



Evaluation of Wound-Healing Activity of *Eupatorium Adenophorum* Spreng Leaf Extract in Adult Wistar Rats

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ABSTRACT:

Use of plant extracts in the treatment of several ailments is a science known to the mankind since time immemorial. Yet, its popularity is limited owing to the lack of recorded experimental evidence to determine their potential health benefits. Medicinal plants play indispensable roles to treat various ailments. *Eupatorium adenophorum* Spreng belonging to family Asteraceae used traditional as blood coagulation in different kinds of haemorrhage condition and the leaves of this plant are used in Ayurveda and other folk medicines for the treatment of cut and wounds.

Objectives: Evaluation of wound healing activity of *Eupatorium adenophorum* Spreng leaf extract in adult albino rats.

Materials and Methods: Dried alcoholic and aqueous extracts of the leaves of *E. adenophorum* Spreng dissolved in normal saline with the help of tween-80, & The blood coagulation activity was determined by tail bleeding method and the alcoholic extract shows more significant when compared with aqueous extract and control group.

Results: The extract of *E. adenophorum* Spreng exhibit good coagulation activity, pharmacological studies of the *E. adenophorum* Spreng extract shows that the alcoholic extract is more potent than aqueous extracts.

1. Introduction

Blood Coagulation Activity

Blood coagulation is the process which causes blood to clot and helps prevent excessive blood loss when a vein or artery is ruptured^[1]. Coagulation is an important part of hemostasis (the cessation of blood loss from a damaged vessel), wherein a damaged blood vessel wall is covered by a platelet and fibrin-containing clot to stop bleeding and being repair of the damaged vessel^[2].

Blood coagulation is a series of coordinated and Ca²⁺-dependent proenzyme to serine protease conversions likely to be localized on the surfaces of activated cells *in vivo*, and culminating in the conversion (activation) of prothrombin to thrombin by the coagulant enzyme complex known as prothrombinase. Blood coagulation is initiated after the serine protease; factor VIIa, in the blood is exposed to and binds to tissue factor, a

transmembrane receptor for factor VII/VIIa. Tissue factor is constitutively expressed on subendothelial cells and also becomes expressed on the surface of activated monocytes and other inflammatory cells which accumulate at sites of injury^[3-6]. The apparent end point of blood coagulation, namely the conversion of the soluble plasma protein fibrinogen into insoluble fibrin, is only one of several necessary reactions of hemostasis catalyzed by thrombin.

As is true for most of the enzyme complexes that activate clotting factor zymogens into their respective serine proteases, prothrombinase consists of a serine protease (factor Xa) and its cofactor (factor Va) bound to (artificial or) activated cell surfaces in acidic phospholipids and Ca²⁺ dependent manner. The cofactor factor Va and the surfaces on which prothrombinase assembles jointly accelerate, by several orders of magnitude, the rate of prothrombin activation by factor



Xa^[7-9]. Cell surfaces such as activated platelets, monocytes, endothelial cells and lymphocytes which also serve as assembly sites (i.e. coagulant surfaces) for coagulation enzyme complexes. Coagulant surfaces also increase the turnover numbers of the proenzyme to enzyme conversions. These combined effects of coagulant surfaces and cofactors optimize the rates of clotting factor zymogens activation by their respective proteolytic enzyme cofactor complexes ^[10, 11]. Both thrombin (generated during blood coagulation) and platelets (after their activation by thrombin) participate in the maintenance of hemostasis and formation of thrombi. In addition to its well known functions in blood coagulation, hemostasis, and thrombosis, thrombin has several direct and indirect roles in inflammation, wound healing, embryogenesis, angiogenesis, and morphogenesis.^[12]

Coagulation begins almost after an injury to the blood vessel has damaged the endothelium, platelets immediately form a plug at the site of injury, these metabolic changes are usually termed aggregation ^[13]. The coagulation proteins play a major role in the formation of the fibrin clot. The protein which are involved in blood coagulation are listed in Table C-1

Table 1: Blood clotting proteins:

Name of protein	Function
Fibrinogen (factor I)	Adhesive protein that forms the fibrin clot
Prothrombin (factor II)	Activated form is main enzyme of coagulation
Tissue factor (factor IV)	Lipoprotein initiator of extrinsic pathway
Calcium ions (factor IV)	Metal cation necessary for coagulation reaction
Factor V (Labile factor)	Cofactor for activation of prothrombin to thrombin
Factor VII (proconvertin)	With tissue factor, initiates extrinsic pathway
Factor VIII (antihemophilic factor)	Cofactor for intrinsic activation of factor X

Factor IX (Christmas factor)	Activated form is enzyme for intrinsic activation of factor X
Factor X (Stuart-prower factor)	Activated form is enzyme for final common pathway activation of prothrombin
Factor XI (Plasma thromboplastin antecedent)	Activated form is intrinsic activator of factor IX
Factor XII (Hageman factor)	Factor starts intrinsic pathway

Mechanism of blood coagulation

Blood coagulation is a complex procedure and is important part of hemostasis (the cessation of blood loss from a damaged vessel). Blood clotting results in the formation of an insoluble thread-like mesh of *fibrin* which traps blood cells and is much stronger than the rapidly formed platelet plug. In the final stages of this process *prothrombin activator* acts on the plasma protein *prothrombin* converting it to *thrombin*. *Thrombin* then acts on another plasma protein *fibrinogen* and converts it to *fibrin*.¹⁵

Blood coagulation cascade involves two equal important pathways, *intrinsic pathway* (contact activation) and *extrinsic pathway* (tissue factor). These pathways involve a series of reactions and a large number of endogenous coagulant molecules.¹⁴

These pathways are a series of reactions, in which a *zymogen* (inactive enzyme precursor) of a *serin protease* and its *glycoprotein* co-factor are activated to become active components that catalyze the next reaction in the cascade, resulting in the cross-linked fibrin.^{1,17}

A cut or break caused on the skin surface leads to disruption of normal integrity of the skin, in turn causing impairment in the anatomic and cellular functionality. They are mainly classified based on their symptoms, Aetiology, location, depth and clinical appearance of the wound. It involves complex biological processes such as inflammation, matrix



synthesis and deposition, coagulation, angiogenesis, fibroplasia, epithelialization, contraction, and Remodelling working in tandem to bring about wound healing. Delayed wound healing has important clinical implications in patients with diabetes, Anaemia, obesity, and other co morbidities. It can be caused due to various factors such as malnourishment, ischemia, immunosuppressive drugs, reactive oxygen species, and so on. Wounds that fail to heal under normal circumstances reach a stage of pathological inflammation owing to uncoordinated healing. Such chronic wounds have affected several million people across the globe, with over 85% occurring in patients over the age of 65. Agents with enhancing wound healing potential are intended to cause speedy recovery, thereby preventing amputation and other complications. Attempts to identify such chemical and herbal agents in the past have led to identification of several herbal remedies. Indian medicinal system relies on traditional medicinal formulations for the treatment of several diseases.

Wound healing is an important but complicated process in human or animal, containing a multifaceted process governed by sequential yet overlapping phases, including hemostasis/ inflammation phase, proliferation phase, and remodelling phase. After an injury to skin, the exposed sub-endothelium, collagen and tissue factor will activate platelet aggregation, which results in degranulation and releasing chemotactic factors (chemokines) and growth factors (GFs) to form the clot, and all above-mentioned procedures will achieve successful hemostasis. Neutrophils, the first cells to appear at the injury site, cleanse debris and bacteria to provide a good environment for wound healing. In the following, macrophages accumulate and facilitate phagocytosis of bacteria and damage tissue.

The hemostasis and inflammatory phase often takes 72 h to finish. The following proliferative phase is characterized with an accumulation of lots of cells and profuse connective tissue. The wound encompasses fibroblasts, keratinocytes, and endothelial cells. Extracellular matrix (ECM), including proteoglycans, hyaluronic acid, collagen, and elastin forms a granulation tissue to replace the original formation of clot. Many kinds of cytokines and GFs participate this phase, such as transforming growth factor- β family (TGF- β , including TGF- β 1, TGF- β 2, and TGF- β 3),

interleukin (IL) family and angiogenesis factors (i.e., vascular epidermal growth factor). This phase continues days and weeks. The last step of wound healing is a remodelling phase, which needs a precise balance between apoptosis of existing cells and production of new cells. Gradual degradation of profuse ECM and the immature type III collagen and formation of mature type I collagen are critical in this phase, which continues a few months and years. Any aberration in this phase may lead to excessive wound healing or chronic wound. Since a better understanding of the mechanism of wound Healing can be presumed from the increased number of in vitro or in vivo experiments and a better treatment algorithm to maintain a regulated and orchestrated inflammatory response will be developed, the following is an update of wound healing.

In pursuance of the above objectives, in the present investigations, *Eupatorium adenophorum* Spreng was selected on the basis of their traditional use in blood coagulation in different kinds of hemorrhage condition.

Eupatorium Linn (Fam Asteraceae) is a large genus of herbs, shrubs or undershrubs, distributed chiefly in tropical America, a few species occurring in Europe, Africa and Asia and India.

The various species of *Eupatorium* growing in India are *E. cannabinum* Linn –var. Heamp Agrimony, *E. triplinerve* Vahl (syn: *E. ayapana* Vent), *E. odoratum* Linn. (Syn: Chromolaena), *E. capillifolium* Linn. (Syn: *E. foeniculaceum* Willd), *E. birmanicum* DC.

E. adenophorum Spreng is pantropic weed with erect, branched herb with woody base, is commonly known as Crofton weed, a variety of this plant, known as *Eupatorium adenophorum* Spreng - var. Banmara (Syn: *Eupatorium gl andulosum* Kunth, *Ageratina adenophora* (Spreng.) Kings-Robin). It is panatropic weed native in Mexico found in Sikkim, Meghalaya, Tirpura, West Bengal, Uttar Pradesh, Uttarakhand and Tamilnadu.

The various parts of this plant are used in Ayurveda and other folk medicines for the treatment of cut and wounds. Leaves are used as an application to unhealthy ulcers. A decoction of the plant and the juice of the leaves are traditionally used as popular haemostatic remedy for various kinds of hemorrhage. Traditionally the leaves paste mix with mustard oil is useful for ulcer.



Every part of the plant either alone or in combination has also been recommended for snake bite

2. Objectives

To investigate the potential therapeutic effects of *Eupatorium adenophorum* Spreng leaf extract on wound healing processes in adult Wistar rats, with the goal of identifying its efficacy and mechanisms of action in promoting tissue repair and regeneration

3. Methods

Plant material preparation

Leaf samples of *E. adenophorum* Spreng were procured from the forest of Nagdhar Pokhari Chamoli (Uttarakhand) and identified. Its authentication was performed by Botanical Survey of India Dehradun, and its voucher specimen (Acc No.1127802) was deposited at the herbarium.

The dried alcoholic and aqueous extracts of the leaves of *E. adenophorum* Spreng were prepared and dissolved in normal saline with the help of tween-80.



Fig 1: *Eupatorium adenophorum* Spreng

Animals

Healthy mature wistar rats procured from the central animal house were used to study the various parameters of wound repair. After availing the animal ethical committee clearance (CPSCEA No.196)

Mature Wistar rats of either sex weighing 150-250 g were procured from Laboratory Animal Resource, Division of Animal Genetics, IVRI, Izatnagar, (Reg. No. CPC – 196) and acclimatized to laboratory condition at Animal House in College of Pharmacy,

IFTM, Moradabad at room temperature $24\pm 2^{\circ}\text{C}$ with a 12h/12h/light/dark cycle and 70% RH).

All rats were treated in accordance with the guideline for the Care and Use of Laboratory Animals (NIH Publication No.86-23, revised 1985) with the permission of institute ethical committee. The animals were kept in polypropylene cages and maintained on balanced ration provided by Feed Technology Unit, Division of Animal Nutrition, IVRI, of following composition

Table 2- Animal Diet Formula

Ingredients	%
Wheat (crude)	60
Maize (crushed)	30
Wheat bran	07
Mineral mixture	02
Common salt	01

Milk 10 ml/rat/day or 5% skimmed milk powder was added at mixture. Twenty g/rat/day feed was given. The animals had free access to clean drinking water.

Tail-bleeding Method:

24 animals were divided into 4 groups and each group comprised of six rats.

Group I- Normal saline was given (served as Normal Control).

Group II- Standard drug Rutin, 0.375mg /kg body weight orally, 30 minute before induction bleeding.

Group III- Received *E. adenophorum* Spreng aqueous Ext. 0.375mg /kg body weight, orally 30 minutes before induction bleeding,

Group IV- Received *E. adenophorum* Spreng alcoholic Ext. 0.375mg /kg body weight, orally 30 min before induction bleeding.

Hemorrhagic activity was measured by "Tail bleeding model" as described by Chatopadhyay et al.²⁸ Tail injury was done by surgical blade and total bleeding time and total blood loss were estimated. (The total bleeding time is defined as the duration of bleeding episode after induction of tail injury). Blood loss was



determined by measurement of the accumulated amount of hemoglobin in saline from the time of injection of the drug. The hemoglobin measurement was performed by addition of hemoglobin reagent (1 g Sodium bicarbonate and 0.200 g Potassium ferricyanide in 1L distilled water) and resulting Cyanomethaemoglobin was measured spectrophotometrically at 540 nm²⁹.

Clotting Time:

Clotting time was measured as described by Salawa et al.³⁰, A cut was made on the distal part of each rat tail using sterile scissors. The blood was placed on a grease-free glass slide. A stop clock was started immediately. A needle was passed through the blood on the glass slide every 15 sec. until a thread – like structure was seen. The time taken to form the thread – like structure were taken as a clotting time.

Statistical analysis:

All values were expressed as Mean±S.D. differences in mean values were compared using Graph Pad in State by Mann-Whitney Test and Kruskal-Wallis Test (Nonparametric ANOVA).

4. Results

Data of hemoglobin content and total bleeding measured after 30 min given in table no. C-2 & table no. C-6. Data after 1hr of dosing are given in table no. C-3 & table no. C-7. Data for the clotting time measured after 30 min and after 1hr given in table C-4 & table C-5.

Table no.C-2: Total bleeding time after 30 Min.

S.no	Control	Rutin	E.a.Aq. Ext.	E.a.Alc. Ext.
1	110	81	99	89
2	107	84	101	7083
3	112	79	94	8186
4	109	73	89	7291
5	119	76	93	7980
6	121	77	91	83
Mean	113.0	78.6667	94.5	85.33333

SD	5.6921	3.829708	4.636809	4.13182
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After 30 min. of dosing aqueous extract of *E. adenophorum* Spreng decreases the bleeding time as compared to control group animal ($p > 0.05$). Alcoholic extract was found much more potent and decrease the clotting time as compared to aqueous extract ($p > 0.05$).

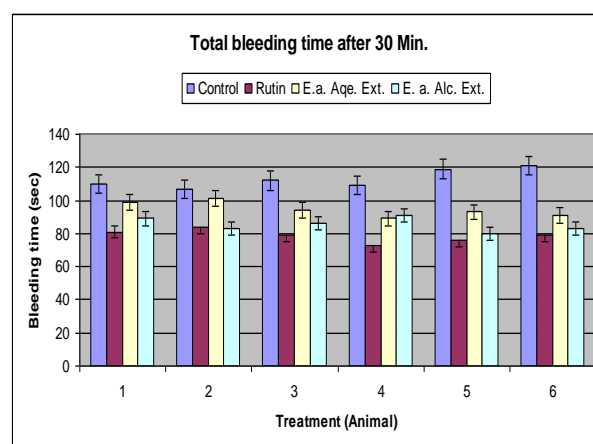


Image 2- Total bleeding time after 30 min

Table no C-3: Total bleeding time after 1 Hr

S.no.	Control	Rutin	E.a.Aq. Ext	E.a.Alc. Ext
1	118	79	90	74
2	109	81	83	70
3	111	81	87	81
4	113	76	84	72
5	108	79	89	79
6	121	73	91	83
Mean	113.3333	78.16667	87.333333	76.5
SD	5.163978	3.125167	3.265986	5.244044

After 1 Hr. of dosing aqueous extract of *E. adenophorum* Spreng decreases the bleeding time more



effectively in comparison to the control group animal. Alcoholic extract of *E. adenophorum* Spreng was as effective as standard drug Rutin after 1 hr. aqueous extract showed significantly reduction in the total bleeding time but less potent as comparison to standard and alcoholic extract.

Image 3- Total bleeding time after 60 min

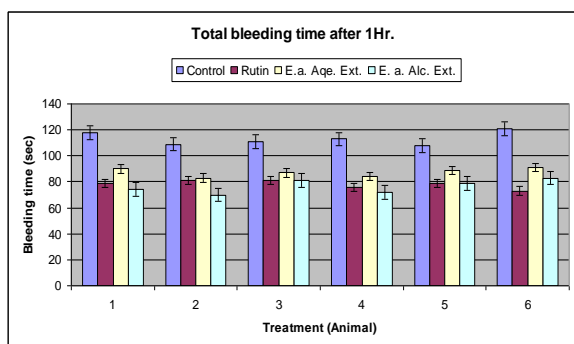


Table no: C-4 Clotting time after 30 Min.

S.no	Control	Rutin	E.a.Aq,Ext	E.a.Alc,Ext
1	75	51	60	55
2	70	59	55	50
3	65	57	60	60
4	65	52	5	55
5	70	53	65	55
6	75	50	65	65
Mean	70	53.667	60.0	56.667
SD	4.472136	3.559026	4.472136	5.163978

Bleeding time and clotting time are parameter that unidirectional parameters, smaller the clotting time smaller the bleeding time. In clotting time results, after 30 Min. of dosing alcoholic extract of *E. adenophorum* Spreng decreases the clotting time significantly ($p>0.05$) as compared to the control group data. Alcoholic extract was again more potent and effective decrease the clotting time but not as effective as standard drug.

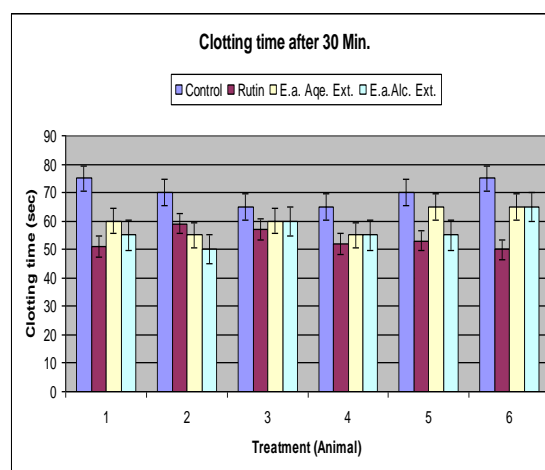


Image 4 - Clotting time after 30 min

Table no: C- 5 clotting time after 1 Hr.

S.no	Control	Rutin	E.a.Aq,Ext	E.a.Alc,Ext
1	75	50	55	50
2	65	49	50	50
3	70	57	60	45
4	70	52	60	55
5	75	53	50	50
6	80	49	65	55
Mean	72.5	51.66667	56.66667	50.83333
SD	5.244044	3.076795	6.055301	3.763863

After 1Hr. of dosing alcoholic extract of *E. adenophorum* Spreng decreases the clotting time significantly ($p>0.01$) as compared control group and equipotent to that of standard drug treated animals. Both of the extract showed significantly decreased the clotting time as compared to previous data (after 30 min. of dosing).

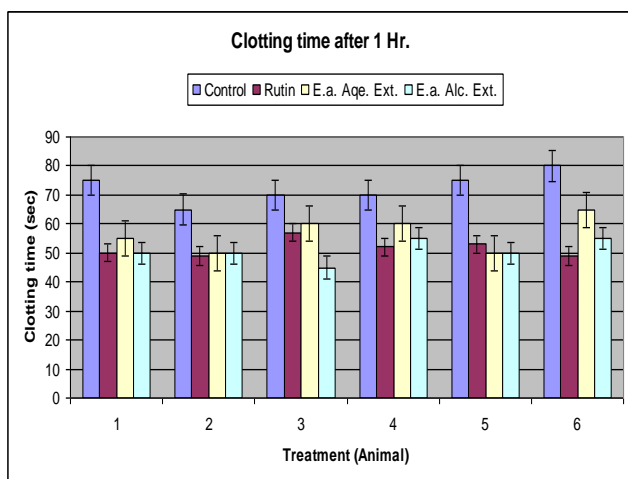


Image 5- Clotting time after 60 min

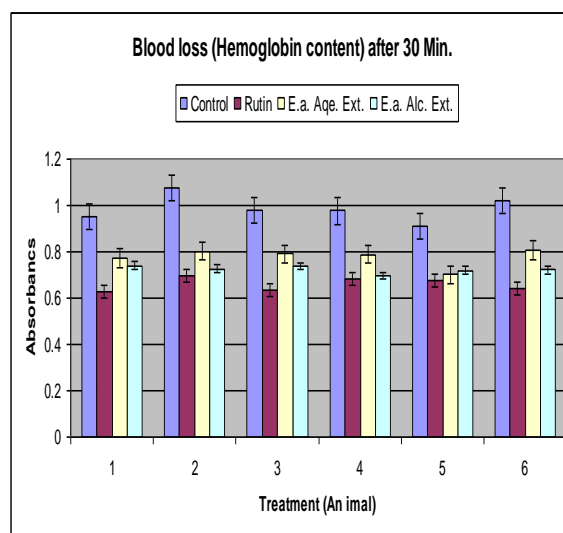


Image 6- Blood loss after 30 min

Table no: C-6 Blood loss (Hemoglobin content) after 30 Min.

S.no.	Control	Rutin	E.a.Aq.Ext	E.a.Alc.Ext
1	0.951	0.625	0.773	0.741
2	1.073	0.694	0.803	0.726
3	0.981	0.635	0.79	0.739
4	0.976	0.681	0.789	0.697
5	0.913	0.673	0.701	0.719
6	1.021	0.641	0.807	0.721
Mean	0.985833	0.65816	0.777167	0.72833
SD	0.0556288	0.02813	0.0391939	0.0160052

Table no: C-7 Blood loss (Hemoglobin content) after 1 Hr.

S.no.	Control	Rutin	E.a.Aq.Ext	E.a.Alc.Ext
1	0.948	0.697	0.623	0.591
2	0.957	0.68	0.679	0.603
3	0.963	0.701	0.701	0.589
4	1.061	0.711	0.709	0.595
5	0.941	0.679	0.663	0.583
6	0.9855	0.69	0.697	0.581
Mean	0.9855	0.693	0.67867	0.59033
SD	0.05236	0.0124	0.03193	0.008066

Plant extract decreases the clotting time, blood loss will decrease also. After 30 minutes of dosing aqueous and alcoholic extract of *E. adenophorum* Spreng total blood loss data were significantly different from control group animal ($p > 0.01$) but less potent than that of standard drug. Alcoholic extract treatments give less absorbance as compared to the aqueous extract. So, there was a significant decrement in blood loss in case of alcoholic extract and aqueous extract treatment comparison to control group.

Similarly after 1hr. of dosing alcoholic and aqueous extract of *E. adenophorum* Spreng gives much less absorbance as compared to control group. Alcoholic extract was found equipotent to standard drug, Rutin. Both extract significantly decreases in the absorbance as compared to previous data (after 30 min. of dosing).

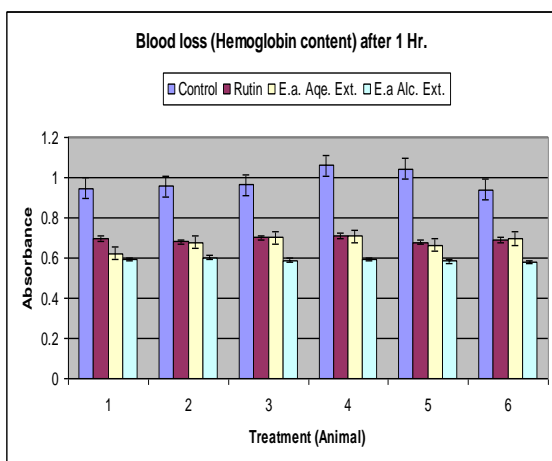


Image 7- Blood loss after 60 min

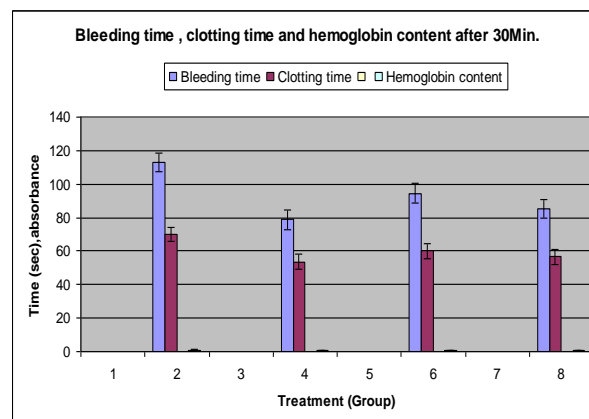


Image 8- Bleeding time, clotting time and haemoglobin content after 30 min

Table no. C-8 Effect of aqueous and alcoholic extracts of *E. adenophorum* Spreng on Bleeding time, clotting time and blood loss (hemoglobin content) after 30 Min.

Treatment	Bleeding time	Clotting time	Hemoglobin content
Control group	113 ± 5.6921	70 ± 4.472136	0.9858333 ± 0.055628829
Rutin treated group*	78.6666 ± 3.829708	53.667 ± 3.559026	0.6581667 ± 0.02813124
Aqueous ext. treated group*#	94.5 ± 4.636809	60.0 ± 4.472136	0.7771667 ± 0.039193962
Alcoholic ext. treated group*\$	85.333 ± 4.13182	56.667 ± 5.163978	0.72833 ± 0.016005207

All data presented as Mean±S.D, n= 6 * significantly different from control group, # significantly different from Rutin treated group, \$ significantly different from aqueous treated group.

Table no. C-9 Effect of aqueous and alcoholic extracts of *E. adenophorum* Spreng on Bleeding time, clotting time and blood loss (hemoglobin content) after 1 Hr.

Treatments	Bleeding time	Clotting time	Hemoglobin content
Control group	113.3333 ± 5.163978	72.5 ± 5.244044	0.9855 ± 0.052366974
Rutin treated group*	78.166667 ± 3.125167	51.66667 ± 3.076795	0.693 ± 0.012473973
Aqueous ext. treated group*#	78.166667 ± 3.125167	56.66667 ± 6.055301	0.67866667 ± 0.031935351
Alcoholic ext. treated group*\$	87.333333 ± 3.265986	50.833333 ± 3.763863	0.59033333 ± 0.008066391

All data presented as Mean±S.D, n= 6 * significantly different from control group, # significantly different from Rutin treated group, \$ significantly different from aqueous treated group.

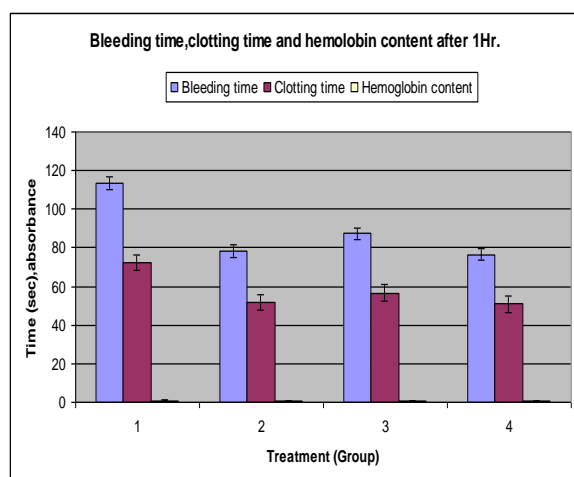


Image 9- Bleeding time, clotting time and haemoglobin content after 60 min

5. Discussion

The dried leaf extracts of *E. adenophorum* Spreng (alcoholic and aqueous) were tested for blood coagulation activity on Wister rats. Alcoholic and aqueous leaf extracts of *E. adenophorum* Spreng shows positive chemical reaction for alkaloids, tannins and flavonoids.

E. adenophorum Spreng, flavanoids are reported main constituents could be responsible for the important pharmacological activities. Orally administered of these flavanoids have also been observed to inhibit vascular permeability and prevent pulmonary haemorrhage.

Pharmacological evaluation of the *E. adenophorum* Spreng alcoholic and aqueous extract shows that the rats which are treated with alcoholic extract there are significant decreased in bleeding time and clotting time as compared to aqueous extract. Total blood loss was also decreased significantly by the alcoholic extract.

Aqueous extract similarly potent but not as effective as of alcoholic extract, and significantly reduces the bleeding and clotting time after 30 min of dosing.

All data of bleeding time, clotting time and haemoglobin loss were significantly reduced after 1hr of doing as in comparison of 30 minutes dosing data of both aqueous and alcoholic extract. After 1 hr of dosing alcoholic extract data becomes equipotent to standard drug animal group data. Which shows that onset of

action may be faster for plant but it takes time to achieve maximum therapeutic effectiveness.

Higher potency of alcoholic extract may be due to higher alcohol solubility of plant constituents responsible for the coagulant activity.

Conclusion

Blood coagulation is a complex procedure and is an important part of hemostasis (the cessation of blood loss from a damaged vessel). Blood clotting results in the formation of an insoluble thread-like mesh of fibrin which traps blood cells and is much stronger than the rapidly formed platelet plug. Wound healing is an important but complicated process in human or animal, containing a multifaceted process governed by sequential yet overlapping phases, including hemostasis/ inflammation phase, proliferation phase, and remodelling phase. A cut or break caused on the skin surface leads to disruption of normal integrity of the skin, in turn causing impairment in the anatomic and cellular functionality. The present study demonstrated that the extract of *E. adenophorum* Spreng exhibits good coagulation activity. Pharmacological studies of the *E. Adenophorum* Spreng extract show that the alcoholic extract is more potent than aqueous extracts.

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