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In-vitro Experimental Study on the Procoagulant Activity of *Rakta Sthapana Churna*

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ABSTRACT

Haemorrhagic disorders are characterized by an increased tendency to bleed due to defects in the clotting process, and can manifest as spontaneous bleeding or prolonged bleeding after injury or surgery. *Rakta Sthapana Churna* (RSC) is an Ayurvedic formulation designed for external application during *Rakta Mokshana* to address excessive bleeding tendencies. **Aims & Objectives**: The in-vitro study aims to evaluate its procoagulant activity using blood samples from healthy human volunteers. **Materials & Methods**: Coagulation time is determined by a modified Lee and White's Method. The clotting time of 20 blood samples treated with aqueous extracts of *RSC* at 5% & 10% concentration(AG1 & AQ2), along with normal saline as the control is recorded. The in-vitro pro-coagulant activity is evaluated by comparing the clotting time of test and control groups. **Results**: On comparing the average clotting times with the control, it was found that AQ1 had 13.71% higher CT, while AQ2 had 43.1% higher CT (highly significant). **Discussion**: RSC in 5% concentration showed reduction in clotting time in few samples (sample 9,11,12,14,20), whereas in 10% concentration it did not show any reduction. **Conclusion**: The study showed dose-dependent anticoagulant effects of RSC at higher concentrations, as well as pro-coagulant activity at lower concentrations. The unique clotting patterns observed highlight the complex nature of clot initiation and clot completion, which need further research & validation.

Key Words *Rakta sthapana churna* (RSC), procoagulant activity, clotting time, dose-dependent, clot initiation, clot completion

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INTRODUCTION

Ayurveda system of medicine has elaborate discussions on *Rakta dhatu* (blood), its functions in normalcy, signs and symptoms on vitiation, its disorders, their treatment strategies, and efficient therapeutic formulations. In this context, *Rakta mokshana* (controlled therapeutic blood-letting) is stated to be a prime modality of treatment for *raktaja vyadhis*[1], and to ensure that *raktamokshana* itself is not hindered by preexisting bleeding tendencies due to *rakta dushti*, a set of herbo-mineral drugs for *Rakta sthapana* activity has been mentioned by Acharya Sushrutha[2]. This formulation is prepared in the form of *churna* and intended for external application on the site of *raktamokshana*. Termed

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as Rakta sthapana *churna*, the formulation is tested for its probable coagulation modifying activity in the present in-vitro evaluation.

The ingredients of *Rakta Sthapana Churna* includes *churnas* of *Lodhra, Madhuka, Priyangu, Raktachandana, Shuddha Gairika, Sarjarasa, Rasanjana, Shalmali, Shuddha Shankha, Shuddha Shukti, Masha, Yava, Godhuma* taken in equal quantity and made into a homogenous compound churna preparation which is to be applied on the site of *rakta srava.*

Various studies that have been carried out previously on procoagulant activities of Ayurvedic and herbal drugs were referred[3-5]. In this study the coagulation time is determined by a modified Lee and White's Method. The clotting time of blood samples treated with aqueous extracts of *Rakta Sthapana churna* at two different concentrations along with normal saline as the control is recorded. The in-vitro pro-coagulant activity is evaluated by comparing the clotting time of test and control groups.

Lee and white's method[6]

Principle: Whole blood, when removed from the vascular system and exposed to a foreign surface, will form a solid clot. Within limits the time required for the formation of the solid clot is a measure of the coagulation system.

OBJECTIVES

- To evaluate the coagulant activity of Rakta Sthapana Churna (RSC)
- To find the clotting time.
- Comparison of clotting time of test drug with control group

Criteria of the Study

- **Number of samples:** 20 samples
- **Inclusion criteria:** Healthy human volunteers of age 20-30 yrs. are selected randomly
- **Exclusion criteria:** Oral contraceptive, anti-coagulant therapy, bleeding disorders

Grouping: The grouping is done as mentioned in Table 1

Table 1 Grouping for procoagulant study of RSC

Group	Drug	No. of samples
Group 1	Control group with Normal saline	20
Group 2	5% conc. of Aqueous extract of RSC **(AQ1)**	20
Group 3	10% conc. of Aqueous extract of RSC **(AQ2)**	20

MATERIALS AND METHODS

Equipment: 20 healthy volunteers in the age group of 20-30 years of either sex, Aqueous extracts of RSC and its testing solution at 5% and 10% concentration, Normal saline**,** Test tubes, Eppendorf tube, test tube stands**,** Micropipette, Microtips**,** Hot water bath**,** Cyclo mixer, Orbital shaker, Hot air oven**,** Syringes, Gloves, Cotton**,** Stop-watch

Preparation of drug extract: Preparation of drug extract: Rakta Sthapana churna is taken and aqueous solution is prepared with 20gm of churna dissolved in 200ml of distilled water. The solution is kept mixed in orbital shakers for 10-12 hours (Figure 1). Then the solution is filtered (Figure 2 & 3) and evaporated to dryness in a hot air oven at 57ºC (Figure 4), further concentrated

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in heating mantles such that the solvent is completely removed (Figure 5). The aqueous extract thus obtained is weighed and stored in Eppendorf tubes for further use (Figure 6).

Figure 3 Aqueous solutions of RSC

Figure 2 Filtering the drug solutions

Figure 4 Drying the solutions in hot air oven at 57ºC

Figure 5 Concentrating the extracts in heating mantle

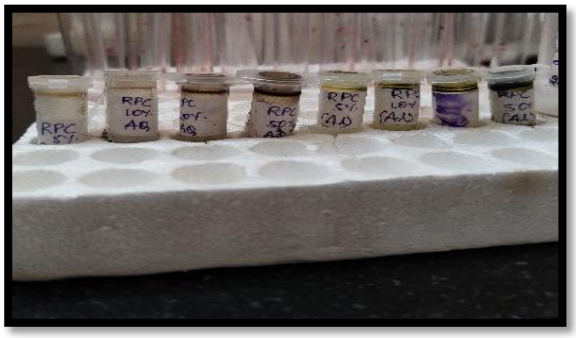

Figure 6 Prepared extracts of RSC stored in eppendorf tubes

Selection of volunteers: Healthy human volunteers of age between 20-30 yrs. of age of either sex are considered for this study. Volunteers should not be suffering from any bleeding disorders or taking oral contraceptives. Females who are menstruating are also excluded.

Specimen: 3 empty test tubes were added with 250µl of test drugs(AQ1, AQ2) and control(NS). Whole blood (2.5ml) was drawn from healthy volunteers, 0.5ml blood was transferred to each of the test tube.

Preparation of solutions

- Control: 250µl of Normal saline solution is used as the control.
- Test drug: The aqueous extract of RSC is dissolved in distilled water in 5% and 10% concentrations. These serve as test drug solutions and 250 µl is used per test tube.

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PROCEDURE: To begin with, 3 empty sterile test tubes were taken and filled with 250 µl of AQ1, AQ2, and Control. About 2-3ml of venous blood was drawn from the healthy volunteers. Once the blood came to the barrel of the syringe the stop watch was switched on. The blood drawn was quickly filled into the 3 test tubes separately with 500 µl of blood pipetted into each tube. All the test tubes were incubated in a water bath at 37ºC (Figure 7). After 5 mins all the tubes were tested carefully for the clots by inclining them slowly up to 45 degrees (Figure 8). Once the clot was seen in any test tube (Figure 9), the time from the stop watch was recorded as clotting time for that tube. The clotting time of all 3 test tubes (AQ1, AQ2, Control) were recorded. The experiment was repeated 20 times with blood samples of 20 volunteers. This experiment provides the clotting time of blood exposed to the test drug and control, which will be compared to get the measure of pro-coagulant activity.

Figure 7 Incubating blood samples in water bath at 37ºC

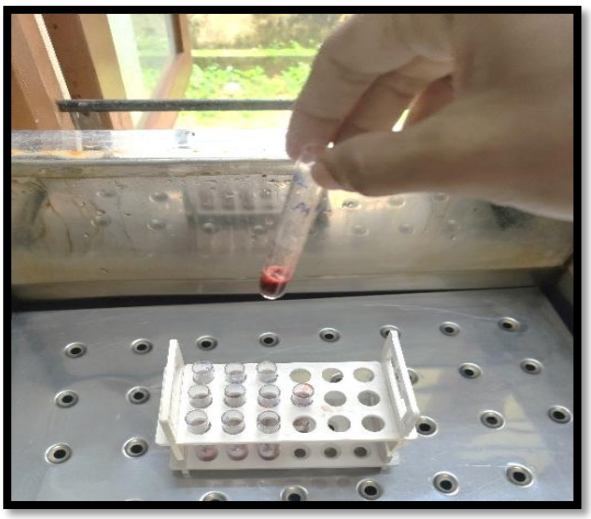

Figure 8 Testing the tubes for clotting

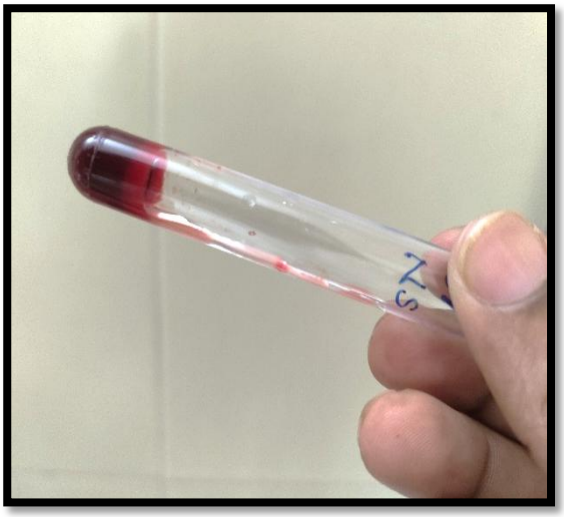

Figure 9 Tube with NS showing complete clotting

RESULTS

The experimental results of coagulation time in 20 samples treated with normal saline, aqueous extracts of *Rakta sthapana churna* in 5% and 10% concentrations are tabulated in Table 2. The descriptive statistics of the study is tabulated in Table 3. Percentage change in coagulation time is tabulated in Table 4.

Table 2 Results of Coagulation time with RSC

SL No.	Clotting time with N.S (in mins)	Clotting time with AQ1(in mins)	Clotting time with AQ2 (in mins)
1.	10.33	13	15.5

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2.	9	12	14
3.	9	10	13
4.	8.75	10.50	12.75
5.	8.50	11	14
6.	12	13	15.75
7.	11.50	12.75	14.50
8.	13	14	16.50
9.	12.50	12	15
10.	10.50	13.50	15.50
11.	12.50	12	14.50
12.	11	10.67	15
13.	9.50	13	14.75
14.	9.33	8.75	12.25
15.	7	8.25	10
16.	7.50	9.67	13
17.	7	9	15
18.	6	9	12
19.	9.25	9.50	12.50
20.	8.50	7.50	10

As per the results tabulated in Table 2, in comparison with Normal saline, aqueous extract of *Rakta Sthapana Churna* taken in 5% concentration (AQ1) showed reduction in clotting time in few samples (sample 9,11,12,14,20), whereas the aqueous extract taken in 10% concentration did not show any reduction in the clotting time.

Table 3 Descriptive statistics of RSC experimental study

Sample	N	Minimum CT	Maximum CT	Mean CT (with SE)	Standard Deviation	Variance
A (NS)	20	6	12.50	9.63 ± 0.45	1.9998	3.999
B (AQ1)	20	7.50	14	10.95 ± 0.43	1.9402	3.764
C (AQ2)	20	10	16.50	13.78 ± 0.40	1.8026	3.249

Table 4 % change in CT in RSC experimental study

Sample	% change in CT compared to NS	% change in CT compared to AQ1	% change in CT compared to AQ2
A (NS)	-	12.1%↓	30.11%↓
B (AQ1)	13.71%↑	-	20.5%↓
C (AQ2)	43.1%↑	25.84%↑	-

A vs B **ns** **P > 0.05** P value > 0.05 can be considered as not significant
A vs C ******* **P < 0.001** P value < 0.001 can be considered highly significant
B vs C ******* **P < 0.001**
(P value: Mean ± SEM of clotting time)

The mean clotting time for normal saline group was found to be 9.63 ± 0.45, while that of 5% RSC and 10% RSC were found to be 10.95 ± 0.43 and 13.78 ± 0.40 respectively, as shown in Table 3.

The percentage change in clotting time of 5% RSC when compared with normal saline group was found to be 13.71% higher, while that of 10% RSC was found to be 43.1% higher, as shown in Table 4.

The graphical representation of clotting time (in minutes) of the 20 samples treated with Normal saline, 5% solution of RSC and 10% solution of RSC is shown in Graph 1.

DISCUSSION

The term *Rakta Sthapana* can be translated to mean "arresting or stopping the flow of blood.". In this context, RSC is envisioned as a formulation designed to facilitate the cessation or reduction of blood flow at a specific site. It is geared towards controlling bleeding by potentially enhancing the coagulation process and minimizing blood loss. The current study explores the potential of RSC extracts in the context of clotting and haemostasis. By evaluating its effects on blood clot formation and stability, the study aims to shed light on the

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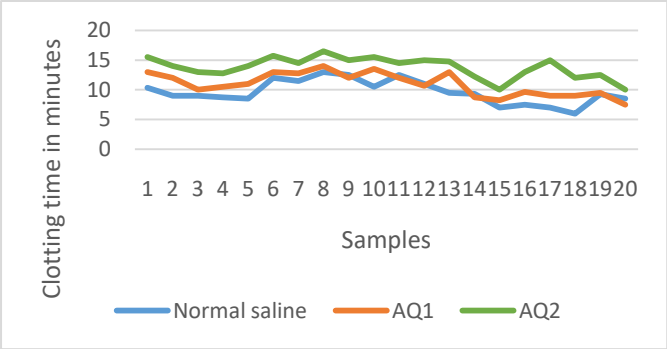
formulation's potential to facilitate the cessation of bleeding.

The modified Lee and White's Method is employed, determining the clotting time of blood samples treated with the test drug alongside a control. Clotting time serves as a measure of the pro-coagulation activity.

Table 5 Consolidated comparison of clotting time in RSC study

Sample	Mean CT (with SE)	Standard Deviation	% change in CT compared to NS	% change in CT compared to AQ1	% change in CT compared to AQ2
NS	9.63 ± 0.45	1.9998	-	12.1%↓	30.11%↓
AQ1	10.95 ± 0.43	1.9402	13.71%↑	-	20.5%↓
AQ2	13.78 ± 0.40	1.8026	43.1%↑***	25.84%↑***	-



Graph 1 In-vitro coagulation activity of *Rakta Sthapana Churna*

Table 5 shows the consolidated comparison of clotting time in the study. The results showed that the average clotting time of Aqueous extract of RSC at 5 % concentration i.e., AQ1 (10.95 ± 0.43) was slightly higher than that of the NS control group (9.63 ± 0.45), but the situation was statistically insignificant (P > 0.05). The average clotting time of Aqueous extract of RSC at 10 % concentration i.e., AQ2 (13.78 ± 0.40) was higher than that of the NS control group (9.63 ± 0.45), and the situation was statistically highly significant (P < 0.001).

On comparing the average clotting times with the control, it was found that AQ1 had 13.71% higher CT, while AQ2 had 43.1% higher CT (highly significant). On comparing the average clotting times with AQ1, it was found that control had 12.1% lesser CT, while AQ2 had 25.84 % higher CT, which is highly significant. On comparing the average clotting times with AQ2, it was found that control had 30.11% lesser CT, while AQ1 had 20.5 % lesser CT.

The inter-group comparisons reveal interesting insights. The 5% aqueous extract of RSC (AQ1) had similar but slightly elevated clotting time compared to the control drug NS (Figure 10), which was statistically insignificant. This means that RSC in 5% conc. had negligible coagulation enhancing activity but suggests possible anticoagulant action. The 10% conc. of RSC (AQ2) had very high clotting time in comparison to the control (NS) which was statistically highly significant. It implies that AQ2 delays the coagulation considerably. This also indicates that RSC may in fact exhibit some form of dose-dependent anticoagulant activity which becomes evident in higher concentrations.

At this juncture, it is important to highlight that during the experiment, the control group exhibited a gradual and complete clot formation of the blood within the test tube (Figure 9). However, samples in test groups AQ1 and AQ2

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demonstrated a distinct pattern: a small portion of the blood coagulated at the base of the tube, while the remaining portion remained in a liquid state for extended periods (Figure 11 & Figure 12).

Figure 10 5% Aqueous solution RSC showing complete clot

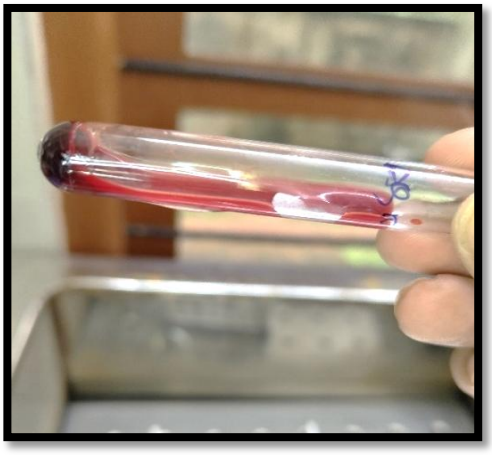

Figure 11 10 % Aqueous solution of RSC showing incomplete clotting

Figure 12 10% aqueous solution of RSC showing clot initiation without clot completion

This observation indicated a significant disparity between the time of clot initiation and the time of clot completion within the test groups. In some instances, the exact moment of clot initiation could not be discerned, and similarly, the precise time of clot completion was unattainable in few samples. Consequently, the recorded clotting time values for the test group predominantly reflect the time of clot initiation for all practical purposes. It is therefore important to note that while these values are often labelled here as clotting time, such a generalization is not entirely accurate because clot initiation and clot completion are governed by different physiological processes.

Clot initiation/appearance time refers to the time it takes for a visible clot to form after the initiation of the coagulation process. It gives an indication of the initial phase of coagulation, where factors like platelet activation and the activation of the intrinsic and extrinsic coagulation pathways contribute to the formation of a loose clot. Clot completion time refers to the time it takes for a clot to reach its maximum stability and firmness. It is the point at which the clot has undergone sufficient reinforcement through fibrin cross-linking and platelet aggregation to become fully stabilized. It indicates the later stages of coagulation when the clot is more resistant to disruption.

On finer scrutiny of test results another point of discussion is that the extract of RSC in 5% concentration showed reduced clotting time than the control group in few samples (sample

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9,11,12,14,20). Table 6 gives enlists this sample subset with a comparison of clotting time in Normal saline and 5% RSC solution. Considering these 5 samples, the mean clotting time for NS group was (10.76 ± 0.81) whereas the mean clotting time for the drug AQ1 was (10.18 ± 0.8). Mean clotting time is slightly reduced. In comparison to NS, AQ1 showed 5.39% lesser clotting time in these 5 samples. Lesser clotting time of AQ1 than NS suggests a coagulation enhancing activity of the drug. Statistically this is insignificant, however, this shows that there could be some pro-coagulant activity by RSC.

Table 6 Sample subset comparison of clotting time in RSC study

Group	Sample 9	Sample 11	Sample 12	Sample 14	Sample 20	Mean CT	% change w.r.t NS
NS	12.50	12.50	11	9.33	8.50	10.76 ± 0.81	-
AQ1	12	12	10.67	8.75	7.50	10.18 ± 0.8	5.39%↓

CONCLUSION

The experimental study yielded insightful results regarding the effects of Aqueous extract of Rakta Sthapana Churna on clotting time. The study presents compelling evidence of dose-dependent anticoagulant effects of RSC at higher concentrations, as well as intriguing hints at pro-coagulant activity at lower concentrations. The unique clotting patterns observed in the test groups highlight the complex nature of clot initiation and clot completion.

It is also important to recognize that in vitro testing, while valuable, may not comprehensively reflect the full range of coagulation activity exhibited by a drug. Coagulation is a complex process influenced by numerous factors, including the intricate interplay of blood vessels, blood flow dynamics, and various physiological conditions. Therefore, relying solely on in vitro studies might not provide a complete understanding of a drug's effect on coagulation. In-depth investigations that account for these multifaceted variables are necessary to gain a more accurate assessment of the drug's impact on the coagulation cascade, mechanisms underlying these effects and to explore the potential therapeutic implications.

Conflicts of interest: There are no conflicts of interest.

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