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COMPARATIVE ANALYSIS OF THROMBOLYTIC ACTIVITY OF ETHANOL EXTRACT AND ITS DIFFERENT FRACTIONS OF *ANOGEISSUS ACUMINATA* LEAVES

Mahmudul Hasan*[1,2], Syed Md. Abdul Kader[1,2], Md Imdadul Haque[1,2], Ebrahim Khan Samrat[1,2], Nishat Rahman[2], Mohammad Ikram Uddin[1], Md. Golam Sarowar[1] and Jackie Barua[2,3]

[1]Department of Pharmacy, International Islamic University Chittagong, Chittagong, Bangladesh.

[2]GUSTO A Research Group, Chittagong, Bangladesh.

[3]Comilla Medical College, Faculty of Medicine, University of Chittagong, Bangladesh.

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***Corresponding Author**
Mahmudul Hasan
Department of Pharmacy, International Islamic University Chittagong, Chittagong, Bangladesh.

ABSTRACT

This study shows the thrombolytic effect of different ethanol extracts and fractions of the leaves of *Anogeissus acuminata* by thrombolysis which is also known as thrombolytic therapy, we mean a treatment to dissolve dangerous clots in blood vessels, improve blood flow, and prevent damage to tissues and organs. In this experiment fraction of leaves of *A. acuminata*, as this is a medicinal plant. These were used in the treatment of thrombolytic therapy because it shows remarkable result in thrombolytic activity. Using an *in vitro* antithrombotic model, all extracts, fractions, and Streptokinase exhibited significant ($P < 0.0001$) clot lysis. EEAA extract exhibited highest % of clot lysis 44.14 ± 3.26 ($P < 0.0001$, $P < 0.001$). So this plant can further be used in cardiovascular diseases. One of the main causes of multiple cardiovascular diseases is intravascular thrombosis and current agents, which are used for the treatment and prevention of thrombosis but have some serious side effects. Therefore, new antithrombotic and thrombolytic agents are still needed. So this experiment can be referred over the drugs with hazardous side effects.

KEYWORDS: *Anogeissus acuminata*, extract, leaves, antithrombotic, fraction.

INTRODUCTION

Many human diseases like cancer, diabetes, cardiovascular diseases are caused by oxidative stress which is initiated by free radicals that play a vital role in damaging various cellular macromolecules.[1,2] Antioxidants scavenge the free radicals and help to prevent the damage caused by them.[3] Endogenous antioxidants are produced in the body itself to neutralize free radicals. However, the body relies on external (exogenous) sources, primarily the diet, to obtain the rest of the antioxidants it needs. These exogenous antioxidants are commonly called dietary antioxidants. Fruits, vegetables, and grains are rich sources of dietary antioxidants. Some dietary antioxidants are also available as dietary supplements.[4,5]

Thrombosis is the fundamental pathophysiological process that underlies the acute coronary disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks; which are the main causes of morbidity and mortality in developed countries.[6] This disease due to the formation of thrombus or embolus which hinders the blood flow by blocking the blood vessel, therefore, depriving tissues of normal blood flow and oxygen. These leads to necrosis of the tissue in that area. Thrombin formed blood clot from fibrinogen and is lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator (tPA). The purpose of a fibrinolytic drug is to dissolve thrombin in acutely occluded coronary arteries thereby to restore blood supply to ischemic myocardium, to limit necrosis and to improve prognosis.[7]

Acute myocardial infarction and ischemic stroke have become the leading causes of death nowadays that result from thrombosis. Other than surgical interventions to remove or bypass the blockage or the generation of collateral vessels to provide a new blood supply, the only treatment available is the administration of antithrombotic agents to dissolve the blood clot.[8] In thrombolysis the breaking of clots occurs, secondary fibrinolysis is stimulated by activation and catalyzing of plasminogen into plasmin by tPA (tissue plasminogen activator) which degrades the fibrin network.

The plant under investigation *Anogeissus acuminata* (Family: *Combretaceae*). It is an indigenous plant distributed in the Chittagong Hill Tracts and Cox's Bazaar and with tribal name Phul jhumuri gaas (Chakma). The plant is rich in tannins and flavonoids. The plant material used for this study was collected from Bandarban district, Bangladesh. The tannoid principles of the plant possess antioxidant activity which was proven to reduce microbial infection.[9,10]

The main objective of the study was to investigate whether the ethanol extract of *Anogeissus acuminata* leaves and its different fractions possess thrombolytic activities or not.

MATERIALS AND METHODS

Plant materials

The leaves of *A. acuminata* were collected from Bandarban, Bangladesh in March 2015 at a mature stage. The leaves were cut into small pieces and then dried in shade at 21-30ºC for 7 days. Then the materials were dried in an oven at low temperature to improve grinding. Then the pieces were ground by a mechanical grinder and then passed through a size 60 mesh screen to obtain a fine powder of the leaf material. This was stored in an air-tight container.

Preparation of sample

The fine powder of leaves of *A. acuminata* (800 g) was taken in a clean round-bottom flask (5 L) and soaked in 4 L of Ethanol for 15 days at room temperature with occasional shaking and stirring. Then the mixture was first filtered with cotton plug followed by Whatman No. 1 filter paper. The filtrate is evaporated to dryness in Heidorph rotary evaporator at 45ºC to obtain a concentrated extract. This was then air dried to obtain a solid residue. Thus the Ethanolic extract of the leaves of *A. acuminata* was prepared and then four solvents chloroform, n-hexane, ethyl acetate and methanol was used for solvent-solvent partitioning from ethanol solution. To prepare test samples and the standards were suspended in distilled water using Tween 80 for *in vivo* test.

Chemicals and reagents

The chemicals used were: ethanol, methanol, n-hexane, chloroform (Merck, Germany). Lyophilized streptokinase's (SK) vial (Square Pharmaceuticals Ltd. Bangladesh). All chemicals used were analytical grade.

In vitro antithrombotic activity

Blood specimen

Whole blood (6 ml) was drawn from healthy human volunteers (n = 12) without a history of oral contraceptive or anticoagulant therapy. A new consent, approved by Mohammed Abu Sayeed, Assistant Professor & Head of Department of Pharmacy, International Islamic University Chittagong (IIUC), Bangladesh, for the collection of blood samples from Human volunteers. Blood collection was conducted by Md. Shariful Islam (Lab technician, Department of Pharmacy, IIUC) and preservation were conducted by Abdul Karim (Lab

technician, Department of Pharmacy, IIUC), who stored the clot containing Eppendorf tube in the refrigerator in Microbiology lab, Department of Pharmacy, IIUC. A 500 µL of blood was transferred to each of the 12 previously weighed Eppendorf tube tubes to form clots.

Statement on informed consent of the donors

The volunteer donors were supplied a consent form which informed the title of the research project, name and detail contact of investigators as well as purpose of the research. Description of the research mentioning step-by-step brief of the proposed research, inclusion and exclusion criteria of the donors, whether donors will receive any therapy or not, the volume of blood to be taken, possible discomfort at the puncture sites, the time required for the blood sampling. Benefits of the volunteer described. It was indicated to the consent form that the volunteers might refuse to donate blood at any time. The donor, whether could withdraw his sample data, was disclosed. The sample was restricted to that individual study, not for future research projects was presented in the consent form. Potential harm, injuries, discomforts or inconvenience associated with donors in this study was added as informed consent statement. If there was known harm to the donors, the potential harm, current knowledge regarding the probability of the occurrence of the harm, clinical importance of the harm; and any relevant knowledge regarding the probability of reversibility. Treatment alternative and possibility of the research was described. Confidentiality statement was included in the consent form in the way that "confidentiality will be respected and no information that discloses the identity of the participant will be released or published without consent unless required by law of states. Finally, identification of investigators was provided in case of further query. The consent form was concluded with major questions on above disclosures in Yes/NO form followed by the signature (with date) of the donor.

***In vitro* antithrombotic study procedure**

Experiments for clot lysis were carried as reported earlier. Briefly, 3.5 mL venous blood drawn from the healthy volunteers was distributed in 7 different pre-weighed sterile Eppendorf tube (0.5 mLtube) and incubated at 37°C for 45 min. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube - the weight of tube alone). To each Eppendorf tube containing pre-weighed clot, 100 µL of different extracts and fractions of *A. acuminata* leaves were added separately. As a positive control, 100 µL of SK and as a negative non-antithrombotic control, 100 µL of distilled water

were separately added to the control tubes numbered. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. The difference obtained in weight taken before and after clot lysis was expressed as a percentage of clot lysis. The experiment was repeated with the blood samples of the 12 volunteers.

Statistical Analysis

All results are expressed as Mean ± Standard error of the mean (SEM). The results were statistically analyzed using repeated measures analysis of variance with Tukey test for thrombolytic activity. $P<0.05$, $P<0.01$ and $P<0.001$ were considered as statistically significant. Statistical programs used were SPSS (Statistical Package for Social Science, version 22.0, IBM Corporation, NY) and for graphical presentation GRAPHPAD PRISM® (version 6.00; GraphPad Software Inc., San Diego, CA, USA) were used.

RESULTS AND DISCUSSION

***In vitro* thrombolytic activity assay**

In our present study, different fractions of *A. acuminate* showed antithrombotic activity. Among them, ethanolic and methanolic fraction showed maximum clot lysis signifying ethanol and methanol soluble compounds are mainly responsible for clot lysis.

In an antithrombotic approach with a human blood sample, the addition of 100 μL streptokinase (a positive control), to the clots and subsequent incubation for 90 minutes at 37°C 79.8 ± 1.38 % clot lysis. On the other hand, distilled water was treated as negative control which showed negligible 6.60 ± 1.84 % clot lysis. EEAA showed the highest significant 44.14 ± 3.26 %, clot lysis activity among all the extracts (P values <0.001). MFEEAA showed 41.04 ± 3.14 % of clot lysis (P > 0.0001 compared to negative control and P > 0.001 compared to positive control). Percentages of clot lysis obtained after treating the clots with different extracts, fractions and appropriate controls are shown in **Table 1 and Figure 1**.

Table. 1: *In vitro* clot lysis activity of the extracts and fractions of *A. acuminata* leaves and Streptokinase on human blood.

Drug/Extracts	% of clot lysis
Water	6.60±1.84
Streptokinase	79.8 ±1.38[a]
EEAA	44.14 ± 3.26[a, b]
NHFEEAA	37.39 ± 3.64[a, b]
CHFEEAA	36.68 ± 3.07[a,b]
EAFEEAA	39.55 ± 2.63[a,b]
MFEEAA	41.04 ± 3.14[a,b]

Values are mean ± SEM (n = 12); [a]P<0.0001, Tukey test as compared to negative control, [b]P< 0.001, compared to positive control. Statistical representation of the effective clot lysis percentage by extracts and fractions preparations, positive antithrombotic control (streptokinase), and negative control (sterile distilled water) processed by Tukey test by using SPSC for windows, version 22.0.

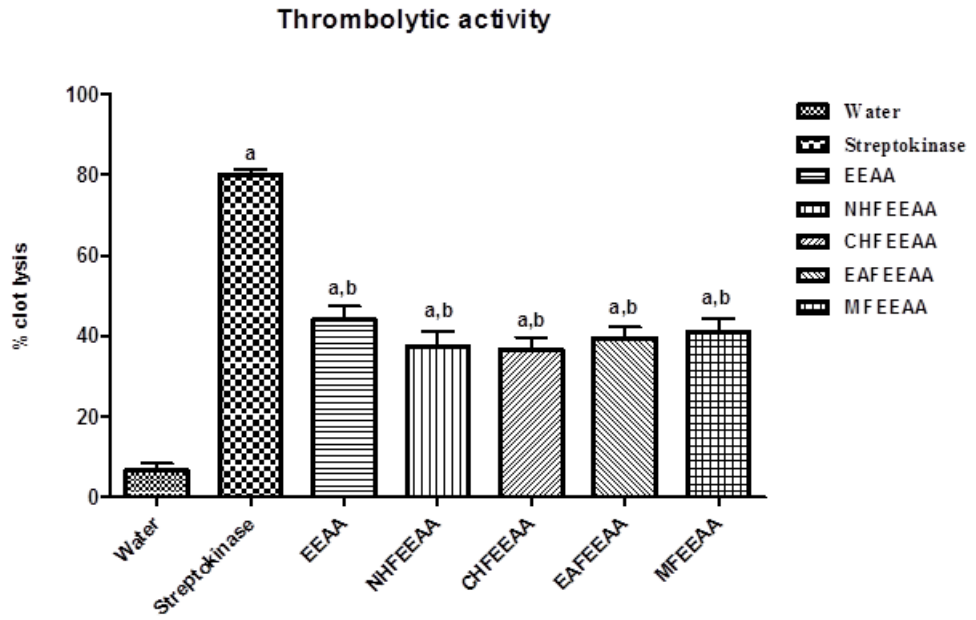

Figure 1: *In vitro* clot lysis activity of the extracts and fractions of *A. acuminata* leaves and Streptokinase on human blood. Values are mean ± SEM (n = 12); [a]P< 0.0001, Tukey test as compared to negative control, [b]P< 0.001, compared to positive control. Statistical representation of the effective clot lysis percentage by extracts and fractions preparations, positive antithrombotic control (streptokinase), and negative control (sterile distilled water) processed by Tukey test by using SPSC for windows, version 22.0.

CONCLUSIONS

In conclusion, this well-informed study evaluated significant thrombolytic activity of Ethanol extract and its different fractions of *A. acuminata* leaves. It can be expected that distinctive dynamic secondary metabolites are available in this concentrate and maybe some of these mixes may work in a synergistic way. On the other hand, further studies are important to illustrate the component lying with these impacts. On the other hand, this is the first write about this example and it may serve as a stride with respect to the natural and pharmacological exercises of this specimen.

ABBREVIATIONS

EEAA = Ethanolic extract; NHFEEAA = n-hexane fraction of ethanolic extract; CFEEAA = Chloroform fraction; EAFEEAA = Ethyl acetate fraction; MFEEAA = Methanol fraction of ethanolic extract of *A. acuminata* leaves; µg: Microgram; L= liter; mL= Milliliter; µL= Micro liter; µg/mL= Microgram per Milliliter; *etss al*.= et alliori (and others); SEM: Standard error for mean.

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Competing interests

The authors declare that they have no competing interests.

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