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# STUDIES ON THE ANTICOAGULANT ACTION OF ASPILIA AFRICANA

Magdy M. Hanna and Julian Niemetz*

Department of Physiology and Hematology,
A.M. Dogliotti Medical College, University of
Liberia, Monrovia, Liberia; and V.A. Medical Center
130 W. Kingsbridge Rd., Bronx, N.Y. 10468*
and Mt. Sinai School of Medicine, New York, N.Y.
USA

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ABSTRACT

An anticoagulant activity was identified and isolated from the leaves of a West African plant, Aspilia africana by gel filtration on Sephadex G-100. The anticoagulant factor had an apparent molecular weight of approximately 60,000 d. Upon incubation with plasma, it prolonged the partial thromboplastin time, prothrombin time, thrombin and reptilase time. The factor decreased the fibrinogen content of plasma as well as the activity of coagulation factors V, VIII and IX but not factor VII, X or XI activities. After incubation with fibrinogen, the thrombin clotting time was prolonged and the quantity of clottable fibrinogen reduced. The action on fibrinogen was characterized by sequential lytic breakdown of the A-alpha-chain and B-beta-chain, the gamma-chain being lysed last, after prolonged incubation. Benzamidine, Epsilon aminocaproic acid or soybean trypsin inhibitor did not impede lysis.

## INTRODUCTION

The leaves of Aspilia africana have been used for a long time in Africa as a native medicine to stop bleeding from wounds (1-4). Dalziel quotes Roberts ex Holmes (in Pharm. Jour. wer 3,8, 1878: 563) that "in Liberia, it is said to be preferred as a haemostatic to any European remedy and to be extraordinarily effective in stopping bleeding, even from a severed artery, as well as rapid healing of the wound. The fresh plant is always used. A decoction has also been recommended for pulmonary hemorrhage." (2).

However, in our laboratory, attempts to study this hemostatic activity showed that a saline extract of dry leaves of Aspilia africana had a powerful anticoagulant activity. Our studies of this anticoagulant activity are presented in this report.

Key words: Aspilia africana, Anticoagulant, Fibrinogen, Fibrinogenolysis
*Address correspondence

## MATERIALS AND METHODS

Dry leaves of Aspilia africana were supplied by the University of Liberia. One gram of the powdered leaves was soaked in 40 ml veronal buffered saline: 0.10 M NaCl, 0.05 M Na barbiturate, pH 7.25 (VBS), at 4°C for 18 hrs. After centrifugation for 10 min at 12,000 g, the supernatant was collected and used as "crude Aspilia extract".

Human fibrinogen, Grade L. from A.B. Kabi (Sweden), was used at a concentration of 4.0 mg/ml in VBS unless otherwise specified. Thrombin, Parke Davis (Morris Plains, N.J.), was used at a concentration which clotted fibrinogen in the control system in 18-22 sec.

Gel filtration was carried out using Sephadex G-100 (Pharmacia, Piscataway, N.J.). Ten ml of crude Aspilia extract were lyophilized, redissolved in 2.0 ml distilled water, centrifuged for 10 min at 12,000 g and the supernate applied to a 1.5x47 cm column at 4°C. 3.5 ml fractions were collected at a flow rate of 10 ml/hr. The most active fractions were pooled after measuring the activity and optical density at 280 nm, and stored in 1.0 ml aliquots at -70°C. This solution, appropriately diluted was used as a standard reference stock solution. One unit of activity was arbitrarily defined as the quantity present in 0.1 ml, which when incubated at 37°C for one hr with an equal volume of a fibrinogen solution containing 4.0 mg/ml, prolonged the thrombin time from 19±2 sec to 36±2 sec. In the following experiments, an Aspilia solution containing 25 U/ml was used unless stated otherwise.

### Coagulation tests:

Plasma Recalcification Time was performed as described by Biggs and MacFarlane (5), Thrombin Time as described by Rapaport and Ames (6), Prothrombin Time using Simplastin (General Diagnostics, Morris Plains, N.J.) and the Reptilase Time using Atroxin (Sigma, St. Louis, MO.) as recommended by the manufacturer. Plasma was incubated with equal volumes of Aspilia colution (or VBS as a control) at 37°C for 1.0 hr before proceeding with the test.

To test the effects of Aspilia solution on the coagulation factors V, VII, VIII, IX, X and XI activities, normal human plasma was incubated with an equal volume of the solution at 37°C for 1.0 hr. The factor's activities were then assayed in a one stage test as described by Biggs (5), using the original plasma similarly incubated with VBS as a reference. At least 4 dilutions were tested. Congenitally factor-deficient plasmas were used as substrate in these assays (General Diagnostics).

The fibrinogenolytic activity of Aspilia solution was also studied by incubating a fibrinogen solution containing 4.0 mg/ml with an equal volume of Aspilia solution for different periods of time, the remaining fibrinogen was clotted by thrombin, or reptilase, or heat precipitated at 56°C for 15 min and measured by the Ratnoff and Menzie method (7).

To study the effects of different procedures on the fibrinogenolytic activity, Aspilia solution was subjected to the following procedures: a. Changes in pH: the extract was incubated at a chosen pH for 5 hrs before pH neutralization and activity testing. 1.0 N. HCl and 1.0 N. NaOH were used to change the pH and for subsequent neutralization. b. Trichloroacetic acid (TCA): 10% TCA was added to an equal volume of the extract, centrifuged and both the supernatant and the precipitate tested after restoring the pH to 7.3. c. Benzamidine (Sigma, St. Louis, MO.) at a final concentration of 25mM; Epsilon amino caproic acid, EACA, (Calbiochem, San Diego, CA) at a final concentration of 12.5mM; and Soybean trypsin inhibitor, SBTI, (Sigma) at 20 mg/ml were incubated with Aspilia solution for 1 hr and the activity measured and compared to appropriate controls substituting VBS for the inhibitor.

Polyacrylamide Gel Electrophoresis:

Disc Electrophoresis after reduction with dimercaptoethanol was carried out in the presence of SDS as described by Laemmli (8) using 7.5% running gels, 3% upper gels.

## RESULTS

Extraction of the dried Aspilia leaves with VBS yields a yellowish-brown solution endowed with a fibrinogenolytic-anticoagulant activity. The extract of 1 gm of leaves was fractionated by gel filtration on a Sephadex G-100 column. The fibrinogenolytic activity eluted, showed little absorbance at 280 nM, appearing ahead of the major colored components which were devoid of anticoagulant activity. The activity appeared between bovine albumin and ovalbumin which were used as M.W. markers (Fig. 1) suggesting a M.W. of approximately 60,000 Daltons. The three most active tubes were pooled and used as stock Aspilia solution.

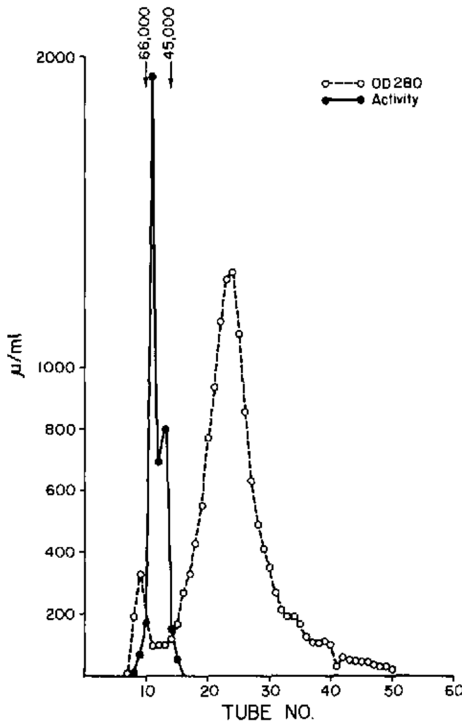


Fig. 1
Elution profile of Aspilia africana VBS extract from Sephadex G-100. The arrows at the top represent elution of bovine albumin (66,000) and ovalbumin (45,000) M.W. standards. Tubes 11, 12 and 13 were pooled and used as stock Aspilia solution.

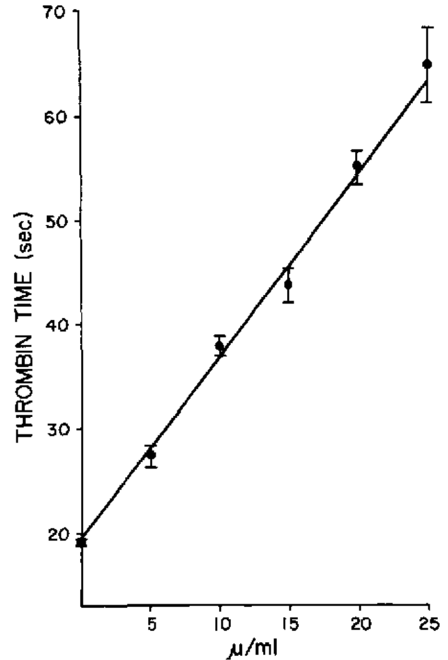


Fig. 2
The effect of different concentrations of stock Aspilia solution on the thrombin clotting time of fibrinogen after one hr incubation at 37°C. (mean ± SEM).

Fig 2 shows the effect of different concentrations of the Aspilia stock solution on the fibrinogen-thrombin clotting time. The relationship is linear from 19 to approximately 65 sec. The 20% concentration, which gave a

thrombin time of 36±2 sec was assigned an activity of 1 U per 0.1 ml.

When incubated with human plasma at 37°C for 60 min, Aspilia solution prolonged the plasma recalcification time, prothrombin time, thrombin time and reptilase time (Table 1).

Table 1
Effects of Aspilia solution on plasma recalcification time, prothrombin time, thrombin time and reptilase time.

| Test | Control mean ± SEM sec | Aspilia mean ± SEM sec |
|---|---|---|
| Plasma recalcification time | 194 ± 7.6 | 434 ± 19.6 |
| Prothrombin time | 10.9 ± 1.0 | 47.6 ± 2.8 |
| Thrombin time | 18.7 ± 0.8 | 68.8 ± 4.6 |
| Reptilase time | 17.7 ± 1.4 | 53.9 ± 4.7 |

One ml of Aspilia solution (25U) was incubated for 1 hr at 37°C with 1 ml of citrated plasma. Incubation was terminated by chilling to 0°C, prior to testing. Plasma incubated with VBS was used as a control.

Specific assays of coagulation factor activities were then performed. Aspilia decreased the activity of Factor V to 16%, Factor VIII to 25% and Factor IX to 43%, as compared to the same plasma incubated with VBS and used as control. No detectable effect on the activity of Factors VII, Factor X or Factor XI was observed.

Aspilia solution was incubated with fibrinogen at 37°C and the activity was followed by thrombin times. Significant prolongation of the thrombin time was observed after 15 min. This prolongation increased with time of incubation. The relationship is linear between 5 min and one hr (Fig. 3).

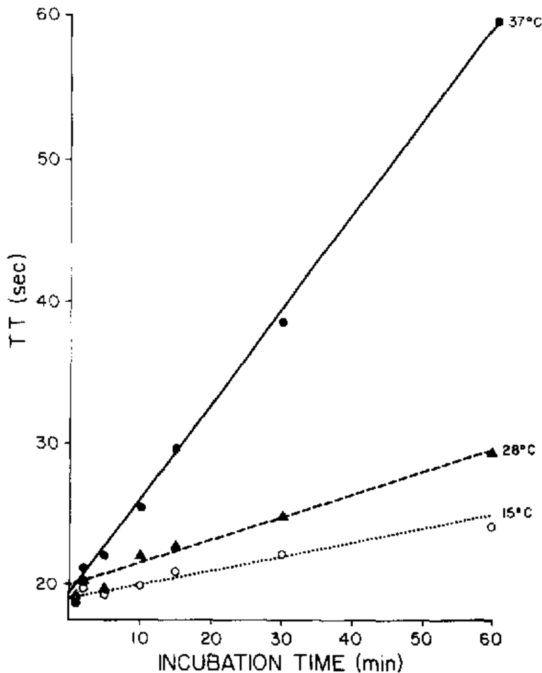


Fig. 3
Effects of temperature on the anticoagulant activity of Aspilica. Equal volumes of Aspilia solution (25 U/ml) and fibrinogen solution (4.0 mg/ml) were incubated at the above temperatures for the indicated time before performing the test.

The effect of Aspilia on the fibrinogen-thrombin time was temperature dependent. At 15°C, the activity was modest. There was an increase of activity at 28°C with further substantial increase of the activity at 37°C (Fig. 3).

The effect of Aspilia on fibrinogen was also quantified by assaying residual clottable fibrinogen. Fibrinogen solution was incubated with Aspilia solution at 37°C. The concentration of thrombin-clottable fibrinogen decreased gradually to 47±7.5, 38±10, 24±5.5 and 18±5% of the original concentration at 30, 60, 90 and 120 min respectively. In contrast, heat-precipitable (56°C) material was 85±8.5% of control after 2 hrs, suggesting that a major part of the degradation products although incoagulable by thrombin were heat precipitable. Reptilase-clottable fibrinogen was decreased following a pattern similar to thrombin-clottable fibrinogen (results not shown).

When the extract was kept for 5 hrs at pH 5 it lost its anticoagulant activity, as evidenced by lack of prolongation of the thrombin fibrinogen clotting time. At pH 6.3, it lost 20% of activity but at pH 7 and above, and up to pH 10.5, no activity was lost.

Further characterization indicated that the active factor was not dialyzable. Complete loss of the activity occurred after treatment with 10% trichloroacetic acid. Freezing, lyophilization had no adverse effects on the activity for at least 6 months. Furthermore, when 25 units of Aspilia solution were incubated with Benzamidine (25mM), EACA (12.5mM), or Soybean trypsin inhibitor (20 mg/ml) for 60 min at 37°C, the activity was not significantly impeded, being 24.0±.6, 23.8±1.1 and 25.4±0.8 U respectively.

The effect of Aspilia on fibrinogen was further investigated at the molecular level. Fig. 4 shows polyacrylamide gel electrophoresis patterns of fibrinogen before and after incubation with Aspilia solution for 1 hr at 37°C. Tube A (control) shows the characteristic three bands of fibrinogen after reduction of disulfide bonds. When treated with Aspilia solution the A-alpha-chain and B-beta-chain were degraded and a new band appeared distal to the gamma-chain (Fig. 4,B). When the Aspilia solution was heated at 100°C for 5 min only the A-alpha-chain was degraded (Fig. 4,C), however, after heating Aspilia for 15 min at 100°C some intact A-alpha-chain remained (Fig. 4,D).

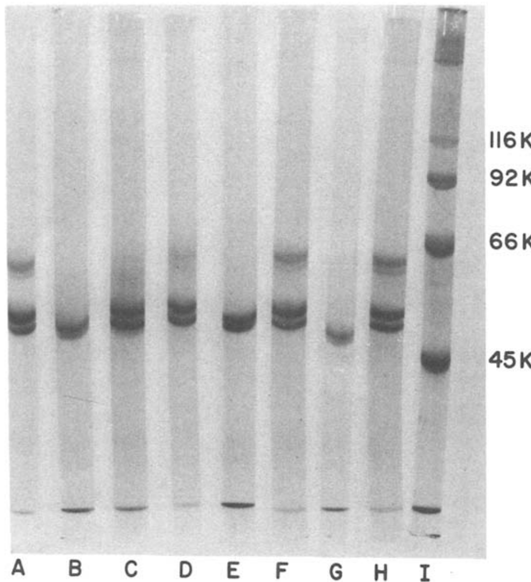


Fig. 4
The effect of Aspilia extract on fibrinogen.

0.1 ml of fibrinogen was incubated for 1 hr at 37°C as indicated below. After reduction with mercaptoethanol, the samples were electrophoresed on 7.5% acrylamide gels, in 0.1% SDS. (A) 0.1 ml fibrinogen + 0.2 ml VBS. (B) Fibrinogen + Aspilia + VBS. (C) Fibrinogen + Aspilia extract previously heated in boiling water bath for 15 min. (E) Fibrinogen + Benzamidine + Aspilia. (F) Fibrinogen + Benzamidine + VBS. (G) Fibrinogen + EACA + Aspilia. (H) Fibrinogen + EACA + VBS. (I) M.W. standards.

The digestion of fibrinogen over a 3 hr period was also followed (Fig.5). Loss of intact A-alpha-chain occurs first and appears complete at 30 minutes. B-beta-chain degradation proceeds at a slower rate and loss of intact B-beta-chain is nearly complete at 2 hrs. Gamma chain degradation is slowest.

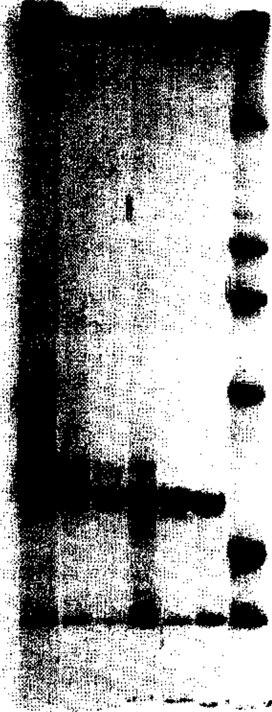

Fig. 5
Time course of fibrinogen digestion.

Incubation of fibrinogen with Aspilia solution at 37°C. 0 min, 15 min, 30 min, 60 min, 120 min, 180 min, Mol. Wt. Standards.

## DISCUSSION

We report the novel findings that solution of Aspilia africana prolonged the recalcification time, prothrombin time, thrombin time and reptilase clotting time of human plasma.

The anticoagulant activity, after partial purification by molecular sieving on sephadex G-100, had an apparent molecular weight of 60,000 D (assuming a globular shape and non adherence to the gel. Upon incubation with plasma, Aspilia solution decreased Factor V, Factor VIII and Factor IX activities of human plasma but not Factor VII, Factor X or Factor XI activities.

Aspilia solution also digested fibrinogen as suggested by prolongation of the thrombin time and reptilase time, and demonstrated by acrylamide gels.

The A-alpha-chain of fibrinogen was the most susceptible to lysis. It was digested first; B-beta-chain degradation occured at a slower rate; and gamma chain lysis was the slowest. This pattern is similar to that of plasmic hydrolysis of fibrinogen described by Mosesson et al (9). However, the activity of Aspilia solution was not impeded by plasmin inhibitors. The action of Aspilia solution on fibrinogen had also some points of similarity with platelet derived-calcium activated protease in that it was not inhibited by benzamidine, EACA and SBTI (10).

The action on fibrinogen was time and temperature dependent markedly decreasing when the temperature was lowered from 37°C to 28°C and further diminishing at 15°C. The results are also compatible with the presence of more

than one fibrinolytic enzyme, similar to the alpha and beta fibrinogenases of T. gramineus venom described by Quyang and Huang (11).

The above findings were unanticipated, since Aspilia leaves have been, and are still used in Africa to stop bleeding. However, it should be noted that fresh leaves are used as a hemostatic (2). In the present work the extract was prepared from dried leaves which could have lost their hemostatic activity during drying. Alternatively, the hemostatic effect may be due to vasoconstriction, or action on platelets. Indeed, Akabue et al (4), have recently reported that topically applied alcoholic extract of Aspilia was potently vasoconstrictive. Furthermore, these extracts also induced a marked prolongation of the whole blood clotting time of rabbit, rat, and ox.

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