Evaluation of Anti-Thrombotic Activity of *Astragalus Membranaceus* Var. *Mongholicus*) Root Extract: *In Vitro*

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Abstract

Thrombotic diseases, especially heart diseases and cerebrovascular thrombosis, are primary causes of death and their incidence has been increasing annually. Traditional Indian medicine and the use of plant drugs against various diseases are receiving considerable attention nowadays. *Astragalus membranaceus* var. is very much used as a therapeutic agent in traditional and folklore medicine. Thus, this present research work was conducted to appraise the antithrombotic activity of aqueous root extract of *A. membranaceus* (AMRE) using *in vitro* experiments such as: Clotting Time Assay, Thrombolytic Activity, Determination of *in-vitro* Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT), Calcium chloride induced clotting time assay by ELISA, and Anti-coagulant activity by plasma re-calcification method. The results were analysed by one-way Analysis of Variance (ANOVA). The results showed more significant anti-thrombotic activity by preventing the formation of blood clots and thromboembolic disorders. The results are tabulated and evaluated for their therapeutic efficacy in comparison to a standard drug (Aspirin). AMRE produced significant ($P<0.01$- $P<0.001$) and dose-dependent increases in all the above-mentioned methods, with maximum effect at the highest concentration of 100 mg/ml. This work ascertains that AMRE possesses potential in vitro antithrombotic activity and, hence, has proved to be therapeutically beneficial in patients with vascular diseases.

Keywords: Astragalus membranaceus, in vitro, antithrombotic activity, vascular diseases

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Introduction:

Cardiovascular diseases like ischemic heart disease, acute myocardial infarction, and high blood pressure account for 29% of the total global mortality rate. One of the significant factors contributing to the aetiology of cardiovascular diseases is the imbalance between fibrin formation and fibrinolysis. This pathophysiological condition results in intravascular thrombus formation in the blood vessels (known as thrombosis), which poses a clinical challenge in its treatment. Moreover, the higher levels of blood fibrinogen (hyperfibrinogenaemia), besides stimulating thrombotic events, enhance the risk of myocardial infarction through blood coagulation [1]. The coagulation system is distinguished into two pathways: extrinsic and intrinsic. Activation of the extrinsic pathway is generally considered to initiate both haemostasis and thrombosis. Haemostasis is initiated when blood is exposed to tissue factors located in the adventitia of blood vessels, and thrombosis is initiated when blood is exposed to tissue factors in the necrotic core of the ruptured atherosclerotic plaques, in the sub endothelium of injured vessels, and on the surface of activated leucocytes attracted to the damaged vessel. Anticoagulation is a very common form of medical intervention which is increasingly used for primary or secondary prevention of thromboembolic complications of vascular disease. Oral anticoagulation with

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vitamin K antagonists (VKAs), mainly warfarin, is almost the only tool for chronic anticoagulant treatment in clinical practice, although newer anticoagulants have already been approved or evaluated in clinical studies [2]. *Astragalus membranaceus* (of the family *Fabaceae*), or more commonly known as Astragalus, is a medicinal herb used in Traditional Chinese Medicine. According to Zhang et al., it "is the dry root of *Astragalus membranaceus* (Fisch.) Bge. *Astragalus membranaceus* var. Mongholicus (Bge.) Hsiao is a member of the Fabaceae family and grows mainly in northern China, Mongolia, and Siberia. It is also known as Huang-Qi (Chinese), membranous milk-vetch root (English), ogi (Japanese) and Hwanggi (Korean). These roots are also sometimes referred to as *Astragalus Radix*, a mostly synonymous term. It tends to have a slightly warm and sweet taste when administered as a beverage made from root powder. This herb has already been shown to have anti-inflammatory, anti-tumor, analgesic, anti-diabetic, anti-aging, antioxidant, and other beneficial properties. This in vitro study was carried out to demonstrate that this herb also has potential anti-thrombotic activity, which includes reducing the risk of complications from blood clots and enhancing the turbulence of the blood flow without obstructions, inhibiting the formation of new clots, and preventing the growth of existing clots [3].

Materials and Methods:

Plant Material:

The root extract powder of Astragalus membranaceus plant was procured from the certified supplier Navchetana Kendra Health Care Provider Limited (an ISO 9001: 2008 & WHO-GMP certified company), E-138/A, Shastri Nagar, New Delhi-110052.

Chemicals and equipment

Aspirin, EDTA, Prothrombin Time Reagent (PT), and Activated Partial Thromboplastin Time Reagent (APTT). ELISA reader, Micro Centrifuge, BOD Incubator.

In vitro Screening:

Clotting time assay

Clotting time assay was used to determine the anticoagulant effects of test extract powder on human plasma. The inhibitory activity of the evaluating agent was tested on thrombin, ADP, epinephrine, Trypsin, bromelain, and papain induced platelet aggregation. The evaluating agent (40 µl) is added to rat platelets (100 µl) and left at room temperature for 5 min. Thrombin/ADP/epinephrine (20µl) is added and the inhibition of blood clots is measured at 412nm using a plate reader for 20 min at 30 second intervals. The activity of the evaluating agent is expressed as a percentage of mean inhibition [4].

Clot lysis method/Thrombolytic assay

Healthy volunteer venous blood is taken, placed in a pre-weighed, sterile micro centrifuge tube (500 l/tube), and then incubated for 45 minutes at 37°C. After clot formation, completely remove the serum, and each tube containing a clot is again weighed to determine the clot weight. Each micro centrifuge tube containing clot is labelled and 100µl of streptokinase along with various dilutions in sterile distilled water (undiluted, 3:4, 1:2, and 1:3) is added to the tubes. Water is also added to one of the tubes containing clots, and this serves as a negative control. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, the fluid obtained is removed and the tubes are weighed again to observe the difference in weight after clot disruption. The difference obtained in weight taken before and after clot lysis was expressed as % of clot lysis [5].

Determination of in vitro prothrombin time and activated partial thromboplastin time

Trisodium citrate was added to each centrifuge tube, followed by 3 mL of blood sample from the rabbit (n = 5) and centrifugation at 3,000 rpm for 5 minutes. The plasma was separated using a micropipette into the Eppendorf tubes. 250 uL of the *M. longifolia* extract solution of different concentrations (2.5, 5 and 10%) was added into the Eppendorf tubes separately. To determine prothrombin time, samples were incubated at 37°C for 5 min. Afterward, 200 uL of prothrombin time reagent was added to 100 uL of test plasma and clotting time was measured as prothrombin time. Moreover, for appraisal of activated partial thromboplastin time, 100 uL of activated partial thromboplastin reagent was added to 100 uL of test plasma (platelet poor plasma + plant extract of various concentrations) and the mixture was incubated for 1 min, after which 100 uL of calcium chloride (25 mmol) was

added and the mixture was again incubated for 15 sec. Then clotting time was recorded as activated partial thromboplastin time [6].

Calcium chloride induced clotting time assay

This assay allows for the determination of a 50% clotting time and effect on fibrin formation. The assay is performed by adding a solution of the evaluating agent (40µl) to human plasma (100µl). The reaction was mixed and left to incubate for 5 min at room temperature. Clotting is induced by the addition of 20µl 0.16 M CaCl2, and the reaction is followed at 412 nm with a microtiter plate reader for 2 hrs. at 3 min intervals [7].

Anticoagulant activity by plasma recalcification method

Blood was collected from normal rabbits through the ear vein in EDTA (0.1 M) added tubes. The plasma was separated by centrifugation (1000 rpm, 5 min). To 100 l of pre-warmed plasma at 37 C, 200 microliters of M/100 CaCl2 were added. The time taken for the formation of a firm clot was noted immediately with the help of a stopwatch [8].

Statistical Analysis

The outcomes are expressed as the Mean ± SEM. Statistical evaluation was carried out by using one-way ANOVA followed by Tukey; compare all pairs of columns. P<0.05 was significant.

Results and Discussion

The vascular endothelium provides several important functions to maintain adequate blood supply to vital organs. These functions include prevention of coagulation, regulation of vascular tone, orchestration of the migration of blood cells by the expression of adhesion molecules, and regulation of Vasopermeability. Dysregulation of thrombotic and fibrinolytic potential of endothelial cells is actively involved in the development of thrombus[9]. The present study is aimed at screening the *in-vitro* anti-thrombotic activity by using the root extract powder of *Astragalus membranaceus* plant. The blood samples were collected into Eppendorf tubes from the male albino rats and centrifuged, then separated the serum.

The obtained plasma is subjected to various *in vitro anti-thrombotic* activity screening methods by adding the root extract powder of *Astragalus membranaceus* plant. This includes clotting time assay to evaluate the increase in clotting time of blood, thrombolytic assay or clot lysis method for ascertaining lysis of the formed blood clot, determination of *in vitro* prothrombin time and activated partial thromboplastin time to know the increase in clotting time by APTT & PT reagents, calcium chloride induced clotting time assay for an increase in clotting time by the addition of calcium chloride and anticoagulant activity by plasma recalcification method to observe an increase in plasma recalcification time upon administration of AMRE.

By screening the root extract powder of *A. membranaceus* through all the methods described above, the results are tabulated below

Table 1 includes data on *in vitro* clotting time, thrombolytic activity, prothrombin time, activated partial thromboplastin time, and plasma recalcification time.

Group	Clotting time assay (Time in seconds*)	Thrombolytic activity (% clot lysis*)	Prothrombin Time (PT) (clotting time in sec*)	Activated partial thromboplastin time (clotting time in sec.*)	plasma recalcification time (in sec*)
Normal	179.3 ± 1.76	0.00±0.00	119.7 ± 0.33	123.3 ± 3.33	48.33 ± 4.41
Test 1(60 mg/ml)	205.0 ± 2.88**, ###	10.76 ± 1.94***	156.7 ± 2.84ns, ###	240.0 ± 5.77***, ###	--
Test 2(80 mg/ml)	270.0 ± 5.7***, ###	14.83 ± 2.58**	208.3 ± 15.90**, ###	353.3 ± 17.64***, ns	--
Test 3(100 mg/ml)	362.0 ± 4.16***, ns	29.00 ± 2.30ns	353.3 ± 17.64***, ns	346.7 ± 12.02***	130.3 ± 0.88***, ns
Standard 100 mg/ml)	359 ± 3.78***	31.00 ± 2.08	350.0 ± 11.55***	190.0 ± 5.77**, ###	138.0 ± 1.52***

Along with test and standard treatments, invitro prothrombin time (plasma + PT reagent), activated partial thromboplastin time (plasma + APT reagent), and plasma recalcification (plasma + cacl2) were added. *All values are expressed as Mean ± SEM: **P<0.01 when compared to normal; ***P<0.001 when compared to normal; ###P<0.001 when compared to standard; NS: Non-significant when compared to the standard.

In the clotting time assay, the test drug at a concentration of 60 mg/ml, 80 mg/ml, and 100 mg/ml showed significant differences (**P<0.01, ***P<0.001, ***P<0.001 respectively) compared to normal. While the standard drug at

a concentration of 100 mg/ml showed a significant difference (**P<0.001) compared to normal. In comparison to standard, the test drug at concentrations of 60 mg/ml and 80 mg/showed significant differences (###P<0.001) and (###P<0.001). However, at a concentration of 100 mg/ml, the test drug showed no significant difference from the standard.

In thrombolytic activity as well as the test drug at a concentration of 60 mg/ml and 80 mg/ml showed significant differences (***P<0.001, **P<0.01) respectively, compared to the standard. No significance was observed when testing the extract at a concentration of 100 mg/ml compared to the standard.

The determination of the invitro prothrombin time for the test drug at a concentration of 60 mg/ml showed no significant difference compared to normal. While at a concentration of 80 mg/ml showed significant differences of (**P<0.01), 100 mg/ml (***P<0.001), and standard drug at a concentration of 100 mg/ml showed a significant difference (***P<0.001) compared to normal. Test doses of 60 mg/ml (###P 0.001), 80 mg/ml (###P 0.001), and 100 mg/ml showed no significant difference compared to the standard.

In-vitro Activated partial thromboplastin time, the test drug at a concentration of 60 mg/ml, 80 mg/ml, 100 mg/ml and standard drug at a concentration of 100 mg/ml showed significant differences (**P<0.01, ***P<0.001, ***P<0.001 and (###P<0.001) respectively compared to normal. Test concentrations 60 mg/ml and 80 mg/ml both showed a significant difference of ###P<0.001 compared to the standard. While there was no significant difference between the test drug and the standard at a concentration of 100 mg/ml.

Anticoagulant activity by plasma recalcification method: the test drug at a concentration of 100 mg/ml and the standard drug at a concentration of 100 mg/ml showed a significant difference (***P<0.001) compared to normal. whereas the test drug at a concentration of 100 mg/ml showed no difference when compared to the standard.

Table 2. Calcium chloride induced clotting time assay.

S. No	Absorbance at various time intervals*					
	5 min	10 min	15 min	20 min	25 min	30 min
Control	1.658 nm	1.614 nm	1.586 nm	1.414 nm	1.212 nm	0.921 nm
Test A (80 mg/ml)	0.835 nm	0.848 nm	0.872 nm	0.916 nm	0.991 nm	1.021 nm
Test B (100 mg/ml)	0.989 nm	0.999 nm	1.010 nm	1.116 nm	1.216 nm	1.226 nm
Test C (150 mg/ml)	0.821 nm	0.831 nm	0.841 nm	0.849 nm	0.914 nm	0.921 nm
Test D (200 mg/ml)	0.811 nm	0.816 nm	0.819 nm	0.829 nm	0.831 nm	0.839 nm
Std.(100mg/ml)	0.993 nm	0.998 nm	1.008 nm	1.120 nm	1.210 nm	1.260 nm

In the control group of the calcium chloride-induced clotting time assay, the absorbance initially increased and subsequently decreased with time. In contrast, absorbance increased over time in the test and standard groups after initially decreasing in both.

Conclusion

Newer drugs for thrombosis are urgently needed as the incidence of complications from thromboembolic disorders is increasing gradually every year. Unfortunately, there are few new drugs in the pipeline. Our present study shows that the root extract powder of *Astragalus membranaceus* is effective as an anti-coagulant and anti-thrombotic agent for thromboembolic disorders. These findings suggest that AMRE may be a promising anti-thrombotic agent for the treatment of a variety of thromboembolic disorders. So, hopefully, this research will continue to shed light on ways to improve the therapeutic agents.

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Conflicts of interest:
The authors declare no conflicts of interest.

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Supplementary Data

Figure: 1 Thrombolytic Activity of *Astragalus membranaceus*

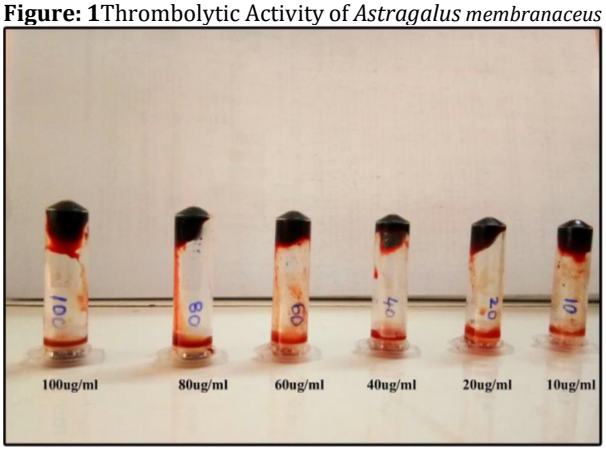

Figure: 2 Determination of *invitro* Prothrombin Time (PT)

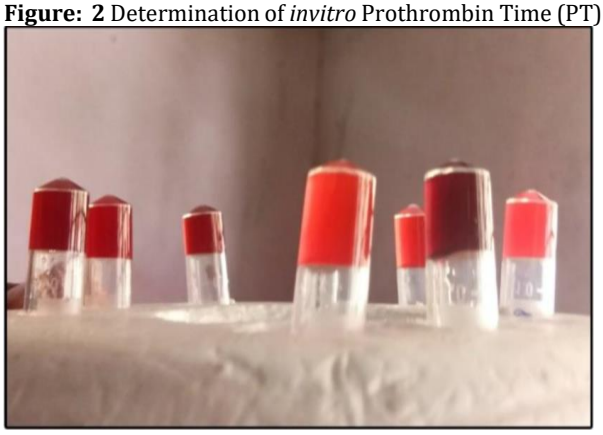

Figure: 3 Anticoagulant activity by plasma recalcification method.