid chain length and the degree of saturation (McGregor et al., 1980; Hornstra and Starrenburg, 1973). There is evidence that dietary long-chain saturated fatty acids appear to increase platelet aggregation whereas intake of short and medium-chain fatty acids has been negatively correlated with platelet aggregation (Takachi et al., 2004). Although polyunsaturated fatty acids have been reported to reduce platelet aggregation (Phang et al., 2013; Gao et al., 2013; Moertl et al., 2011), but available evidence on this is equivocal (Mutanen and Freese, 1996; Rand et al., 1988). There are reports which suggest a possible relationship between dietary fat, lipid peroxidation and thrombus formation (Barrowcliffe et al., 1984).

Apart from fatty acids, antioxidant components present in dietary oil may indirectly inhibit platelets through scavenging of reactive oxygen species (ROS) (Petroni et al., 1994). Previous studies from our laboratory have reported that consumption of virgin coconut oil (VCO) exerts significant antithrombotic effect compared to copra oil (CO) in cholesterol fed rats. In the present study, we investigated the comparative effect of consumption of VCO with CO, monounsaturated fatty acid (MUFA) rich olive oil (OO) and polyunsaturated fatty acid (PUFA) rich sunflower oil (SFO) on thrombotic factors and platelet aggregation in rats fed normal diet.

Materials and methods

Chemicals

Adenosine 5′-diphosphate, factor II, fibrinogen, hemoglobin, and other biochemicals were purchased from Sigma Aldrich Co. (St Louis, MO, USA). All the other chemicals used were of analytical grade.11-dehydro TX B2 immunoassay kit was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA).

Extraction of virgin coconut oil and copra oil

The solid endosperm of mature coconut (West coast tall variety) was crushed, made in to viscous slurry and squeezed through cheese cloth to obtain coconut milk, which was refrigerated for 48 hours, then subjected to mild heating (50° C) in a thermostat oven. The obtained VCO filtered through cheese cloth was used for the present study (Nevin and Rajamohan, 2004). CO was extracted from coconut meat, which was dried in sunlight continuously for 4 days to remove moisture and the resulting copra was pressed in a mill to obtain CO (Nevin and Rajamohan, 2008).

Olive oil and sunflower oil

Olive oil and sunflower oil were purchased from the local market.

Animals and diet

Male Sprague-Dawley rats (100 –120 g body weight) bred in our department animal house were used for the study. The animals were individually housed under hygienic conditions in polypropylene cages in a room maintained at an ambient temperature of 25°± 10°C with a 12 h light and 12 h dark cycle. The rats were randomly divided into four groups of six rats each. Each rat was given a synthetic diet containing 8% dietary oils daily for 45 days (Table 1). Experimental groups were as follows: Group I rats given CO, Group II rats given VCO, Group III rats given OO and Group IV rats given SFO. All the animal cares and procedures were according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. The entire experimental protocol was approved by Institutional Animal Ethics Committee (IAEC), University of Kerala. Food intake was monitored routinely and body weight was determined weekly. After 45 days, animals were fasted overnight and sacrificed by thiopentone sodium injection (>40 mg per kg body weight) and the blood was collected for various estimations.

Table 1. Formulation of synthetic diet.

Ingredients [a]	Group I	Group II	Group III	Group IV
Corn starch	71	71	71	71
Casein	16	16	16	16
Copra oil	8	--	--	--
Virgin coconut oil	--	8	--	--
Olive oil	--	--	8	--
Sunflower oil	--	--	--	8
Salt mixture	4	4	4	4
Vitamin mixture	1	1	1	1

[a] g per 100 g wet weight.

Biochemical investigation

Citrated plasma was used for the determination of coagulation parameters *viz.*, prothrombin time, activated partial thromboplastin time, fibrinogen, fibrin, factor V and 11-dehydro thromboxane B2. Prothrombin time (PT) was estimated using liquiplastin kit from Tulip Diagnostics (P) Ltd, Goa, India (Hull et al., 1982). Activated partial thromboplastin time (aPTT) was estimated using Liquicelin kit from Tulip Diagnostics (P) Ltd, Goa, India (Hoffmann

and Meulendijk, 1978). Citrated plasma was diluted with 2 mL of isotonic saline and from this fibrin was estimated according to the method described by King and Wootton (1959). Plasma fibrinogen levels were estimated as described by Clauss (1957) using fibrinogen kit from Tulip Diagnostics (P) Ltd, Goa, India. Factor V was assayed by the method of Daniel (1955). The concentration of 11-dehydro thromboxane B2 in plasma was determined by EIA kit purchased from Cayman Chemical Co.USA (Takasaki, 1991).The absorbance was read at 412 nm and concentration of each sample was obtained from the standard curve.

Platelet preparation and platelet aggregation test

Blood was collected in anticoagulant solution (2.4% sodium citrate, 1.5% citric acid and 1.8% dextrose). The ratio of the blood to anticoagulant solution was approximately 5:1 and the platelet rich plasma (PRP) was separated by centrifugation at 1850 rpm for 7 minutes. PRP was centrifuged at 4500 rpm for 18 minute to sediment the platelets (Chopra, 1999). The platelet sediment was dispersed in washing buffer composed of 113 mM NaCl, 4.3 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 24.44 mM NaH_2PO_4 and 5.5 mM dextrose (pH 6.5) and the platelets were collected after centrifugation at 900 g for 10 minutes. The platelet aggregating activity was measured by spectrophotometric method as described by Joseph et al. (2005).

Platelet aggregation *in vitro*

In vitro platelet aggregation was performed by the method of Duttaroy and Jorgensen (2004). Polyphenols from test oils were extracted according to the method described previously (Arunima and Rajamohan, 2013). 250 µL of platelet suspension was incubated with 50µg of polyphenol fraction isolated from test oils and allowed to stand for 3 minutes. Absorbance of the sample and control were measured at 600 nm immediately after the addition of agonist (10 µM ADP) and at 120 minutes in a spectrophotometer. The values were expressed as percentage aggregation.

Statistical analysis

Statistical differences were determined using one way ANOVA followed by Duncan's, post-hoc test to identify the differences using SPSS 11.5 (SPSS Inc., Chicago IL, USA). Differences of $p < 0.05$ were considered to be significant. Data are reported as mean ± SEM unless otherwise stated.

Results

Effect of VCO on blood coagulation parameters

Fig. 1 summarizes the levels of prothrombin time (PT) and activated partial thromboplastin time (aPTT) in rats fed test oils. A prolonged PT and aPTT were observed in VCO fed rats when compared to those fed CO. But no significant difference in PT and aPTT were observed among rats fed VCO, OO and SFO. VCO supplementation significantly (p <0.05) decreased the factor V levels when compared to rats fed other test oils. Factor V levels were also significantly (p <0.05) decreased in CO fed rats in comparison to those fed OO and SFO. But supplementation of SFO increased the factor V levels than OO fed rats (Fig. 2). The concentration of fibrinogen was significantly (p <0.05) decreased in VCO fed rats when compared to other oil fed rats. Fibrinogen levels were also found to be decreased in CO fed rats compared to those fed OO and SFO. Rats fed OO showed increased levels of fibrinogen compared to SFO fed rats. The fibrin levels were also significantly decreased in VCO fed rats compared to other oil fed rats. But there was no significant difference in fibrin levels among rats fed CO, OO and SFO (Table 2).

Table 2. Concentration of fibrinogen (mg/dL) and fibrin (mg/dL).

Groups	Fibrinogen	Fibrin
I	273.12 ± 27.76 [a]	17.61 ± 1.6 [a]
II	237.67± 21.69 [b]	15.38 ±1.55 [b]
III	319.68± 29.09 [c]	19.64 ± 1.66 [a,c]
IV	275.26± 28.38 [d]	18.32 ± 1.79 [a,c]

Values are mean of six rats ± SEM, values not sharing a common superscript differs significantly at p <0.05. Group I – 8% CO fed rats; Group II – 8% VCO fed rats; Group III – 8% OO fed rats; Group IV – 8% SFO fed rats.

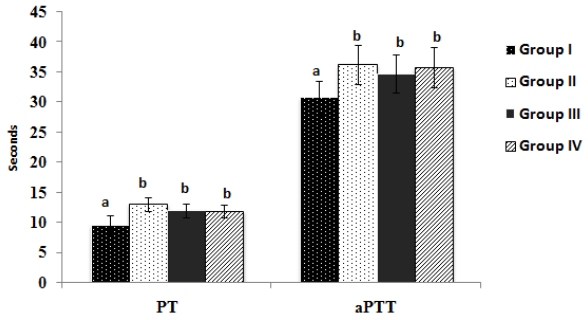

Fig. 1: Prothrombin time and activated partial thromboplastin time**.** Values are mean of six rats ± SEM, values not sharing a common superscript differs significantly at p <0.05.Group I – 8% CO fed rats; Group II – 8% VCO fed rats; Group III – 8% OO fed rats; Group IV – 8% SFO fed rats.

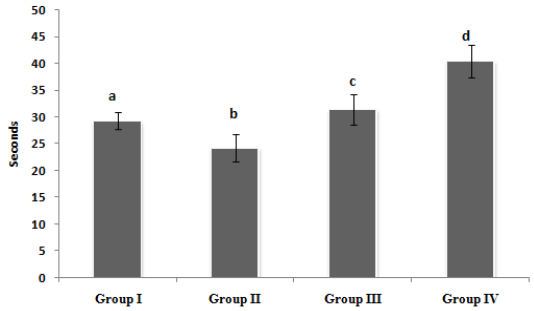

Fig. 2: Concentration of factor V. Values are mean of six rats ± SEM, values not sharing a common superscript differs significantly at p <0.05.Group I – 8% CO fed rats; Group II – 8% VCO fed rats; Group III – 8% OO fed rats; Group IV – 8% SFO fed rats.

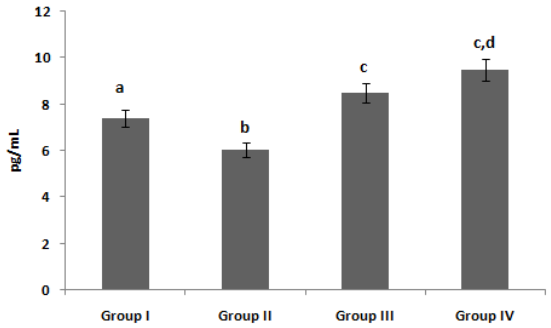

Fig. 3: Concentration of 11-dehydro TX B2 in plasma. Values are mean of six rats ± SEM, values not sharing a common superscript differs significantly at p <0.05.Group I – 8% CO fed rats; Group II – 8% VCO fed rats; Group III – 8% OO fed rats; Group IV – 8% SFO fed rats.

Effect of VCO on 11-dehydro thromboxane B2 in plasma

Concentration of 11-dehydro thromboxane B2 (11-dehydro TX B2) were significantly (p <0.05) decreased in VCO fed rats compared to those fed other test oils. Supplementation of CO decreased the 11-dehydro TX B2 levels when compared to rats fed OO and SFO. But there was no significant difference in 11-dehydro TX B2 levels among OO and SFO fed rats (Fig. 3).

Effect of VCO on platelet aggregation *in vivo* and *in vitro*

The platelet aggregating tendency was significantly lowered in VCO supplementation compared to other oil fed rats (Fig. 4). There was no significant difference in the aggregation tendency among rats fed CO and SFO. Results from *in vitro* analysis indicated that polyphenol fraction (PF) extracted from VCO significantly (p <0.05) decreased ADP induced platelet aggregation than other test oils. But there was no significant difference in platelet aggregating tendency among PF isolated from CO, OO and SFO (Fig. 5).

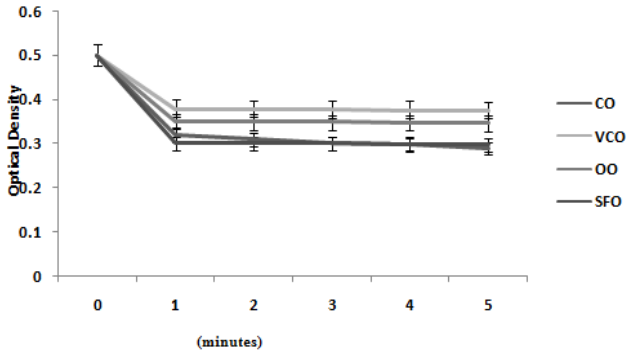

Fig. 4: Platelet aggregation induced by ADP *in vivo*. Values are mean of six rats ± SEM, values not sharing a common superscript differs significantly at p <0.05.Group I – 8% CO fed rats; Group II – 8% VCO fed rats; Group III – 8% OO fed rats; Group IV – 8% SFO fed rats.

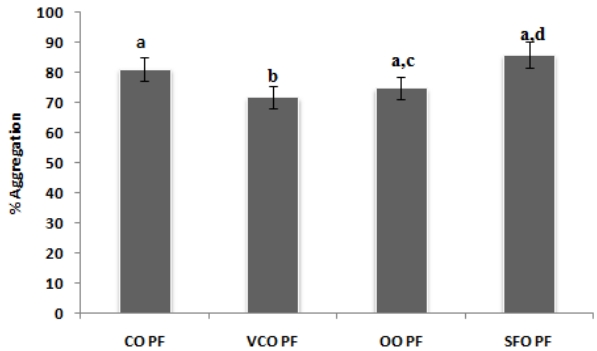

Fig. 5: Platelet aggregation induced by ADP *in vitro*. Values are mean of six experiments. Values not sharing a common superscript differs significantly at p <0.05.Group I-Platelet rich plasma (PRP) + ADP (10µM) + 50 µg CO PF; Group II- PRP + ADP (10µM) + 50 µg VCO PF; Group III- PRP + ADP (10µM) + 50 µg OO PF; Group IV- PRP + ADP (10µM) + 50 µg SFO PF.

Discussion

Results obtained indicate that supplementation of VCO had a significant beneficial effect on blood coagulation factors in rats fed normal diet. PT and the aPTT are global coagulation tests used to assess the coagulation system. PT, which measures the clotting time of plasma in the presence of thromboplastin, was determined to assess the efficiency of extrinsic system, while, aPTT depends on substances normally present in blood for its activity and to assess the intrinsic pathway. Studies suggested that short aPTT probably represent an increase in the procoagulant potential (McKenna et al., 1977) and were associated with an increased risk of thrombosis (Landi et al., 1992; Gallus et al., 1973). In the present study, a prolonged PT and aPTT were observed in VCO fed rats when compared to CO fed rats. Studies revealed that apart from fatty acids, the unsaponifiable components present in dietary oils have a role in regulating the coagulation system. There are reports that polyphenols possess antithrombotic effects, it was evidenced by a prolonged PT and aPTT (Kim et al., 2012). Chemical analysis of the test oils has revealed that VCO by wet processing retains higher amounts of polyphenols and tocopherols than other test oils (Arunima and Rajamohan, 2013). These higher amounts of polyphenols present in VCO may prolong the PT and aPTT and which may be one of the reasons for its antithrombotic potential.

The factor V levels were found to be decreased in VCO fed rats compared to other groups. Factor V is a 330-kDa glycoprotein synthesized in the liver and is released in the bloodstream as a single-chain inactive pro-cofactor. After limited proteolysis by thrombin or factor Xa, factor V is converted to its activated form, factor Va (Monkovic and Tracy, 1990). The factor Va acts as a non enzymatic cofactor of factor Xa in the conversion of prothrombin to thrombin (Nesheim et al., 1979; Rosing et al., 1980). The lesser levels of factor V observed in VCO fed rats reflects reduced thrombotic risk. Moreover, low levels of both fibrin and fibrinogen observed in VCO fed rats are associated with decreased clotting of the blood. A raised plasma fibrinogen concentration is a powerful predictor of risk of fatal CHD (Miller et al., 1996). Clotting response is enhanced by activated blood platelets and the fibrin formed reinforces the fragile platelet mass to a stabilized thrombus of great pathological significance (Mosesson, 2005). Elevated fibrin concentration, lowered prothrombin time and activated partial thromboplastin time are an indication of hypercoagulability (Curnow et al., 2007; McKenna et al., 1977).

Blood clotting is affected by many substances within our body, which depends on a balance between substances that promote coagulation and those that inhibit it (Lipe and Ornstein, 2011). Thromboxane A2 (TX A2) produced by activated platelets, has prothrombotic properties, stimulating activation of new platelets as well as increasing the platelet aggregation. There are reports that thromboxane-dependent platelet activation enhances cerebral thrombosis (Patrono et al., 1991). Moreover, TX A2 acts as both a vasoconstrictor and platelet activator (Ellis et al., 1976). TX A2 has a short half-life in the body and is rapidly hydrolyzed to thromboxane B2 (TX B2).11-dehydro TX B2 is a metabolite of TX B2 with a circulating half-life ($t_{½}$) of 45 minutes; its measurement in plasma or urine will give a time-integrated indication of TX A2 production (Catella et al., 1986). An increased production of TX B2 might be contributory to thrombosis (Saldeen et al., 1983). Our results revealed significant decrease in 11-dehydro TX B2 levels in VCO fed rats as compared to those fed other oils. There are reports that saturated fats have an inhibitory effect on TX B2 production (Steel et al., 1990), while, unsaturated fatty acids *viz.*, oleic, linoleic and arachidonic acid enhances the production of TX B2 (Ishitsuka et al., 2004; Muakkassa et al., 1991; Whelan et al., 1993). But supplementation of n-3 PUFA reduces TX B2 production (Vilaseca et al., 1990).

Blood platelets are known to play a role in the regulation of hemostasis and thrombosis (Véricel et al., 2004) and consequently in the major cardiovascular complications (Massberg et al., 2002). Platelet-dependent thrombus formation plays an essential role in the manifestation of ischemic heart syndrome (Meade et al., 1986; Heinrich et al., 1994). Platelets are thought to initiate a series of intricate reactions by adhering to the injured arterial lining, aggregating irreversibly to form a platelet plug, and releasing vasoactive metabolites and hydrolytic enzymes that might in turn alter both the function and structure of the vessel (Colman, 1975). Platelets mediate both thrombotic occlusion of the entire epicardial coronary artery and also accumulate in the microcirculation resulting in impairment of microcirculation and provoking myocardial ischemia during reperfusion (Gawaz, 2004).

Studies suggest that individual saturated fatty acids differently affect platelet aggregation capacity (Fuhrman et al., 1986; Renaud et al., 1986). There are reports that chain length of fatty acids plays an important role in platelet aggregation and the inhibitory effect on platelet aggregation was increased with increase in chain length

up to C 14 (Kitagawa et al., 1984). Fatty acid analysis of test oils revealed that oil extracted from coconut mostly consists of short and medium chain fatty acids (Arunima and Rajamohan, 2013). *In vivo* experiments have shown that feeding medium chain triglycerides (MCT) decreases thrombosis formation in rats (Kaunitz, 1986). Intervention studies have shown that increased platelet aggregation was found in linoleic acid enriched diets (Mutanen and Freese, 1996). Apart from fatty acids, polyphenols and tocotrienols are known to inhibit platelet aggregation (De Lange et al., 2007; Qureshi et al., 2011). Chemical analysis of these test oils has revealed that VCO by wet processing contain increased polyphenolic contents (84 mg/100 g oil), which is significantly (p <0.05) higher than other test oils *viz.*, CO (64·4 mg/100 g oil), OO (75·63 mg/100 g oil) and SFO (55·26 mg/100 g oil).

HPLC analysis of the phenolic fraction of VCO has revealed the presence of caffeic acid, *p*-coumaric acid, ferulic acid, (+)-catechin hydrate and syringic acid compared with CO, OO and SFO (Arunima and Rajamohan, 2013), which may have a synergistic effect on platelet aggregation. In addition, the nonsaponifiable fraction of VCO contains appreciably higher amounts of antioxidants, namely vitamin E (33·12 mg/100 g oil) and b-carotene (196mg/100 g oil) (Arunima and Rajamohan, 2012). Increased amounts of these non-saponifible components present in VCO may partly be responsible for the decreased platelet aggregation compared with other oils. The inhibitory effect of VCO on platelet aggregation was confirmed by the *in vitro* platelet aggregation assay using polyphenol fraction isolated from test oils. A decreased aggregation rate was observed with VCO polyphenols on ADP induced *in vitro* platelet aggregation.

These results demonstrated that supplementation of VCO have a significant antithrombotic effect compared to those fed CO,OO and SFO, which is characterized by an increased fibrinolytic activity as well as decreased rate of platelet aggregation.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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COMPARISON OF PROTHROMBIN TIME AND ACTIVATED PARTIAL THROMBOPLASTIN TIME BETWEEN PATIENTS WITH DIABETES MELLITUS AND DIABETICS WITH HYPERTENSION

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ABSTRACT

Uncontrolled Diabetes Mellitus Causes both microangiopathy leading to diabetic nephropathy & retinopathy and macroangiopathy leading to coronary heart diseases & also diabetic neuropathy. Hypertension itself is characterized by arteriosclerosis. To assess the coagulation status, we measured PT & APTT in these cases. A 52 Known Type II diabetic patients with hypertension, 32 known Type II Diabetes Mellitus patients & 30 controls were included in the study. Both PT & APTT were measured on CA-50 Sysmex Coagulation Analyzer. The FBS & PPBS were estimated on Humastar-300 complete Auto analyzer. BMI was calculated for all the subjects. The PT was prolonged in Group B (14.86538 + 0.12290) than controls (13.83333 + 0.08419) ,p = 0.0000001 and Group A(13.40 + 1.29) ,p = 0.0000001. The APTT was prolonged in Group B (34.53846 + 0.27933) than controls (30.13333 + 0.32424) p = 0.0000001 and Group A(30.81 + 2.40) ,p = 0.0000001 respectively. The FBS was increased in cases than controls (p = 0.0000001). The PPBS was also increased in cases than controls (p = 0.0000001). The BMI for Group A (26.55 + 3.2) Group B (26.9615 + 2.6515) and for controls (25.05 + 2.7268). This study showed Prolonged PT and APTT in diabetes mellitus with hypertension. Abnormal PT & APTT are the indications of altered activity or absence or decreased levels of clotting factors of intrinsic & extrinsic coagulation pathways respectively. The BMI value indicates that cases are overweight. PT & APTT should be checked in such cases prior to any surgery.

KEYWORDS : Procoagulant, Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT), Tissue Factor, Fibrinolysis.

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INTRODUCTION

Uncontrolled Diabetes mellitus causes both microangiopathy leading to Diabetic nephropathy, retinopathy and macroangiopathy leading to coronary heart disease and also Diabetic neuropathy[9]. Hypertension substantially increases the risk of both microvascular and macrovascular complications including stroke, Coronary artery disease and peripheral vascular disease, retinopathy, nephropathy and possibly neuropathy[7]. Macrovascular disorders such as atherosclerosis are a recognized major cause of morbidity in the diabetic population, and are implicated in the circulatory disturbances that are seen in diabetes[11]. The circulatory disturbances are further compounded by alteration in platelet count and activity, coagulopathy, fibrinolytic aberration, haemorrheological factors, and changes in endothelial metabolism.Thrombotic myocardial infarction may be secondary to complicated or ruptured atherosclerotic plaques with further exposure of procoagulant proteins that initiate blood coagulation or due to contact between blood and damaged endothelium. The purpose of the study is to assay some of the Haemostatic factors (PT & APTT) in patients with Type II Diabetes Mellitus (or) NIDDM and correlate the results with Diabetic patients with Hypertension & controls.

MATERIALS AND METHODS

The present study was carried out at ASRAM Medical College and Teaching Hospital at Eluru, in Andhra Pradesh. 32 known Type II Diabetes Mellitus patients as Group A, 52 known Type II Diabetes Mellitus patients with Hypertension as Group B of both sexes, 30 healthy age and sex matched controls were included in the study. After obtaining ethical committee consent and patient consent, closed in the interview was done to the patients. 35-70 years, Type II diabetic with hypertension, Type II Diabetic patients were included in the study. Vitamin-K deficiency, Pregnancy, Valve replacement was excluded in the study. During questioner, both patient history and Family history was collected. For the Fasting Blood Sugar (FBS) sample, both the patients and controls were instructed to fast for 8-12 hours. During Fasting hours, the patients were allowed to take only water and any other type of food was not allowed. A Fasting venous blood was collected under aseptic condition, by doing vein puncture. The blood was collected in both plain and citrated tubes. A 1.8 ml. of 3.2 % of citrate was used as an Anticoagulant. The citrated blood was centrifuged at 1500 rpm for 10 minutes and plasma was separated immediately. The plasma was used for the estimation of Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) respectively. PT and APTT were measured in plasma on Sysmex 50 coagulation Analyzer immediately. PT was estimated by Liquiplastin Thromboplastin reagent using TULIP Diagnostics Private Limited kits. Similarly APTT was estimated by Liquicelin-E Cephaloplastin reagent using Elagic Acid as an activator using TULIP Diagnostics Private Limited kits. The plain tube blood was allowed to clot for 30 minutes. Serum was separated by centrifuging at 1500 rpm for 10 minutes. The serum was immediately estimated for Fasting Blood Sugar (FBS). After giving, an oral Glucose load of 75 grams of Glucose, Post Prandial Blood Sugar (PPBS) was estimated after 2 hours in serum. The serum Glucose was estimated by G.O.D.-P.O.D. method on Huma star 300 complete Auto analyzer, using Human kits.

RESULTS

The results obtained in this study were from a total of 114 subjects. These 114 subjects have been divided into controls containing (n = 30), Group – A containing (n = 32) and Group-B containing (n = 52).The results are shown inTable-1, Table -2 and Table-3.

Table-1

Comparision of serum FBS , PPBS , BMI , PT ,APTT between Controls & Group A

Groups	FBS	PPBS	BMI	PT	APTT
Controls Mean ±SD	85.8 ± 10.94	124.8 ± 10.45	25.05 ± 2.72	13.83 ± 0.46	30.13 ± 1.77
Group A Mean ±SD	155.62±50.01	235.59 ± 61.9	26.55 ± 3.23	13.40 ± 1.29	30.81 ± 2.40
t – value	42.49	55.11	3.5	-9.680	7.555
p – value	0.001**	0.001**	0.001**	0.5	0.001**
Inference	Highly significant	Highly significant	Highly significant	Not significant	Highly significant

Table 1 shown the Serum levels of FBS, PPBS , APTT and calculated BMI were significantly increased and there was no significant change in PT levels in controls and Group-A

Table -2

Comparision of serum FBS , PPBS , BMI , PT ,APTT between Controls & Group B

Groups	FBS	PPBS	BMI	PT	APTT
Controls Mean ±SD	85.8 ± 10.94	124.8 ± 10.45	25.05 ± 2.72	13.83 ± 0.46	30.13 ± 1.77
Group B Mean ±SD	198.557±73.7	268.25± 67.82	26.94 ± 2.67	14.84 ± 0.84	34.53 ± 2.0
t – value	1.631	22.403	6	11.854	19.52
p – value	0.2	0.001**	0.001**	0.001**	0.001**
Inference	Not significant	Highly significant	Highly significant	Highly significant	Highly significant

Table 2 shown the Serum levels of PPBS , PT ,APTT and calculated BMI were significantly increased and there was in FBS levels and not significant in controls and Group-B

Table – 3

Comparision of serum FBS , PPBS , BMI , PT ,APTT between Group A & Group B

Groups	FBS	PPBS	BMI	PT	APTT
Group A Mean ±SD	155.62±50.01	235.59 ± 61.9	26.55 ± 3.23	13.40 ± 1.29	30.81 ± 2.40
Group B Mean ±SD	198.557±73.7	268.25± 67.82	26.94 ± 2.67	14.84 ± 0.84	34.53 ± 2.0
t – value	5.963	0.8125	1.229	16.22	15.70
p – value	0.001**	0.4	0.2	0.001**	0.001**
Inference	Highly significant	Not significant	Not significant	Highly significant	Highly significant

Table 3 shown the Serum levels of FBS, PT ,APTT were significantly increased and PPBS levels were increased and are not significant and there was no significant change in calculated BMI in Group A and Group-B

DISCUSSION

The present study was done on 32 diabetic patients, and 52 diabetic patients with Hypertension and 30 normals were taken as controls. Various parameters like FBS, PPBS, PT, APTT and BMI of these patients were compared with controls. Diabetic patients comprised Group-A and Diabetic patients with Hypertension comprised Group-B. In comparison with controls FBS, PPBS, BMI and APTT values were highly elevated P< 0.001 indicating that Group A patients had higher BMI, higher Blood glucose levels and prolonged APTT (but still within the normal range) PT did not show any significant variation. Abnormalities in both lipid metabolism and plasma coagulation system have been considered to contribute to the development of Atherosclerotic changes and Thrombotic events in Diabetic patients. The present study did not show any thrombotic tendency in comparison with the controls as seen by a prolonged APTT value in diabetic patients. This finding is correlated to the findings of Fatherlrahman Mahdi Hassan in the year 2009[4] of 50 diabetic patients and 10 controls where there was no difference between APTT values which is not correlated to the present study though the difference is not significant between controls and Diabetics. PT was shortened significantly. Patients with Type-II Diabetes Mellitus also show higher BMI when compared with normal individuals. Hypercoagulation in abdominal obesity is thought to be caused primarly by the synthesis of factors activating coagulation and inhibiting fibrinolysis in adipose tissue. These findings are correlated well with the findings of Acang N, Jalil FD in the year 1993[1], 60 diabetic patients and 60 controls. The mean ± SD of PT is 10.1 ± 1.31 seconds in diabetic patients Vs 11.04 ± 0.93 seconds in controls. Whereas APTT is 29.2 ± 3.69 sec. Vs 32.16 ± 3.77 sec. They found PT and APTT were shorter in diabetic patients when compared to controls.

A similar findings were found in the study done by D.L.Sauls, A.C. Banini, et al., in the year 2006[3] of 41 diabetic patients and 39 controls. They found PT (11.3± 0.5 Vs 11.9 ± 0.6 sec.) and APTT values (26.6 ± 3.7 Vs 29.3 ± 3.4 sec.) were shorter in diabetes when compared with controls. According to their study APTT values were shortened which was not seen in the present study. The study done by OO Alao, DO Damulak, et al. in the year 2010 [6] of 50 Type-II Diabetic patients were compared with 50 control's. PT and APTT values were found to be prolonged (P<0.005). This is not correlated to the present study where PT was shortened in diabetics though APTT was prolonged. The study done by Ritu Madan, Baupta, Sumita Saluja, et al 2009 [8] of 60 type-II diabetic patients were compared with 30 non-diabetic patients. They found no difference in PT (P=0.05) and APTT (P=0.05) values in diabetics verses control's. This indicates that Diabetic patients need not always have a hypercoagulable state PT could be shortened too which is correlated to the present study. Many studies have shown both procoagulant and Anticoagulant status in Diabetics. Because Diabetes Mellitus is multifactorial disease the coagulation profile may be affected by several factors which have yet to be investigated. In the present study on comparison of Group-B with controls parameters like PT, APTT, BMI and PPBS showed a significant elevation in Group-B patients which was statistically significant (P < 0.001). The study done by Cristiana Catena, Laura Zingaro, et al., in the year 2000 [2],coagulation abnormalities were assessed in 382 patients with essential hypertension and compared with controls ,they found that PT and APTT values did not show significant values which is not correlated to the present study. In a study done by R.S.El-Hagracy, G.M. Kamal, et al., in the year 2010[10], 60 Type II Diabetic patients were divided into Group I having 30 patients without history (or) clinically detected heart disease and Group – II having 30 patients with history of Myocardial infarction. These subjects were compared with 20 controls. The levels of tissue factor pathway Inhibitor and factor VIIa were compared b/n these patients and controls. The results showed that the values of TF, TFPI and Factor VIIa were higher in diabetic group when compared to controls. These values were significantly higher in hypertensives and dyslipidemia but not in smokers. This indicates that Diabetic patients with hypertensive patients have procoagulant status when compared with controls. All these studies do not show much change in Hypertensive patients with regards to PT and APTT values. Some studies have shown a procoagulant state which is different from the results obtained in the present study where PT and APTT values are prolonged. This indicates that there is a tendency for bleeding in these patients. In the present study on comparison of PT and APTT values between Group-A and Group – B showed highly significant prolongation in PT and APTT values. P value < 0.001. BMI did not show any significant variation between the two groups. Leonardo A. Sechi, Laura Zingaro, et al., in the year 2003[5], has investigated 352 patients with Mild to moderate essential Hypertension and diabetic patients with hypertension on parameters like PT, APTT and BMI. They found that hypertensive patients had greater BMI than controls. There was no significant difference in PT and APTT which is not correlated to the present study.

CONCLUSION

The present study shows significant difference in PT and APTT values between Group-A and Group-B where there is a prolongation in these values in Group-B patients which should be kept in mind before undertaking any surgical procedure for patients with Diabetes mellitus and hypertension. These findings suggest that haemorrhagic tendencies and complications should not be entirely ruled out among diabetics, and should born in mind during the management of these patients. It would also be helpful to incorporate coagulation screening (PT, APTT) as routine tests for better management of Diabetic patients. Obesity enhances thrombotic tendency through up regulation of Tissue Factor, altered expression of proteins participating in the coagulation cascade as well as atherosclerosis. The positive correlation of procoagulant markers (PT & APTT) and BMI reinforces the importance of Glucose optimal control in T2DM patients.

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