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A Possible Pro-Oxidative Triggered Anti-Atherothrombotic Effect of *Dysophylla Auricularia* Hydro-Alcoholic Extract

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ABSTRACT

Oxidative stress can trigger inflammation and atherosclerosis. Nowadays, the management of atherothrombotic syndromes has gained much attention to defeat atherosclerosis. However, the cost and potential side effects of the existing thrombolytic drugs limiting their use. This study aims to develop a new protocol to screen and say possible action mechanism of a thrombolytic agent trialed and evaluated an anti-atherothrombosis effect of the methanol extract of *Dysophylla auricularia* (MDA). For this, hydrogen peroxide (H_2O_2)-induced hemolysis (HL) test was conducted to check its antioxidant capacity, while clotlysis (CL) for anti-atherothrombosis in human blood. In the latter case clot was treated with an oxidizer (H_2O_2) and/or antioxidant (ascorbic acid) and/or fibrinolytic (SK: streptokinase) and/or MDA to understand the mode of action of MDA. In HL test, MDA concentration-dependently inhibited HL, where highest inhibition was found $67.87 \pm 1.39\%$ at the highest concentration (400 $\mu\text{g/mL}$) tested. In CL test, MDA was found to antagonize H_2O_2 , while potentiated SK activity. However, the MDA + SK exerted effect was further reduced by the application of H_2O_2 . Furthermore, MDA was found to protect the clot by H_2O_2 -induced oxidizing effect. In conclusion, MDA may protect human erythrocytes from H_2O_2 -induced damaging effects due to its antioxidative defense capacity, while thrombolytic activity via antioxidant-mediated pro-oxidative effect. The current protocol can be used in non-clinical thrombolytic drug screening.

Keywords: Antioxidant, Clotlysis, Hemolysis, Human blood

Introduction:

Reactive oxygen and nitrogen species (ROS/RNS) play important physiological functions in our body. However, excessive production is evident to the etiology of the many diseases. Inflammation, chronic human diseases (including aging), and atherosclerosis are some major consequences^{1,2}. The consequences of atherosclerotic vascular disease are the leading cause of morbidity and mortality throughout the world. Based on the pathogenesis and consequences of atheromatous plaque development and progression, nowadays the management of atherothrombotic syndromes has been gained much attention^{3,4}. Generally, a clot inside the blood vessel is called atherombosis, being considered as one of the major consequences of cardiovascular diseases. The clot may be various origins such

as microorganisms, presence of foreign macromolecules/particles, body metabolites, inflammatory process or inflammation in the blood vessel, oxidative stress and so on. Unfortunately, recently used anti-atherothromboic drugs have a number of serious adverse effects⁵, therefore, restricted to the patients having blood disease or bleeding problems, heart or blood vessel disease, stroke, high blood pressure, brain tumor or other brain disease, stomach ulcer or colitis, severe liver disease, active tuberculosis, recent falls, injuries, or blows to the body or head, recent injections into a blood vessel, recent surgery, including dental surgery, tubes recently placed in the body for any reason, and recent delivery of a baby. Thus, the stimulation to search of new and safer thrombolytic agents, eventually, the development of more study protocols for the screening of anti-atherothromboic agents is also needed.

Among others, plants are the potential sources of drugs. Almost, 80% populations of the world rely on plant-based medicaments. Till date, more than 25% pharmaceutically consumed drugs are contributed by the plants. Nonetheless, the use and research on herbal medicaments are growing day by day⁶.

Dysophylla auricularia (L.) Blume. (Syn: *Pogostemon auricularis* Hassk.; Fam: Lamiaceae) is medicinal plant having evidence of traditional usages and some important biological effects such as antifungal, anti-inflammatory, anti-diarrheal, anti-diabetic and anti-pyretic activates⁷. It is also evident exerting a membrane stabilization effect on human erythrocytes (HRBC). Oxidative stresses can trigger inflammatory responses. In severe conditions, both oxidative stresses and/or inflammatory responses can impart detrimental effects on cells. In this context, antioxidant, anti-inflammatory and membrane stabilizing agents can be considered as cytoprotective agents. These kinds of cytoprotective agents can be incorporated in atherothrombosis, especially which is originated from oxidative stress and inflammatory syndromes.

The aim of this study is to develop a de-novo protocol for the screening of anti- atherothrombosis activity upon going through a trial on *D. auricularia* methanol extract for the first-time.

Materials and methods:

Research ethics

This project was approved by the Department of Pharmacy, Southern University Bangladesh (SUB) with the deposition number #SUB (PH) 9991002/2014. For human blood, 9 students of the same/different departments of the SUB were willingly participated in this study. A specialist from a reputed diagnostic centre was invited to collect 4-5 mL blood from each participant through venous puncture.

Collection, identification and extraction of plant materials

The herb *D. auricularia* was collected from the Chittagong division of Bangladesh and was identified by a taxonomist in Forest Research Institute, Bangladesh (FRIH, BD). A voucher specimen was deposited with the accession number BFRIH-4688.

After collection, *D. auricularia* (whole plant) was undergone shade drying (temperature not exceeding 50 °C), following to course grinding. Methanol hot extraction (16 h) was carried out by using the Soxhlet apparatus. The extract was then filtered through a cotton plug followed by Whatman filter paper (No. 1). For a rapid evaporation of solvent, rotary evaporator (temperature not exceeding 50 °C) was used. The yield was 12.93%.

Chemicals and reagents

The ascorbic acid (AA) was kindly given by the Zenith Pharmaceuticals Ltd. Bangladesh. All the other necessary reagents and chemicals were purchased from (Sigma-Aldrich, St. Louis, MO; USA.).

Sample preparation

Required amount of extract (MDA) was soaked in distilled water

(DW) and shaken vigorously for 30 minutes, which was then kept overnight, filtered by using Whatman filter paper and used for the bellow mentioned tests. The AA and STR were dissolved in DW and phosphate buffer saline (PBS: pH 7.4), respectively.

Study design:

Test concentrations for this study were selected according to a recent literature⁷. In this case, MDA up to 400 µg/mL was used. This study is concerned to develop and evaluate possible anti-atherombosis activity of a substance. Therefore, an initial cytoprotective capacity of the test sample (MDA) was screened prior to a sequential treatment with cytotoxic and/or cytoprotective agents. Additionally, a fibrinolytic agent was also used to say a possible action mechanism of the test sample.

H₂O₂-induced hemolysis (HL) test:

This test is an adjustment of the earlier methods described by Ruch et al⁸ and Girish et al⁹. In this context, blood was collected from a healthy man through venous puncture and immediately reconstituted 10% RBC suspension (HRBC) with the phosphate buffer saline (PBS, pH 7.4). To 0.5 mL 10% HRBC suspension 0.1 mL of 40 mM hydrogen peroxide (H₂O₂) was added. The hydrogen peroxide was considered as a stressor (STR), which was prepared in same PBS solution. The tubes were then incubated at 37 °C for 30 minutes. After the incubation period, 0.2 mL of supernatant was withdrawn and absorbance (A_{STR}) was measured at 475 nm by using a UV spectrophotometer after the addition of 2.8 mL PBS. Similarly, the tubes for negative control (NC, vehicle) and test sample (MDA/AA) were treated with 0.1 mL of each, just after the addition of STR, following to incubation similarly and taking the absorbance (A_{TEST}) at 475 nm. The percentage inhibition of hemolysis (%IHL) was calculated as the following equation:

$$\% \text{ IHL} = [(A_{\text{STR}} - A_{\text{TEST}}) \div A_{\text{STR}}] \times 100$$

Clotlysis (CL) test

The CL assay was performed according to the method described by Prasad et al¹⁰ with some modifications. Briefly, five alpine tubes (1 mL capacity) were marked for each treatment, following to take their initial weight (W1). Approximately 5 mL blood was collected from each person (9 healthy persons of either sex with no history of anticoagulant or oral contraceptive within last 6 months) and distributed in the previously marked tubes. To overcome the clotting time's variation, blood was distributed in a manner that, a single person can cover all the groups (Gr. I to IX). Each tube contained 0.5 mL of blood. The tubes were then incubated at 37 °C for 45 min, following to take another weight (W2), which were then followed the following treatments:

Group I: Negative control (vehicle: distilled water)

Group II: Fibrinolytic agent (SK: streptokinase)

Group III: Test sample (MDA: methanol extract of *Dysophylla auricularia*)

Group IV: Stressor (STR: hydrogen peroxide, H₂O₂)

Group V: STR + MDA

Group VI: STR + Antioxidant (AA: ascorbic acid)

Group VII: MDA + SK

Group VIII: AA + SK

Group IX: STR + MDA + SK

After treatment, the tubes were then incubated at the same condition for 1 h and the lysates were discarded, following to take the weight of the tubes (W3). Finally, the percentage of CL was calculated as follows: $\%CL = [(W2 - W3) \div (W2 - W1)] \times 100$.

Results:

Table 1 suggests that, the MDA concentration-dependently inhibited HL in HRBC suspension, where highest IHL was observed at 400 $\mu\text{g/mL}$ by $67.87 \pm 1.39\%$. The standard, AA exhibited strong IHL at 100 $\mu\text{g/mL}$ in comparison to the NC and all the test concentrations of MDA. The NC exhibited negligible %IHL (1.87 ± 0.58). Although, the MDA at 50 $\mu\text{g/mL}$ did not produce significant ($p < 0.05$) %IHL when compared to the 25 $\mu\text{g/mL}$ of it, but in comparison to the NC the effect was significant. The half-minimal inhibitory concentration (IC_{50}) calculated for MDA was $81.59 \pm 0.27 \mu\text{g/mL}$ within a confidence interval (CI) 53.33 – 124.80 $\mu\text{g/mL}$ ($R^2 = 0.96$).

Table 1. Hemolysis inhibition by the test sample and controls

Treatments (0.1 mL)		% IHL	IC_{50} [CI; R^2]
NC (DW)		1.87 ± 0.58	-
AA (100 $\mu\text{g/mL}$)		$78.89 \pm 1.08^{a,b,c,d,e,f}$	-
MDA	25 $\mu\text{g/mL}$	15.67 ± 1.21^a	81.59 ± 0.27 [53.33 – 124.80; 0.96]
	50 $\mu\text{g/mL}$	21.23 ± 1.31^a	
	100 $\mu\text{g/mL}$	$35.10 \pm 2.58^{a,b,c}$	
	200 $\mu\text{g/mL}$	$53.09 \pm 1.10^{a,b,c,d}$	
	400 $\mu\text{g/mL}$	$67.87 \pm 1.39^{a,b,c,d,e}$	

Values are mean $\pm$ standard deviation (SD) ($n = 5$); $p < 0.05$ when compared to the ^aNC, ^bMDA 25, ^cMDA 50, ^dMDA 100, ^eMDA 200 and ^fMDA 400; ANOVA followed by *t*-Student–Newman–Keuls’s as *post-hoc* test; IHL: Inhibition of hemolysis; AA: Ascorbic acid; MDA: Methanol extract of *Dysophylla auricularia*; NC: Negative control (distilled water, DW); IC_{50} : Half minimal inhibitory concentration; CI: Confidence interval; R^2 : Coefficient of determination.

The CL test was carried out with the highest concentration of MDA, observed in HL test. **Table 2** represents the treatment pattern of oxidizer, antioxidant and fibrinolytic agents. The highest CL ($83.57 \pm 2.78\%$) was observed in the co-treatment group of MDA + SK, which was then followed by SK, AA + SK, STR + MDA + SK, MDA, STR + MDA, STR + AA and STR by 78.08 ± 1.67 , 76.03 ± 1.93 , 74.56 ± 2.10 , 71.25 ± 1.29 , 51.01 ± 2.31 , 21.02 ± 1.56 and $11.21 \pm 1.23\%$, respectively. The NC produced negligible CL ($2.06 \pm 0.78\%$).

Table 2. Clotlysis capacity of the treatments

Groups	Concentration (0.1 mL)	%CL
NC	-	2.06 ± 0.78
SK	100 IU	$78.08 \pm 1.67^{a,b,c,d,e}$
MDA	400 $\mu\text{g/mL}$	$71.25 \pm 1.29^{a,c,d,e}$
STR	40 mM	11.21 ± 1.23^a
STR + MDA	40 mM + 400 $\mu\text{g/mL}$	$51.01 \pm 2.31^{a,c,e}$
STR + AA	40 mM + 100 $\mu\text{g/mL}$	$21.02 \pm 1.56^{a,c}$
MDA + SK	400 $\mu\text{g/mL}$ + 100 IU	$83.57 \pm 2.78^{a,b,c,d,e,f,g}$
AA + SK	100 $\mu\text{g/mL}$ + 100 IU	$76.03 \pm 1.93^{a,c,d,e}$
STR + MDA + SK	100 $\mu\text{g/mL}$ + 400 $\mu\text{g/mL}$ + 100 IU	$74.56 \pm 2.10^{a,c,d,e}$

Values are mean $\pm$ standard deviation (SD) (n = 5); p < 0.05 when compared to the ^aNC, ^bMDA, ^cSTR, ^d(STR + MDA), ^e(STR + AA), ^f(AA + SK) and ^g(STR + MDA + SK); ANOVA followed by t-Student–Newman–Keuls's as *post-hoc* test; IHL: Inhibition of hemolysis; AA: Ascorbic acid; CL: Clotlysis; MDA: Methanol extract of *Dysophylla auricularia*; NC: Negative control (distilled water, DW).

Discussion:

The H₂O₂ can also generate free hydroxyl radical ($\cdot$ OH). Both H₂O₂ and $\cdot$ OH then react with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes cellular destruction¹¹. This may be due to the ability of changing membrane permeability and potential, loss of cell inclusions, and damaging of cellular macromolecules. In the H₂O₂ (STR)-induced HL assay, MDA concentration-dependently inhibited HL in HRBC. Although, the activity was lower than the standard antioxidant, AA; but the effect should be considered significant (p < 0.05) when compared to the NC group. In a recent study, Nur et al⁷ also demonstrated a membrane stabilization effect of ethanol extract of *D. auricularia* (EDA) in HRBC, where EDA at 500 showed 79.66 $\pm$ 0.58% protestant capacities. Thus, the finding in this study is an agreement to the previous report.

The SK is a widely used fibrinolytic drug, which binds and hydrolytically activates human plasminogen. Both SK and MDA alone produced significant (p < 0.05) CL as compared to the NC. The activity of their co-treatment (MDA + SK) was accelerated and was found above their individual effect. In seems there may be a synergistic effect of MDA + SK. The STR also exhibited a significant (p < 0.05) CL in comparison to the NC. It may be due to its oxidative-stress-mediated damaging effect of HRBC, which was further confirmed by the co-treatment groups STR + MDA and STR + AA. Most reduction in CL was found in the STR + AA group in comparison to the STR + MDA. There may be a strong redox reaction between STR and AA. On the other hand, MDA continued CL after the neutralization of $\cdot$ OH coming from the STR. The co-treatment group AA + SK exhibited almost similar CL that of the SK, suggesting AA has no significant interference in the fibrinolytic activity of SK. Furthermore, a reduction in %CL with the co-treatment group STR + MDA + SK confirmed that MDA antagonizing the effect of STR, while SK continued CL in HRBC.

The overall findings in the CL assay report that MDA has STR suppressing effect, which was also seen by AA. The CL effect of MDA may not be via fibrinolytic pathway, as MDA did not interfere the activity of SK other than STR. By considering the HL test, and STR and STR + MDA treated groups in CL test, it can be said that MDA has antioxidant effects. Furthermore, in MDA + SK, when STR added it slightly decreased the %CL, which was found the reverse in the MDA + SK group. It seems, SK and MDA worked different ways. Antioxidants at high concentration may act as pro-oxidants¹². Thus in the HL test, in the presence of STR (H₂O₂) MDA after scavenging of $\cdot$ OH imparting a cytoprotective effect on HRBC which may link to its higher HL inhibition rate. In the CL test, the situation is altered, which can be easily confirmed by checking %CL in MDA, STR and MDA + STR treated groups.

CONCLUSION

The MDA can antagonize H₂O₂ activity and scavenged $\cdot$ OH. Both of these activities can be linked to its cytoprotective effect on HRBC. However, the CL effect of MDA may be due to its antioxidant-mediated pro-oxidative effect. Although, the

responsible phytochemicals should be found out yet, but the protocol can be used as a tool to screen thrombolytic activity of substances; especially those have antioxidant or pro-oxidant capacity.

Conflict of interest:

None declared.

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