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PHARMACOLOGICAL INVESTIGATION STUDIES OF ETHANOLIC PLANT EXTRACTS OF ADIANTUM INCISUM AND SAGITTARIA TRIFOLIA IN THROMBOSIS AND INFLAMMATION USING K-CARRAGEENEAN ANIMAL MODEL

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Abstract:
The present study was undertaken to investigate the anti-thrombotic and thrombolytic activity of Adiantum incisum forsk leaves extracts and Sagittaria trifolia linn root extracts.
Male Albino Wistar rats were isolated in six groups comprising six creatures in each group. In order to induce thrombosis and test the activity of both the plant extracts in vivo, the carrageenan induced tail thrombosis model was utilized. K-carrageenan dissolved in saline and the right hind paw of each rat was injected subplantar with 20mg/kg B.W of k-carrageenan 1 hour after each dose of both plants extracts200mg/kg of AI and 200mg/kg of ST respectively) separately and in combination (200mg/kg AI+200 mg/kg ST). The tail thrombus lengths were measured in centimeters with scale and photographed at 24, 48, and 72 hrs after carrageenan injections. After appearance of the thrombus in rat tail, the animals were administered with the respective treatment as per the assigned group for 29 days.
After the completion of study, the blood sample of animals was collected by retroorbital puncture and the serum was separated and send to laboratory for assessment of haematological parameters, the rats were sacrificed and their tails were extracted for histopathological examination.
In this study, the extracts of Adiantum incisum(200mg/kg) showed substantial decrease in the tail thrombus lengths and more significant response compared to Sagittaria trifolia extracts(200mg/kg) and combination of both extracts (200mg/kg of AI + 200mg/kg of ST). The results were obtained from both the extracts respectively and in combination but the extract of Adiantum incisum were more potent than others.
The results of the selected showed marked decrease in CRP levels with Adiantum incisum being most evident among all. CRP being a main biomarker for inflammation and thrombosis
Key Words: *Thrombosis, k-carrageenan model, Adiantum incisum forsk, Sagittaria trifolia linn, Thrombolytic activity, Inflammation, CRP.*

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INTRODUCTION:

THROMBOSIS: Thrombosis is the formation of a blood clot (thrombus) inside a blood vessel, interrupting blood supply to the tissues. The risk of a thrombus developing within a blood vessel is increased by:
Slow blood flow, Damage to the blood vessel intima, Increased blood coagulability.

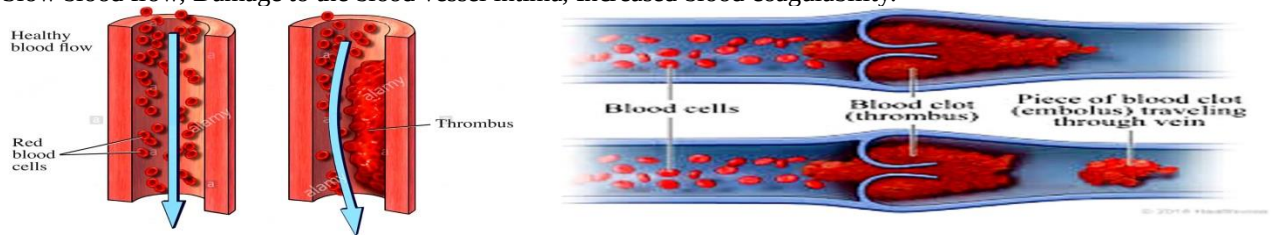


Fig 1 Interruption of blood supply because of thrombus and Representation of embolus travelling in veins or arteries.

Embolism is the blocking of a blood vessel by any mass of material (an embolus) travelling in the blood. Emboli originating in an artery travel away from the heart until they reach an artery too narrow to let them pass, and lodge there, partly or completely blocking blood supply to distal tissues. This is a common cause of stroke, myocardial infarction and gangrenous limbs.

Emboli originating in veins (DVT) travel towards the heart, and from there to the lungs in the pulmonary artery. They then lodge in the first branch narrower than they are (pulmonary embolism).[1]

Venous thrombosis, including deep vein thrombosis, (DVT) and pulmonary embolism (PE), occurs at an annual incidence of about 1 per 1,000 adults. Rates increase sharply after about age 45 years, and are slightly higher in men than women in older age. [2]
DEEP VEIN thrombosis (DVT) and pulmonary embolus (PE), collectively referred to as venous thromboembolism (VTE), are major sources of morbidity and mortality.[3]

INFLAMMATION: Inflammation is the physiological response to tissue damage and is accompanied by a characteristic series of local changes. Its purpose is protective: to isolate, inactivate and remove both the causative agent and damaged tissue, so that healing can take place. The cardinal signs of inflammation are redness, heat, swelling and pain.

Causes of inflammation: Any form of tissue damage stimulates the inflammatory response, even in the absence of infection. The wide range of causative agents includes extremes of temperature,trauma, corrosive chemicals including extremes of pH,abrasion and infection by pathogens.

Acute inflammation: Acute inflammation is typically of short duration. The acute inflammatory response is described here for convenience as a collection of separate events: increased blood flow, accumulation of tissue fluid, migration of leukocytes, increased core temperature, pain and sup-puration.

Chronic inflammation: Tuberculosis is an example of an infection that frequently becomes chronic, leading to granuloma formation. Granulomas form when the immune system attempts to wall off substances it perceives as foreign but is unable to eliminate.[4]

CORRELATION BETWEEN THROMBOSIS AND INFLAMMATION: C-reactive protein (CRP) is an acute phase reactant plasma protein that is present in plasma of healthy humans and whose plasma concentration increases significantly during acute and chronic inflammation. CRP increases the risk of ischemic vascular events, such as myocardial infarction, not by promoting atherosclerotic plaque size, but rather by activating the blood coagulation system and increasing the risk of thrombosis. The regulatory systems that control hemostasis and thrombosis, although functioning in a highly coordinated manner, can be subdivided into three major components, namely: (1) blood platelets; (2) blood coagulation proteins present in plasma and the vascular wall; and (3) the fibrinolytic system.[5]

MATERIALS AND METHODS:

CHEMICALS:

Toxicant – kappa-Carrageenan, Extractor-ethanol, Standard-heparin (low molecular weight), Anaesthetic-chloroform, distilled water and saline was used for this study.

PLANT MATERIAL:

The dried leaves of *Adiantum incisum Forsk* and dried tubers of *Sagittaria trifolia Linn* was collected, identified and authenticated by botanist Dr. Madhava Chetty, Assistant Professor of Botany, (Dept. of

Pharmacognosy), Sri Venkateshwara College, Tirupathi

PREPARATION OF EXTRACTS:
(THROUGH MACERATION): The crude powder of Adiantum incisum forsk leaves and crude powder of Saggittaria trifolia linn tubers were taken and then mixed with 500ml of ethanol each in separate ceramic containers and packed air tight through alluminium foil. Then the mixtures were stirred 2 times a day for 5 days by addition of ethanol in parts of 125ml. After those mixtures were then kept still for 2 days and on the 8th day they were filtered through a clean muslin cloth thoroughly. The filtrates of both the plants were then kept for evaporation until thick creamy mixtures were formed, then taken out in seperate bowls and weighed.[6]

Fig: 2 Maceration process

PHYTOCHEMICAL ANALYSIS OF EXTRACT:
Phytochemical screening of the crude extracts of *Adiantum incisum* and *Sagittaria trifolia* was performed qualitatively for the presence of alkaloids, flavonoids, coumarins, saponins, glycosides etc, according to standard methods. [7,8,9]
The extracts were also sent for GC-MS analysis.

EXPERIMENTAL ANIMALS:
The experimental study was conducted at Shadan Women's College of Pharmacy, Khairtabad, Hyderabad. Forty-two albino wistar rats of about 8-12 weeks of age were procured from VAB Bio sciences, Musheerabad, Hyderabad (282/PO/Bt/S/2000 CPCSEA) and acclimatized for 14 days in animal house of Shadan Women's college of Pharmacy. The selected animals were housed in wire mesh, well aerated cages at normal atmospheric temperature (25±5ºC) and normal 12-hour light/dark cycle. They had free access to water and were supplied with standard agro diet of known composition. All animal procedures were performed in accordance with the CPCSEA guidelines and standard recommendations for care and use of laboratory animals. Our proposal number is IEC-03/SES/2020/41/106.

ACUTE ORAL TOXICITY STUDIES:
The acute toxicity studies of adiantum incisum and sagittaria trifolia were done according to OECD guideline No.425 using 5 animals (i.e., albino wistar rats) for each plant extract. Each animal was administered with the extracts of the plant by oral route (2000mg/kg was used as a starting dose). Animals were observed individually after dosing with attention for first 3-4 hours. At regular intervals, they were observed during the first 48 hours with special attention to first four hours and thereafter daily for the next fourteen days.[10]

EXPERIMENTAL GROUPS:

Animals will be divided into 6 groups, each group containing 6 rats (n=6)

S.no.	GROUPS	AGE OF ANIMALS	DOSAGE SCHEDULE
1.	Normal control	8-12 weeks	Saline.
2.	Thrombotic control	8-12 weeks	k-carrageenan (20mg/kg B.W suboplantar injection).
3.	Standard control	8-12 weeks	k-carrageenan (20mg/kg B.W subplantar injection) + Std heparin 50 IU (I.P).
4.	Plant – I (200mg/kg)	8-12 weeks	k-carrageenan + 200mg/kg of adiantum incisum ethanolic extract (p.o)
5.	Plant- II (200mg/kg)	8-12 weeks	k-carrageenan + 200mg/kg of sagittaria trifolia ethanolic extract (p.o).
6.	Plant – I + Plant – II (200mg/kg + 200mg/kg)	8-12 weeks	k-carrageenan + 200mg/kg of adiantum incisum (p.o) +200mg/kg of sagittaria trifolia ethanolic extract (p.o).

Table-1 Experimental Design

ANIMAL MODEL:

K-CARRAGEENAN INDUCED RAT TAIL THROMBOSIS MODEL:

In order to induce thrombosis and test the activity of adiantum incisum and sagittaria trifolia extracts in vivo, the carrageenan induced tail thrombosis model was utilized. K- carrageenan was dissolved in saline and the right hind paw of each rat was injected subplantar with 20mg/kg B.W of k- carrageenan 1 hour after each dose of extract in each group. The tail thrombus lengths were measured in centimeters with scale an d photographed at 24, 48, 72 hr after carrageenan injection.[11]

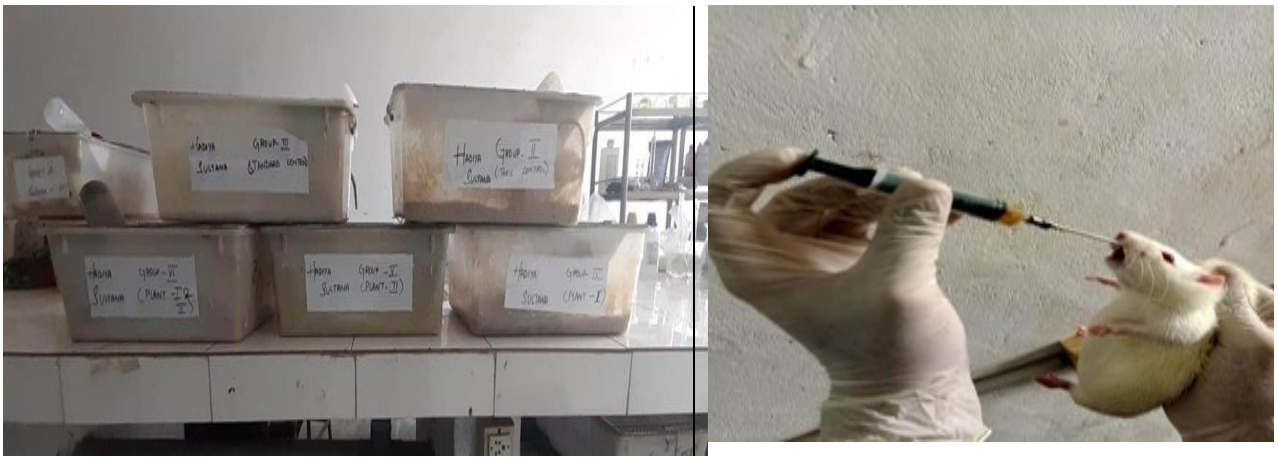


Fig: 3 Grouping and Dosing of animals

After appearance of the thrombus in rat tail, the animals were administered with the respective treatment as per the assigned group for 30 days.

After the completion of study, the rats were sacrificed. The blood sample of the animals were collected through retro orbital puncture and the serum was separated and sent to the laboratory for the assessment of hematological parameter through hematological analysis.

SAMPLE COLLECTION

COLLECTION OF BLOOD SAMPLES:

Blood was withdrawn from retro orbital plexus for serum estimation of the hemoglobin, CRP, WBC, etc. the blood withdrawn from the animals was up to 5ml in volume with the help of capillary tube for serum estimation.

HISTOPATHOLOGICAL ANALYSIS:

The procedure was carried out and on the 29th day of study animals were anaesthetized using diluted chloroform in a dessicating chamber and tails were ecxtracted from the dismembered creatures and sent to lab for parameters estimation like thrombus, vein congestion, epithelial morphology, disruption of cellular matter.

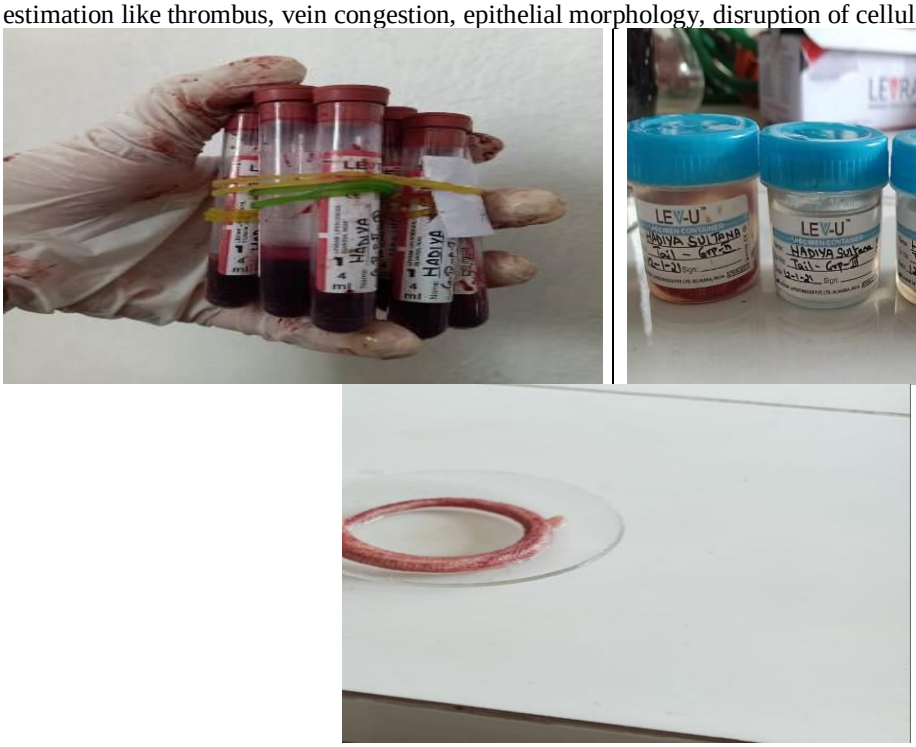


Fig: 4 Sample Collection

PARAMETERS FOR EVALUATION:

a) Length of thrombus.
b) Bleeding time.
c) Clotting time.
d) Haemoglobin levels.
e) Leukocyte count.
f) Platelet count.
g) C-reactive protein levels.
h) Histopathological study of tail.

STATISTICAL ANALYSIS:

The statistical analysis results were depicted as mean of ± S.E.M values and P values are P<0.01**, P<0.001*** were considered significant between standard and various groups. The graphs were drawn using MS-Excel.

RESULTS:

YIELD OF EXTRACT:

Percentage of Extract = wt. of dry sample/ wt. of sample x 100

Plant – I Extract - 130 / 500 x 100 = 26 %

Plant – II Extract - 122 / 500 x 100 = 24.4 %

ACUTE ORAL TOXICITY:

Adiantum incisum and *Sagittaria trifolia* were found to be safe at all doses and no behavioural changes and mortality was encountered at a dose of 2000mg/kg of the ethanolic extracts when given orally. Hence 200mg /kg was taken as a therapeutic dose

GC-MS ANALYSIS:
ADIANTUM INCISUM

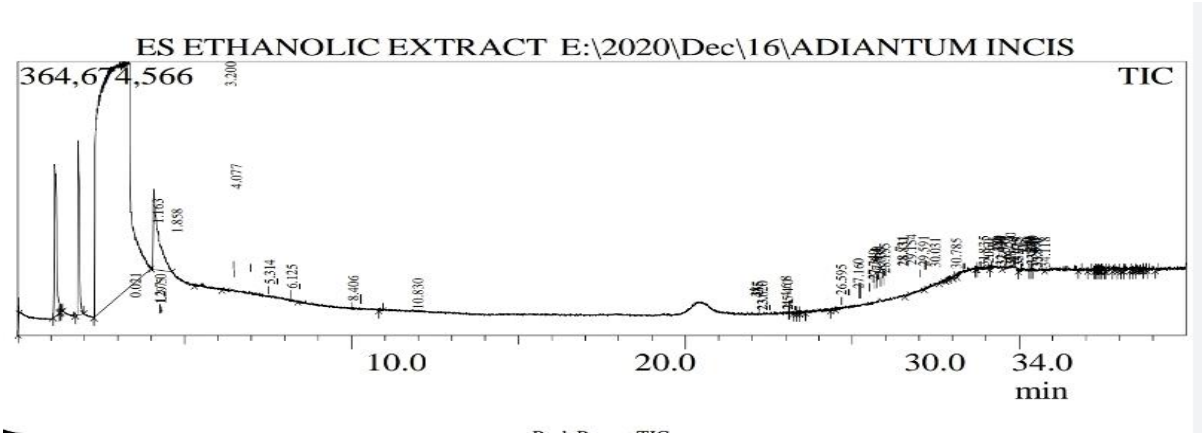


Fig 5 GC-MS Graph Of *Adiantum Incisum Forsk*

Table-2 GCMS analysis of adiantum incisum

S.No	RETENTION TIME	AREA%	CHEMICAL CONSTITUENTS	PHARMACOLOGICAL USES
1.	24.540	0.05	Methyl-4(2,4 dinitrophenylhydrazono) valerate	Antiinflammatory anti pyretic and analgesic, ani septic
2.	4.077	4.85	Methane sulfonic acid	Antiinflammatory, analgesic and anticoagulant, anti tumor
3.	28.035	0.05	Coumarone 3 one 6 methoxy	Anticoagulant, anti inflammatory, antiplatelet
4.	33.320	0.06	5,8 epoxy 3h 2 benzopyran	Antiplatelet,anticoagulant, antiinflammatory, analgesic
5.	33.470	0.07	3,5 Ethanoquinoline 10-ol decahydroxy sulfonic acid	Antimalarial,anticoagulant

SAGITTARIA TRIFOLIA

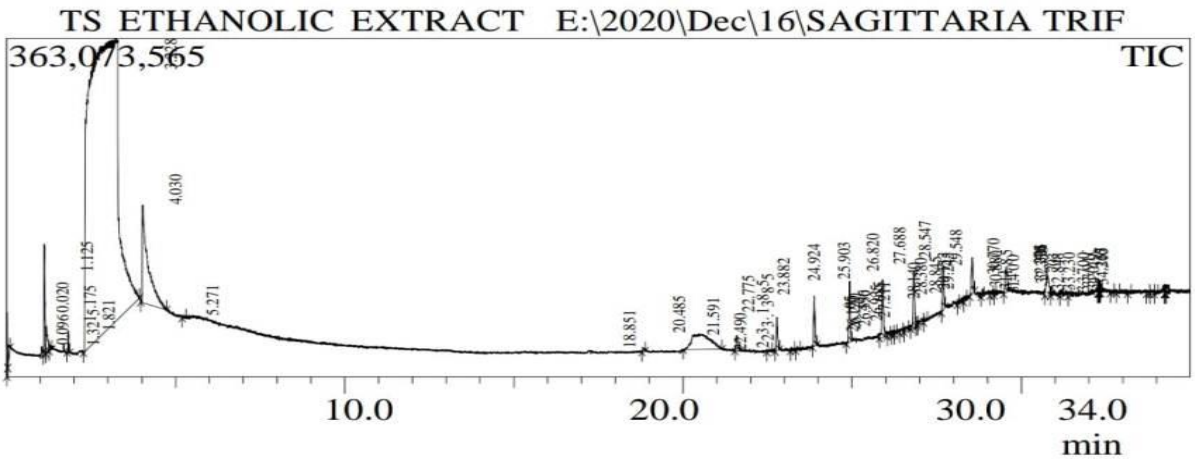


Fig 6 GC-MS Graph Of *Sagittaria Trifolia*

Table-3 GCMS analysis of sagittaria trifolia

S.No	RETENTION TIME	AREA%	CHEMICAL CONSTITUENTS	PHARMACOLOGICAL USES
1.	26.935	0.06	2,4-Di-tert-butylthiophenol	Anti-inflammatory, anticoagulant anti-tumor
2.	34.265	0.07	Coumarin-6-ol,3,4-dihydro-5,7-dinitro	Antiplatelet, antiseptic antihypertensive analgesic, anticoagulant
3.	26.820	0.85	Cyclodecasiloxane, eicosamethyl	Analgesic, anti-inflammatory. Antibiotic
4.	32.275	0.08	7,10 Epoxytricyclo [4.2.1.1(2,5)] decane, 1-trimethylsilyl	Anti-ulcer, emetic
5.	30.880	0.06	Estr-5(10)-ene-6.beta.-methanol, 3.beta.-fluoro-17.beta.-hydroxy-,17-acetate methanesulfonate	Anti-inflammatory, antibiotic,pregnancy termination

PHYTOCHEMICAL SCREENING:

Table -4 Phytochemical Screening Of Compounds

S.NO	PHYTOCONSTITUENTS	PLANT-I (Adiantum incisum)	PLANT-II (Sagittaria trifolia)
1.	Alkaloids	++	-
2.	Carbohydrates	+	++
3.	Reducing sugars	+	++
4.	Glycosides	++	++
5.	Flavonoids	++	+
6.	Phenolic compounds/phenols	++	++
7.	Tannins	-	+
8.	Saponins	++	++
9.	Phytosterols/steroids	+	++
10.	Terpenoids	++	-
11.	Coumarins	++	++

(+) indicates presence, (-) indicates absence

A) **LENGTHOF THROMBUS IN TAIL**:

The thrombus lengths were measured using scale in centimeters and were photographed at24,48 and72 hours and again on last day of study i.e., 29th.

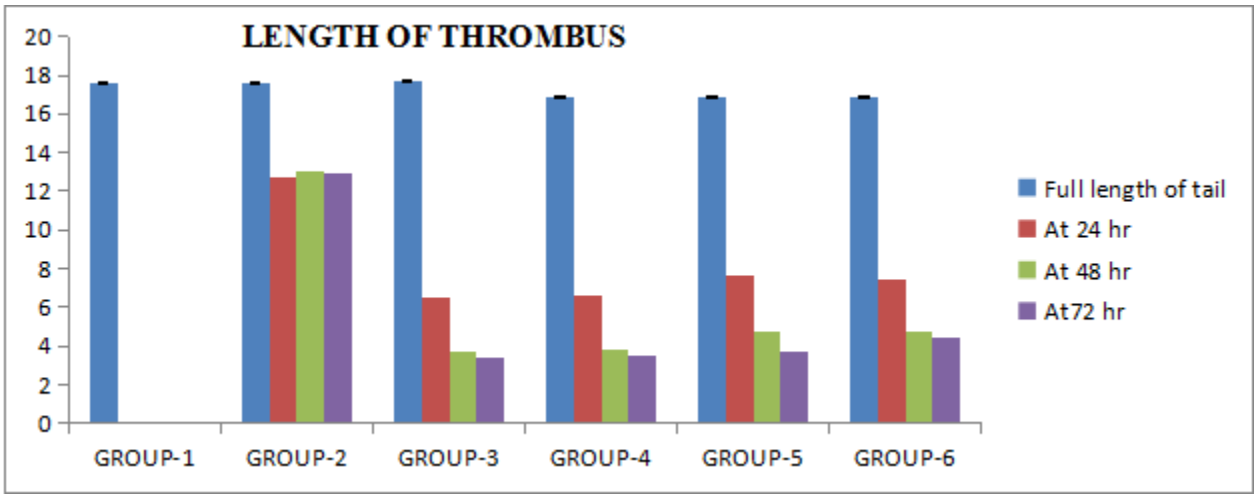
Table-5 length of thrombus

GROUPS	**TAIL FULL LENGTH (cm)**	**THROMBUS LENGTH (cm) AT 24 HR**	**AT 48 HR**	**AT 72 HR**
GROUP-1	17.60±0.06	-	-	-
GROUP-2	17.60 ± 0.09	12.7 ± 0.11	13.0 ± 0.15	12.96 ± 0.11
GROUP-3	17.68 ± 0.08	6.5± 0.04	3.7±0.06	3.4 ± 0.04***
GROUP-4	16.88 ± 0.02	6.6 ± 0.04	3.8 ± 0.07	3.5 ± 0.03***
GROUP-5	16.90 ± 0.02	7.6 ± 0.05	4.8 ± 0.67	3.7 ± 0.03**
GROUP-6	16.89 ± 0.02	7.4 ± 0.06	4.7 ± 0.18	4.4 ± 0.09*

Data was demonstrated as Mean ± S.E.M, n=6 rats in each group

*=P<0.05, **=P<0.001, ***P<0.0001 are considered significant when compared to thrombotic control group. Among all the treatment groups, 3 and 4 were found to be more significant

Fig 7 length of thrombus



Graph-1 length of thrombus

Blood clotting time: It determines the time required to form a clot. Make a bold prick at the tail vein of rat and suck the blood in a capillary tube,15cm long. Approximately 1cm of capillary tube was carefully broken off every 15 seconds until a fine thread of clotted blood appears while the capillary tube is being broken. The period in between appearance of blood from tail vein and formation of thread like fibrin clot was recorded as clotting time

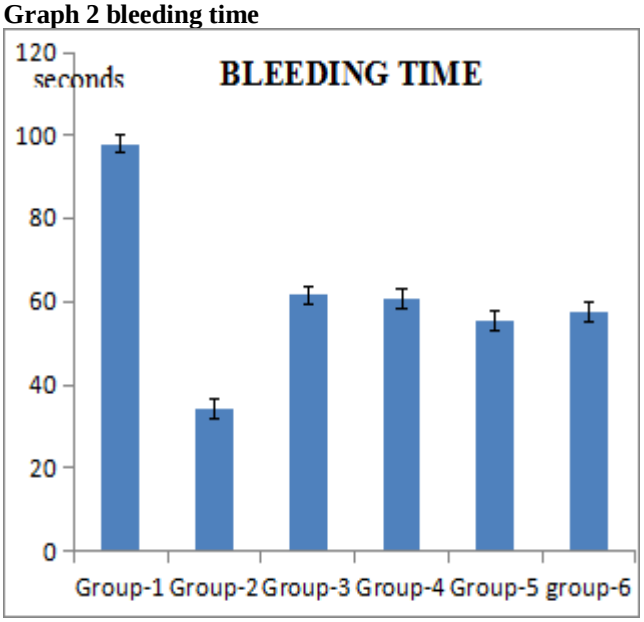
Bleeding time method (BT): Bleeding time was obtained according to reported methods. The tail of the rat was warmed for 1 min in water at 40°C and then dried. A small cut was made in the middle of the tail with a scalpel. Bleeding time started when the first drop touched the circular filter paper. It was checked at 30 s intervals until bleeding stopped.[12]

B) BLEEDING TIME

GROUPS	BLEEDING TIME (s)
GROUP-1	98 ±2.30
GROUP-2	34.3 ± 1.56
GROUP-3	61.5 ± 0.76***
GROUP-4	60.5 ± 0.76***
GROUP-5	55.5 ± 1.17**
GROUP-6	57.5 ± 2.04*
NORMAL RANGE : 60-110 seconds	

Table-6 bleeding time

Graph 2 bleeding time



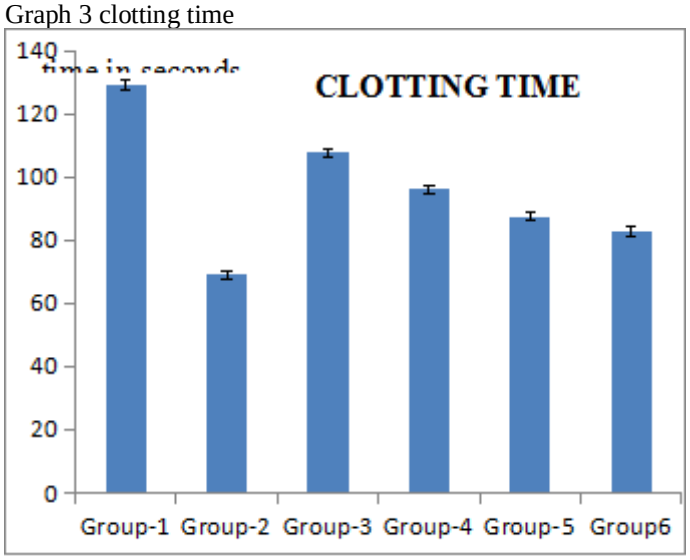
Data was demonstrated as Mean ± S.E.M, n=6 rats in each group
*=P<0.05, **=P<0.001, ***P<0.0001 are considered significant when compared to thrombotic control group. Among all the treatment groups, 3 and 4 were found to be more significant

C) CLOTTING TIME

GROUPS	CLOTTING TIME (s)
GROUP-1	129 ± 1.45
GROUP-2	69 ± 3.44
GROUP-3	107±.6 3.52***
GROUP-4	96 ±0.76***
GROUP-5	87.5 ± 0.76**
GROUP6	82.6 ± 1.86*
NORMALRANGE : 113-136 seconds	

Table-7 clotting time

Graph 3 clotting time



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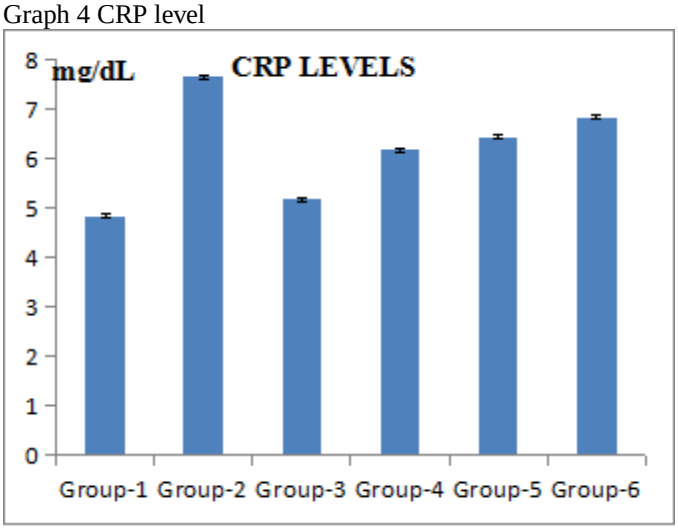
*=P<0.05, **=P<0.001, ***P<0.0001 are considered significant when compared to thrombotic control group. Among all the treatment groups, 3 and 4 were found to be more significant.

D) C-REACTIVE PROTEIN LEVELS

GROUPS	CRP LEVELS (mg/dL)
GROUP-1	4.84 ± 0.04
GROUP-2	7.66 ± 0.15
GROUP-3	5.19 ± 0.06***
GROUP-4	6.18 ± 0.23***
GROUP-5	6.44 ± 0.12**
GROUP-6	6.83 ± 0.12*
NORMAL RANGE : 3-5 mg/dL	

Table-8 C-reactive protein

Graph 4 CRP level



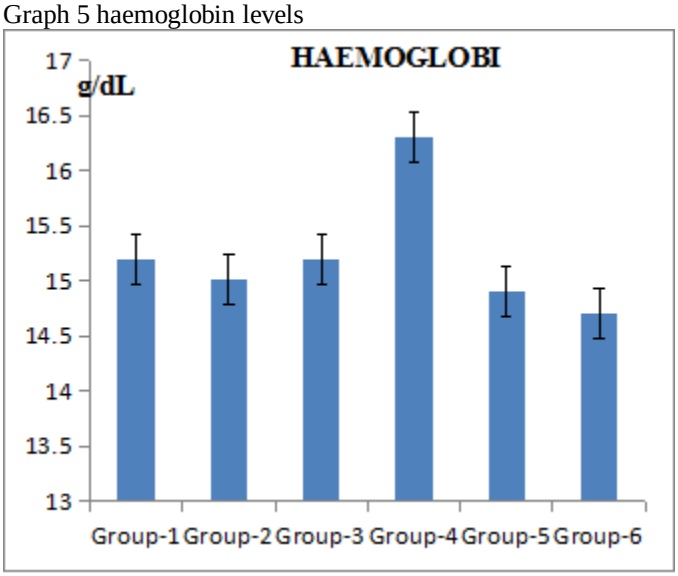
Data was demonstrated as Mean ± S.E.M, n=6 rats in each group
*=P<0.05, **=P<0.001, ***P<0.0001 are considered significant when compared to thrombotic control group. Among all the treatment groups, 3 and 4 were found to be more significant.

E) HAEMOGLOBIN LEVELS

GROUPS	HAEMOGLOBIN(g/dL)
GROUP-1	15.2 ± 0.23
GROUP-2	15.01 ± 0.23
GROUP-3	15.2 ± 0.24**
GROUP-4	16.3 ± 0.10***
GROUP-5	14.9 ± 0.21 **
GROUP-6	14.7 ± 0.04*
NORMAL RANGE: 10.36-13.7 gm/dL	

Table-9 haemoglobin levels

Graph 5 haemoglobin levels



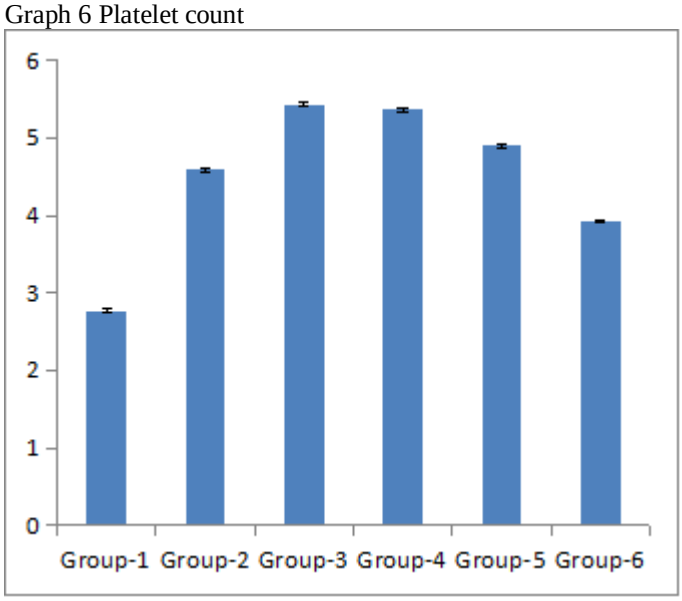
Data was demonstrated as Mean ± S.E.M, n=6 rats in each group
*=P<0.05, **=P<0.001, ***P<0.0001 are considered significant when compared to thrombotic control group. Among all the treatment groups, 4 was found to be more significant.

F) PLATELET COUNT

GROUPS	PLATELET (x 10^5/µL)
GROUP-1	2.77 ± 0.02
GROUP-2	4.59 ± 0.04
GROUP-3	5.43 ± 0.08***
GROUP-4	5.36 ± 0.08***
GROUP-5	4.90 ± 0.03**
GROUP-6	3.93 ± 0.04*
NORMAL RANGE :1.5 - 4.6 (x 10^5/µL)	

Tablr-10 platelet count

Graph 6 Platelet count



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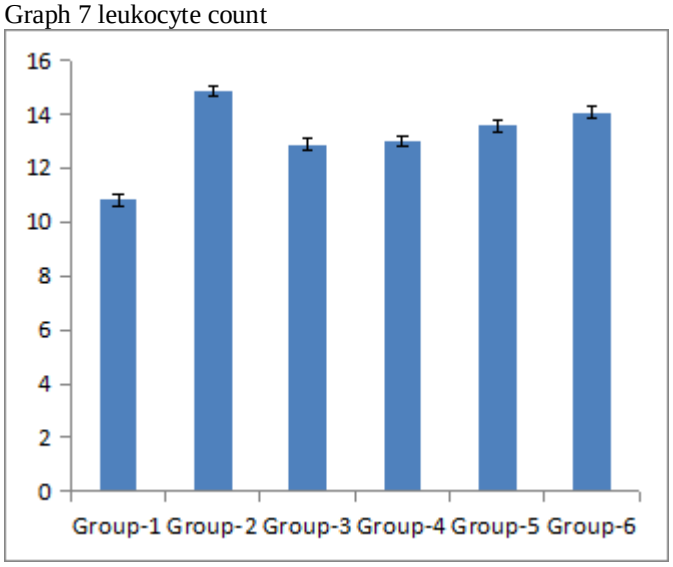
*=P<0.05, **=P<0.001, ***P<0.0001 are considered significant when compared to thrombotic control group. Among all the treatment groups, 3 and 4 were found to be more significant.

G) LEUKOCYTE COUNT

GROUPS	LEUKOCYTES(x 10^3/µL)
GROUP-1	10.82 ± 0.02
GROUP-2	14.86 ± 0.06
GROUP-3	12.89 ± 0.02 ***
GROUP-4	13.01 ± 0.21***
GROUP-5	13.57 ± 0.13**
GROUP-6	14.07 ± 0.12*
NORMAL RANGE : 6.6 - 12.6 (x 10^3/µL)	

Table-11 leukocyte count

Graph 7 leukocyte count



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HISTOPATHOLOGICAL CHANGES IN EXPERIMENTAL RATS:

Fig; 8 normal histology of tail

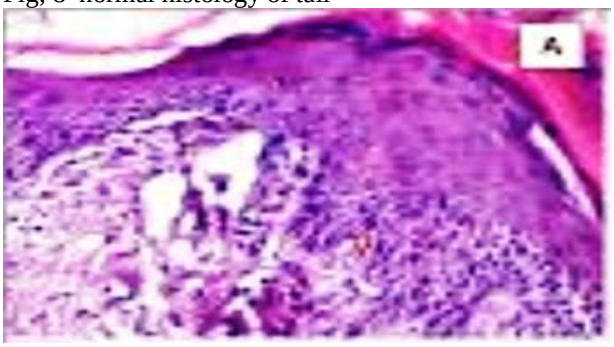


Fig:9 Inflammatorycells and discontinuity of epithelium

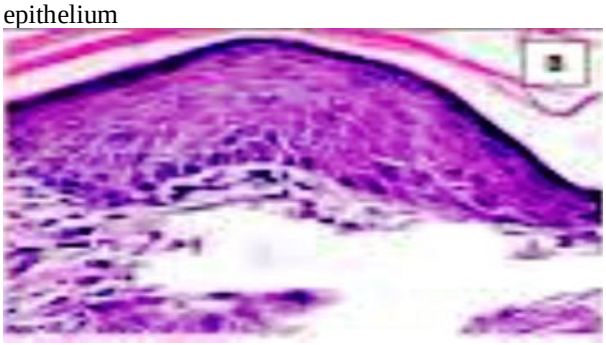


Fig:10 Thickening of capillary epithelium

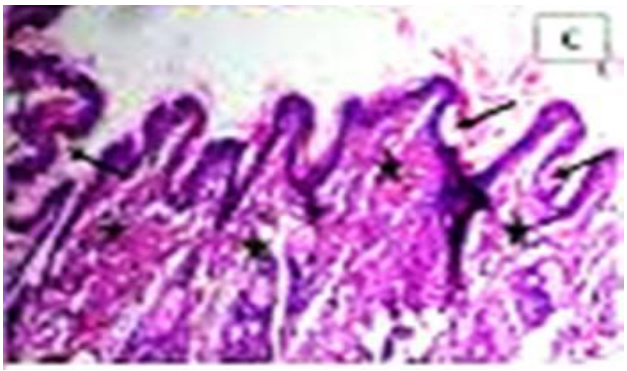


Fig: 11 loss of details in cellular structure

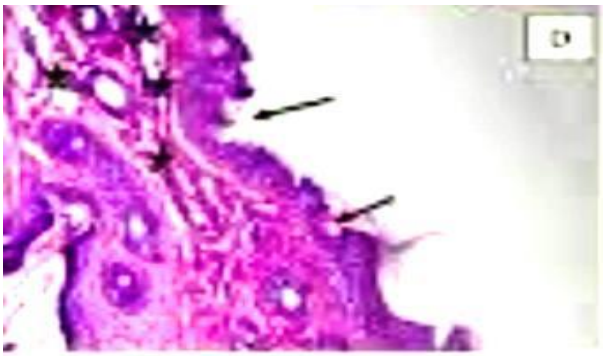


Fig:12 severe congestion endodermal

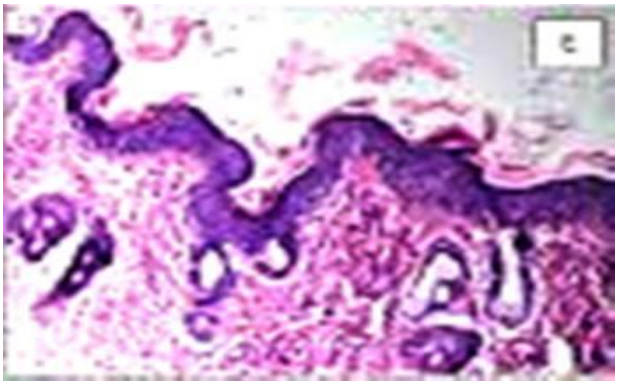
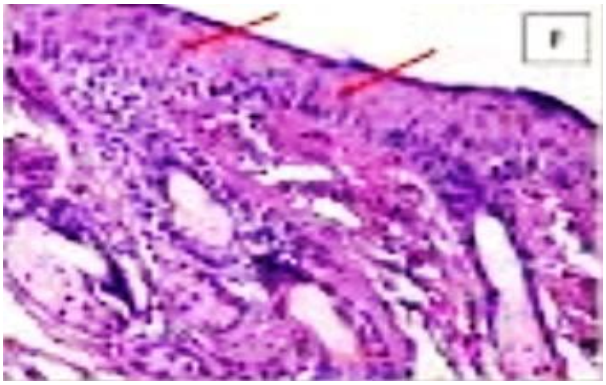


Fig:13 fatty deposit



DISCUSSION:

Medicinal plants and their constituents have been extensively used for their health promoting effect. They have established a role in disease prevention and treatment. In this panorama, adiantum incisum and sagittaria trifolia and its constituents are analysed for their disease prevention ability.

This study was conducted to investigate the protective effect of *Adiantum incisum* and *Sagittaria*

trifolia on k-carrageenan induced animal model. The k-carrageenan induced tail thrombosis model in rat is useful in evaluating the antithrombotic effects in drug discovery stage. The researchers can use this model to observe the progression of thrombosis visually and directly in a time dependent manner. Moreover, this model is simple and noninvasive on lab animals.
In this study we have used type-I/k-carrageenan as a toxicant drug in order to induce thrombosis tails of the rats through sub plantar route at a dose of 20mg/kg B.W. kappa carrageenan belongs to a family of sulphated polysaccharides and has one sulphate group. Kappa carrageenan is known to be most potent amongst the other carrageenan's available. The carrageenan drug was weighed and dissolved saline and desired dose was injected into the right hind paw of rats following which a red wine cultured thrombus appears in the tip of tail, the length of thrombus progresses with time and then pathogenic part demonstrates infarction. Since thrombus formation developed in tail has a clear border with the normal part, it's easy to detect the time it first appeared, the developing process progressing range and length of thrombus formed can be easily and visually measured in vivo. In our study the thrombus formed duet sub plantar injection of k- carrageenan had 100% frequency and thrombosis was succeeded in all control groups of animals.

Heparin (LMW) has been used as a standard drug in 50 I.U dose for comparing the potency of plants as it showed desired effect.

Here, the extracts of both the plants were prepared through maceration process respectively. the animals were divided into six groups each containing six rats the extracts were given orally in two dose first separately and then in combination i.e., 200mg/kg B.W of each plant separately (adiantum and sagittaria) and thenin combination of 400 mg/kg B.W tithe rats after the appearance of thrombus in their tails following carrageenan injection fore period of 29 day.s

The thrombus length were measured regularly with scale and the lengths were compared respectively.
At the end of study , the blood samples were collected through retro orbital puncture, and were send to laboratory for biochemical estimations. Where included CRP as a major biomarker for inflammation and thrombosis in order to evaluate the potency of plant extracts. Then the animals were sacrificed and tails of treated groups were sent for histopathological studies.

The results obtained were promising *adiantum incisum* extracts showed significant thrombolytic effect with visible reduction inthe thrombus lengths and inflammation there was marked decrease in CRP levels with the adiantum incisum extract which demonstrated the potent anti-inflammatory efficiency and extent of reduction in thrombus with signified its thrombolytic effect as compared with the sagittaria and the combination doses.

Apart from this the extracts have elevated haemoglobin levels and a considerable improvement in Bleeding time, Clotting time, Platelet swells leukocyte counts.

The histopathological study demonstrated a substantial enhancement at the tissue level. All extracts suggested a little to amused healing property owing to its anti-inflammatory efficiency.

From our study it can be concluded that the flavonoids and phenols present in both the plants and especially presence of coumarin in adiantum is responsible forth thrombolytic activity. However further investigations needed to be processed for understanding the mechanism involved.

CONCLUSION:

The outcomes of this study explain the substantial antithrombotic and fibrinolytic efficacy of *Adiantum incisum* and for the first time the use of combination of *Sagittaria trifolia* and *Adiantum incisum* has showed and given the idea of its little efficiency in antithrombotic and thrombolytic activity but the extracts of *Adiantum* being the most potent. The exact mechanism of the anti-thrombotic effects of *Adiantum* remains vague. As the literature reveals the flavonoids act by inhibiting platelet aggregation and the coumarins act by in habiting the release of plasma clotting factor VII, hence it can be postulated that flavonoids (in both plants) and coumarins present in *Adiantum* playsa key role in clot lysis and further clotting of blood. However, further studies are required to understand the exact mechanism of the activity of plant extracts.

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