Original Article

Clot Lysis Capacity of *Andrographis paniculata* and its Pure Compound Andrographolide

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ABSTRACT

Background: *Andrographis paniculata* and its main components andrographolide (AGL) have been reported for their promising cardio-protective effects in experimental animals. **Aim:** To investigate clot lysis activity of *A. paniculata* and AGL. **Materials and Methods:** The clot lysis activity of the aqueous *A. paniculata* stem extract (AAP) and AGL was investigated on clotted human blood by using streptokinase (SKE) as a standard drug. **Results:** Both AAP and AGL showed a concentration-dependent clot lysis activity. AAP showed the highest clot lysis activity at 100 µL (eq. 10% w/v) (54.12 ± 1.22%), while AGL showed at 100 µg/mL (58.62 ± 2.02%). The half maximal effective concentration (EC_{50}) values of AAP and AGL were 87.32 ± 0.37 µL (eq. 8.732% w/v) and 79.74 ± 0.31 µg/mL, respectively. The netagive controls showed negligible clot lysis capacities, while the standard SKE showed 81.54 ± 0.78% clot lysis activity. **Conclusion**: AAP and its pure component NGL exhibited significant clot lysis activity on human clotted blood and may be good candidates for the treatment of atherothrombosis.

Key words: *Andrographis paniculata*, Aqueous extract, Andrographolide, Atherothrombosis, Human blood.

INTRODUCTION

Andrographis paniculata (Burm. f.) Wall. ex Nees (Acanthaceae) has many essential medicinal properties and is commonly used in Sri Lanka, China, India, Bangladesh and many other Southeast Asian countries.[1] The plant contains many important biologically active compounds, including diterpene lactones (deoxyandrographolide, andrographolide, neoandrographolide and 14-deoxy-11, 12-didehydroandrographolide), diterpene glucoside (deoxyandrographolide19β-D-glucoside) and flavonoids (5,7,2',3'-tetramethoxyflavanone and 5-hydroxy-7,2',3'-trimethoxyflavone).[2]

Cardiovascular diseases (CVDs) are one of the major consequences of death worldwide. Traditionally, *A. paniculata* is used in cardiac diseases. Several scientific reports also suggest the cardiological activities of this plant.[3-15] *A. paniculata* is evident to act against atherosclerotic in a rabbit model.[9] Moreover, it was seen to prevent constriction of blood vessels and increase blood clotting time in the pre- and post-angioplasty procedures (Wang and Zhao, 1994).[7] Furthermore, it has antihypertensive activity[16] and act against myocardial infarction in experimental animals.[3,4] *A. paniculata* also found to exhibit platelet antiaggregation effect in *in vitro*[12] and *ex vivo*[17] test systems.

Andrographolide (AGL), the bitter diterpene lactone of this plant[18] is known for is diverse biological activities.[19] AGL is evident to act against cardiovascular diseases and many metabolic disorders, including diabetes, hyperlipidemia, hypertension and obesity.[20] It is a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inhibitor. AGL in human platelets was reported to increase in cyclic GMP/GMP-dependent kinase (PKG), subsequent inhibition of the p38 MAPK (mitogen-activated protein kinase)/(·)HO-NF-κB-ERK2 (extracellular signal-regulated

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protein kinase 2) cascade, suggesting a hope to treat thromboembolic disorders.[21] However, to date, there is no scientific evidence of the clot lysis activity of *A. paniculata* and its major component AGL.

Therefore, this study aimed at the evaluation of anti-atherothrombosis activity (*in vitro*) of aqueous A. paniculata stem extract (AAP) and the pure compound AGL on human clotted blood.

MATERIALS AND METHODS

Plant materials

Dried stems of *A. paniculata* collected during winter season from the local market Chittagong was authenticated by a Taxonomist, Herberium section at the Forest Research Institute, Chittagong, Bangladesh.

Extraction

The dried stems were cut into small pieces and washed with running tap water. The small pieces of stems were then dried in the sunlight for three days and ground into coarse powder by using an electric mixer grinder machine (Bajaj, India). Two grams coarse powder was soaked in the 20 mL (10% w/v) distilled water for overnight. The extract was first filtered by using surgical cotton plug, following to the Whatman No. 1 filter paper. The filtrate obtained by this process was directly used for the test of clot lysis activity.

Pure andrographolide (AGL) and other chemicals and reagents

AGL (Cat. No. 365645; Purity: 98%) and SKE (1.5 million unit/vial) were purchased from the Sigma Aldrich (Germany) and Sanofi-aventis Bangladesh Limited (Bangladesh), respectively. Other chemical and reagents were purchased from the Merck India.

Preparation of test and control solutions

AAP was reconstituted in distilled water and tested at 5, 10, 25, 50 and 100 µL, while AGL was dissolved in a vehicle containing 0.05% tween 80 dissolved in solution containing 0.9% NaCl and tested at 5, 10, 25, 50 and 100 µg/mL. The standard (SKE) was prepared according to the guidlines of the manufacturer. Distilled water and the vehicle were taken as negative controls for the AAP and AGL group, respectively.

Clot lysis test (*in vitro*)

This study was carried out according to Prasad *et al.*[22] Approximately 6.5 mL blood was collected from each volunteer (n = 10) having no history of oral contraceptive or anticoagulant medications recently and distributed in the pre-weighed alpin tubes (each containing 0.5 mL). The blood containing tubes were incubated in an incubator for 45 min (at 37 °C), which caused blood coagulation in the tubes. The serum was removed carefully and clot weight was taken. Each concentration in a volume of 100 µL for the AAP and AGL was added to the alpine tubes containing clotted blood. Similarly, 100 µL of distilled water (vehicle) was treated as negative control group. All the tubes were re-incubated at the same temperature for 1.5 h. Finally, the weight was taken after removing the lysed fluid from each tube. The experiment was triplicated with the same individuals. By applying the following formula, percentage of clot lysis was calculated:

$$\% \text{ Clot lysis} = \frac{(W_2\text{-}W_1)\text{-}(W_2\text{-}W_3)}{(W_2\text{-}W_1)} \times 100$$

Where,

W1 = Empty tube weight

W2 = Tube weight before lysis removal

W3 = Tube weight after lysis removal

Statistical analysis

Data analysis was done by one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test (Graph Pad Prism, version: 6.0). Values are mean ± standard deviation (SD) considering $p< 0.05$ at 95% confidence interval.

RESULTS

SKE (standard) at 100 µL showed 81.54 ± 0.78% clot lysis, while the NC1 (negative control 1) showed insignificant clot lysis (2.18 ± 0.58%). The positive control (SKE) showed very significant ($p< 0.05$) clot lysis capacity when compared to the NC1 group. Clots treated with 100 µL of different concentrations of AAP showed concentration-dependent clot lysis activities. AAP at 100 µL (10% w/v) exhibited extract 54.12 ± 1.22% clot lysis compared to the NC1 group. The half-maximal effective concentration (EC_{50}) value determined for AAP was 87.32 ± 0.37 µL (8.732% w/v) (Table 1).

Clots treated with different concentrations of AGL also showed concentration-dependent clot lysis capacities. AGL at 100 µg/mL showed the highest clot lysis capacity (58.62 ± 2.02%), which was higher than the highest concentration of AAP but lower than the SKE group. The EC_{50} value calculated for AGL was 79.74 ± 0.31 µg/mL (Table 2).

DISCUSSION

Atherothrombosis, a result of atherosclerotic plaque disruption and formation of subsequent arterial thrombosis, leading to arterial occlusion, myocardial

Table 1: Clot lysis capacity of aqueous *Andrographis paniculata* stem extract and controls.

Treatments and Concentrations		% Clot lysis	EC_{50} [CI; R^2]
NC1 (100 µL)		2.18 ± 0.58	-
SKE (100 µL from 1.5 million unit/vial)		81.54 ± 0.78*	-
AAP (+ constituted with distilled water)	5 µL (+ 95 µL)	07.08 ± 1.08*	87.32 ± 0.37 µL [31.78 – 103.40 µL; 0.91]
	10 µL (+ 90 µL)	17.33 ± 1.32*	
	25 µL (+ 75 µL)	22.46 ± 0.54*	
	50 µL (+ 50 µL)	46.93 ± 1.36*	
	100 µL (+ 0 µL)	54.12 ± 1.22*	

Values are mean ± SD (n = 10); $p< 0.05$ when compraed to the *NC1 (distilled water) group; one-way ANOVA followed by Tukey post test; AAP: aqueous extract of *Andrographis paniculata*; SKE: streptokinase; EC_{50}: half-maximal effective concentration; CI: confidence of interval; R2: coefficient of determination at 95% confidence intervals.

Table 2: Clot lysis capacity of andrographolide and controls.

Treatments and Concentrations		% Clot lysis	EC_{50} [CI; R^2]
NC2 (100 µL)		3.13 ± 0.28	-
SKE (100 µL from 1.5 million unit/vial)		81.54 ± 0.78#	-
AGL	5 µg/mL	09.18 ± 0.4[8]#	79.74 ± 0.31 µg/mL [28.84 – 85.80 µg/mL; 0.92]
	10 µg/mL	16.23 ± 2.0[8]#	
	25 µg/mL	29.06 ± 1.0[8]#	
	50 µg/mL	49.28 ± 1.5[4]#	
	100 µg/mL	58.62 ± 2.0[2]#	

Values are mean ± SD (n = 10); $p< 0.05$ when compraed to the #NC2 (vehicle: 0.05% Tween 80 in 0.9% NaCl solution) group; one-way ANOVA followed by Tukey post test; AGL: andrographolide; SKE: streptokinase; EC_{50}: half-maximal effective concentration; CI: confidence of interval; R2: coefficient of determination at 95% confidence intervals.

infarction and even stroke. Atherosclerosis leading to atherothrombosis affects both men and women and causes >25% deaths per year in United States.[23] Tissue factor (TF), the elementary component of extrinsic blood coagulation,[24] which binds to its ligand factor VIIa (FVIIa), resulting thrombin and subsequently the cross-linked fibrin.[25] Thrombin may trigger foam cell formation via inducing CD36 expression.[26] In the study, AGL was seen to reduce significantly CD36 expression in atherogenic rabbits.[27]

In physiological and pathological states, reactive oxygen species (ROS), including partially reduced forms of molecular oxygen, such as hydroxyl radical (•OH), superoxide anion (O2−•), hydrogen peroxide (H_2O_2), lipid peroxides and hypochlorous acid (HClO), are essential. An accumulation of ROS may produce reactive nitrogen species (RNS), including highly reactive peroxynitrite anion and nitric oxide (NO). Our cells defend themselves against the damages caused by ROS and RNS under physiological conditions, through physiological antioxidants that remove free radicals and reduces oxidative states. An imbalance between these two systems (e.g., endogenous oxidants and antioxidants) results in oxidative stress, which may contribute to vascular dysfunction and atherogenesis.[28]

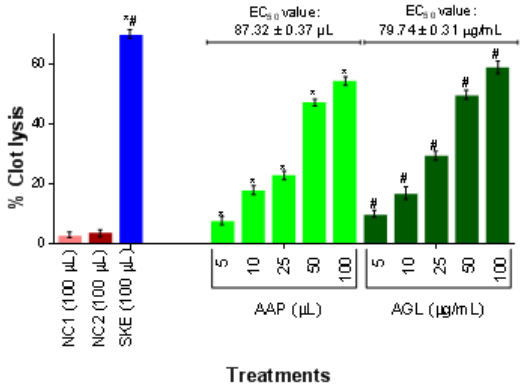

Figure 1: Comparison clot lysis activity AAP, AGL and control groups [Values are mean ± standard deviation (SD) (n = 10); $p< 0.05$ when compraed to the *NC1 (distilled water) and #NC2 (vehicle: 0.05% Tween 80 in 0.9% NaCl solution) group; one-way ANOVA followed by Tukey posttest; AAP: aqueous extract of *Andrographis paniculata*; AGL: andrographolide; SKE: streptokinase]

Scientific reports suggest that *A. paniculata* and AGL has significant antioxidant activity[19,29] that can counteract overproduction of ROS and RNS in experimental animals.

Atherosclerosis have been recognized as an inflammatory disease as inflammation contributes significant role in this process. Interleukin (IL)-1 and

-6 pathways have been implicated in atherogenesis.[30] *A. paniculata* is traditionally known for its potential anti-inflammatory effects. Scientific reports also agreeing to its traditional information.[31] The AGL also have been reported for its anti-inflammatory effects through multiple pathways,[29] such as inhibition of intercellular adhesion molecule-1 expression in cells activated by tumor necrosis factor-α,[32] suppression of inducible nitric oxide synthetase (iNOS),[33] cyclooxygenase-2 (COX-2) expression,[34,35] reduction of ERK-1/2 phosphorylation[36] and interferon gamma (IFN-γ), IL-1β, IL-2 and IL-6 production,[37,38] and inhibition of tumor necrosis factor alpha (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF)[39] and so on.

Patients with elevated certain C-reactive protein (e.g., high-sensitivity C-reactive protein (hsCRP)) has been found to increase cardiovascular risk. The non-soluble monomers C-reactive protein (mCRP) has been found to induce platelet activation and thrombus growth.[40] AGL is evident to reduce CRP in rabbits.[27] Moreover, the transcription factor NF-ϰB has also multiple links to thrombotic processes.[41] Both *A. paniculata* and AGL have been reported to reduce the expression of NF-ϰB in experimental animals and their derived cells and tissues.[42,43]

Anti-atherothrombotic agents are used to treat cerebral venous sinus thrombosis.[22] SKE, a fibrinolytic drug, with human plasminogen hydrolytically activates other free plasminogen molecules through bond cleavage and produces plasmin, results in breakdown of fibrin (a major constituent of blood thrombi), thereby, dissolves the clots inside the blood vessles.[44] In this case, a salt bridge is formed between Ile1 of SKE and Asp740 of plasminogen.[45] SK can be used as a standard clot lysis drug in the anti-atherothrombosis assay. In this study, both AAP and AGL also exerted significant and concentration-dependent clot lysis activity.[22]

In traditional practice, the use of isolated compounds is dominated by crude extract or mixtures. It may be due to at an equivalent dose the latter types often provide better prophylactic effects over the isolated constituents.[46] However, the pure compound AGL showed a better clot lysis capacity than the AAP, which can be confirmed to see the EC_{50}, CI and R^2 values of them. It may be due to AGL present in AAP is mainly responsible for the anti-atherogenic effects.[27] Therefore, this study may be an agreement with the previously reported scientific evidence on the *A. paniculata* and its major component AGL.

CONCLUSION

Both AAP and AGL showed significant clot lysis activity on human clotted blood (*in vitro*). AGL exhibited a better clot lysis capacity than the AAP. Therefore, AAP and its components, for example AGL may be good candidates for treating cardiovascular diseases, such as atherothrombosis. However, this is an *in vitro* study, therefore, further *in vivo* studies must be needed to confirm the clot lysis activity of the test samples with the appropriate mechanism of action.

Ethical Statement

A written informed consent taken from the volunteers was shared to the ethical committee. This study was approved by the Ethical Committee under the Department of Pharmacy, Bangabandhu Sheikh Mujibur Rahman Science and Technology University (BSMRSTU) (Approval No. BSMRSTU-2019/01).

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CONFLICT OF INTEREST

The author declares no conlict of interest.

ABBREVIATIONS

AAP: Aqueous extract of *Andrographis paniculata*; **AGL:** Andrographolide; **•OH:** Hydroxyl radical; **COX-2:** Cyclooxygenase-2; **CVDs:** Cardiovascular diseases; **ERK:** Extracellular signal-regulated protein kinase; **GM-CSF:** Granulocyte-macrophage colony-stimulating factor; **H_2O_2:** Hydrogen peroxide; **HClO:** Hypochlorous acid; **hsCRP:** High-sensitivity C-reactive protein; **IFN-γ:** Interferon gamma; **IL:** Interleukin; **iNOS:** Inducible nitric oxide synthetase; **MAPK:** Mitogen-activated protein kinase; **mCRP:** Non-soluble monomers C-reactive protein; **NF-ϰB:** Nuclear factor kappa-light-chain-enhancer of activated B cells; **NO:** Nitric oxide; **$O_2^{-\bullet}$:** Superoxide anion; **PKG:** GMP-dependent kinase; **RNS**: Reactive nitrogen species; **ROS:** Rreactive oxygen species; **SKE:** Streptokinase; **TF:** Tissue factor; **TNF-α:** Tumor necrosis factor alpha.

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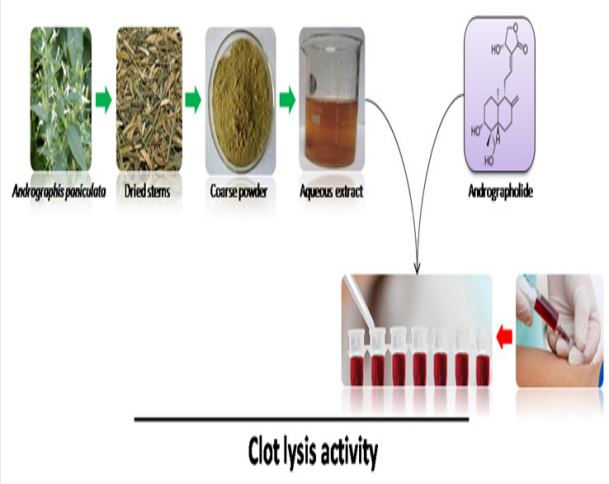
PICTORIAL ABSTRACT



SUMMARY

Clots inside the blood vessels (e.g., veins or in arteries) obstructs blood flow through the circulatory system. Venous thrombosis causes congestion locally in our body, while arterial thrombosis affect systematically, which results an extensive tissue damage and causes ischemia and necrosis. Bleeding is the major side effect of almost all the currently available anti-thrombotic drugs. In this study aqueous stem extract of *Andrographis paniculata* (AAP) and the pure component of *A. paniculata* called andrographolide (AGL) showed significant ($p < 0.05$) clot lysis activity on human clotted blood *in vitro*. AGL exerted better clot lysis activity than the AAP. *A. paniculata* and AGL have been also reported for their cardiological activities in a number of test systems. AGL is reported for its anti-atherogenic effect in rabbits. Therefore, the findings of this present study is an agreement with the previously reported cardiological effects of *A. paniculata* and AGL. In conclusion, this is the first-time *A. paniculata* and AGL underwent for the *in vitro* anti-atherothrombotic effects. *In vivo* studies should be required to understand the molecular mechanism(s) behind this effect.

About Authors

Dr. Muhammad Torequl Islam, working as a part-time researcher at TDTU, Viet Nam. Beside this, he is also working as an Assistant Professor at the Department of Pharmacy at BSMRSTU, Gopalganj, Bangladesh. His research lines are- Drug Discovery and Development, Method Development, Pharmacological and Toxicological Screenings. He is working collaboratively with more than 23 countries (>42 institutions) in the world. RG Profile: https://www.researchgate. net/profile/Muhammad_Islam108

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