Antithrombotic and anticoagulant activities of *Desmodium gyrans* (DC)

Vipin PS¹,²*, Johannah NM¹, Seema Menon¹, Lincy Lawrence¹ and Jose Padikkala¹

¹Department of Biochemistry, Amala Cancer Research Centre (Affiliated to University of Calicut), Amala Nagar, Thrissur, Kerala, India
²Department of Biochemistry Government Arts and Science College, (Affiliated to University of Calicut), Kozhinjampara, Palakkad, Kerala, India

**ABSTRACT**

Antithrombotic, anticoagulant properties of medicinally used *Desmodium gyrans* (DC) was studied using 70% methanolic extract. Clotting time, prothrombin time, plasma recalcification time and platelet aggregation studies were performed in plasma collected from *Desmodium gyrans* extract treated male Sprague Dawley rats. Inhibition of lipid peroxidation was assessed in vitro using platelet rich plasma sample. The treatment with extract gave a delay in coagulation by 14.6% in prothrombin time assay. Plasma recalcification time also was enhanced with treatment. Collagen induced platelet aggregation of platelet rich plasma was brought down significantly in dose dependent manner. On treatment lipid peroxidation was brought down by 29.94% compared to the control.

**INTRODUCTION**

The use of herbal drugs against various diseases is attracting considerable prominence these days. Various plants have been employed in Indian traditional clinical practice and as ingredients in Ayurveda and Unani preparations. There has been a recent upsurge in research in this area after scientific authentication that they are effective for the therapeutic conditions to which they have originally been employed[1].

Thrombosis is the pathological development of blood clots and these clots subsequently may break free and become mobile. Thrombosis typically occurs during myocardial infarction as a result of atherosclerotic plaque rupture[2]. Antithrombotic agent is any medication that prevents clot formation or dissolves already formed clots. They can be used therapeutically for primary and secondary prevention or treatment of acute thrombus [3]. Anticoagulant agents also prevent formation of internal clot in the vessels by affecting either the availability or activation of blood coagulation factors and platelets. Anticoagulants and antiplatelet agents are amongst the most commonly used medications that inhibit activation of clotting factors that prevent venous thrombosis [4].

*Desmodium gyrans* DC, found in Kerala forests belong to the family Fabaceae. *D. gyrans* is popularly used in traditional and folk medicine since its leaves have diuretic, febrifugal and tonic properties and roots are used in Indian medicine as a remedy for asthma, coughs, as antidysenteric and as emollient. It has got a remarkable wound healing effect also. But the main focus of the current study is based on the traditionally assumed cardioprotective effect of the plant [5]. *D. gyrans* has a long history of use in Chinese traditional medicine to treat various ailments[6]. Closely related species *Desmodium gangeticum* has proven cardio protective properties and give protection against cardiac reperfusion injury [7]. The presence of safe and bioactive natural antioxidants raises a substantial interest in deploying these medicinal plants in cardiovascular and other diseases[8].
A number of medicinal plants have been evaluated for their antithrombotic and anticoagulant properties and there have been some significant findings also, Careya arborea, Bauhinia forficata, Gloriosa superba and Jatropha curcas being a few among them [9,10]. The objective of the present study was to evaluate the antithrombotic and anticoagulant potential of methanolic extract of traditionally used medicinal plant Desmodium gangeticum.

EXPERIMENTAL SECTION

2.1 Plant material
Leaves of plant Desmodium gyrans were collected from Ayurvedic Garden maintained in the campus of Amala Institute of Medical Sciences, Thrissur, Kerala, India and were authenticated by Dr. N Sasidharan, scientist, NWFP Division, Kerala Forest Research Centre, Peichi, Kerala, India. A voucher specimen was deposited in the herbarium of Amala Cancer Research Centre (ACRH No.036).

2.2 Preparation of Plant Extract
The dried leaves were powdered and subjected to extraction with 70% methanol using a soxhlet apparatus. The methanolic extract of Desmodium gyrans (MDG) was filtered, concentrated and evaporated to dryness and the dried extract was dissolved again in distilled water was used for the study.

2.3 Animals
Male Sprague Dawley rats (200 gm) were obtained from the Small Animal Breeding Station (SABS), Veterinary and Agriculture University, Mannuthy, Kerala, India. The animals were kept under standardized environmental condition (22-30°C, 60-70% relative humidity, 12 hr dark/light cycle) and fed with standard rat feed (Kerala Feeds, Thrissur, India) and water ad libitum. Animal experiments conducted during the study had prior permission from Institutional Animal Ethics Committee (IAEC) and followed the guideline of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India.

2.4 Determination of total flavonoid content
Colorimetric aluminum chloride method was used for determination of total flavonoid content [11]. Exactly 0.5 ml solution of plant extract was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water, and kept at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm using spectrophotometer. Total flavonoids contents were calculated from a calibration curve using quercetin as standard. Quercetin solutions at serial concentrations 12.5, 25, 37.5, 50, 62.5, 75, 87.5 and 100 mg/ml in methanol were prepared. These standards were run using the same procedure and standard curve was prepared. Total flavonoid values are expressed in terms of quercetin equivalent.

2.5 Determination of total phenol content
Total phenolic compound contents were determined by Folin-Ciocalteau method [11]. Exactly 0.5 ml of extract was added to 5 ml pre diluted Folin-Ciocalteau reagent (1:10 diluted with distilled water), mixed for 5 min and then 4 ml aqueous Na2CO3 (1M) was added. The mixture was allowed to stand for 15 min and the phenols were determined colourimetrically at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200 mg/ml solutions of gallic acid in methanol and water solvent (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent.

2.6 Clotting Time
Clotting time is used as a screening test to monitor all phases of intrinsic coagulation system and to monitor heparin therapy[12]. The coagulation time of whole blood is the time required for blood to clot under normal standard conditions. Blood was collected from normal rat through the tail vein and was transferred directly to capillary tube. The end of the capillary tube was broken at regular intervals and clotting time was noted when a fibrin thread appears between broken pieces of capillary tubes. Time taken for clot to appear is noted. Clotting time was also assayed on rats injected with standard dose heparin injected through tail vein as well as on rats fed with MDG.

2.7 Study groups and sampling
The rats were divided into three groups, normal, standard and extract treated group consisting of 6 male Sprague Dawley rats in each group. The standard group received heparin standard drug injected to tail vein at a dose of 250 U/kg b.wt of the animal for one day. The extract treated group received MDG extract at a dose of 250 mg/kg b.wt of the animal for a continuous period of seven days.

2.8 Prothrombin Time (PTT)
The arrest of bleeding depends upon formation of primary platelet plug formed on site of injury followed by the formation of a stable fibrin network. Formation of this clot involves the sequential interaction of a series of plasma proteins and the interaction of these complexes with blood platelet and components released from tissues. Tissue thromboplastin, in presence of calcium initiates the extrinsic pathway of coagulation, which comprises plasma coagulation factors VII, X, V, Prothrombin and Fibrinogen[13]. During oral anticoagulant therapy specific blood coagulation factors are depressed.

Prothrombin time determination is the preferred method for screening and diagnosis of congenital deficiency of factors II, V, VII and X. PTT is also used for monitoring of patients on oral anticoagulant therapy and as a liver function test.

Plasma collected from normal male Sprague Dawley rat 0.1 ml is taken in a glass test tube and incubated at 37°C for 5 minutes. Liquiplastin 0.2 ml is mixed with plasma and time is noted instantly. Formation of a visually detectable solid plasma clot occurs inside glass tube within a specified period of time. The time required for clot formation is noted in seconds. The experiment is repeated using heparin treated standard plasma and plasma from extract treated group of animals. Tissue thromboplastin in the presence of calcium initiates the extrinsic pathway of coagulation mechanism. When liquiplastin reagent is added to normal plasma, the clotting mechanism is initiated. PTT would be prolonged if there is a deficiency or delay of blood coagulation factor activity in the extrinsic pathway of the coagulation mechanism.

2.9 Plasma Recalcification Time
Plasma recalcification time was calculated by the addition of M/100 CaCl₂ solution to the previously warmed plasma at 37°C. Platelet rich plasma (PRP) was prepared by centrifugation (1000rpm × 5 min) of blood collected from normal aspirin free blood bank donors. 400 µl of PRP was taken in tubes marked control, standard and extract treated, and were incubated for 1 minute at 37°C. To one of these tubes added 20 µl of saline followed by 200 µl of M/100 CaCl₂ and a stopwatch was started immediately. The time taken for formation of a firm plasma clot was noted. The experiment was repeated using 20 µl of heparin in standard tube and 20 µl of MDG in two concentrations of 100 µg and 150 µg in place of saline used in normal control. The procedure was continued to determine the respective plasma recalcification time. The values were noted in each of the tubes and were compared with the value of the normal control.

2.10 Platelet Aggregation Study - Collagen induced
Platelet rich plasma (PRP) was prepared by centrifugation (1000rpm × 5 min) of blood collected from normal aspirin free blood bank donors. Platelet aggregation can be done using specific agents to induce platelet aggregation or cause platelets to release endogenous ADP, or both. Platelet aggregation can be induced in vitro using thrombin, ADP, arachidonic acid, epinephrine or collagen and different mediators can be studied for their inhibition of platelet aggregation [14].

In glass cuvettes 0.45 ml of PRP was taken and incubated with 50 µl of saline. The cuvette was incubated at 37°C for 5 minutes without disturbing the content. Platelet aggregation was initiated by adding 1µg/ml of collagen. Aggregation was recorded for every minute continuously for 5 minutes using spectrophotometer at 340nm. Decreases in optical density were recorded and a graph was plotted against time taken in minutes. The procedure was repeated substituting 50 µl of MDG in place of saline, in two concentrations of 100 µg and 150 µg. The graph obtained using normal saline and plant extract as test material were compared.

2.11 Lipid Peroxidation
Malondialdehyde (MDA) produced during peroxidation can react with thiobarbituric acid (TBA) reagent to form a pink coloured product which has an absorption maximum at 532nm. The assay is calibrated with 1,1,3,3, tetramethoxypropane, which on hydrolysis produces malondialdehyde. The results are expressed in terms of the amount of malondialdehyde produced during the reaction.
PRP sample 0.1ml in Tris buffer was added to a reaction mixture containing KCl (0.1 ml), ascorbic acid (0.1ml), ferrous ammonium sulphate (0.1 ml) and Tris buffer (0.1 ml). Final volume is 0.5 ml. The reaction mixture was incubated for 1hr at 37°C. To 400µl of this reaction mixture added 0.2ml SDS, 1.5 ml of acetic acid and 1.5 ml TBA and incubated for 1 hour at 95°C. After incubation, the reaction mixture was cooled and added 1 ml distilled water. To this mixture 5 ml of butanol-pyridine mixture (15:1, v/v) was added, mixed thoroughly and centrifuged at 3000 rpm for 10 minutes. Absorbance of upper layer containing the chromophore was measured at 532 nm against pyridine butanol mixture. In control sample, 0.1ml of PRP sample (25%) in Tris buffer was added to a reaction mixture containing KCl (0.1 ml), ascorbic acid (0.1ml), ferrous ammonium sulphate (0.1 ml) and Tris buffer (0.05 ml). In addition 0.05 ml collagen is also added. In test solution 0.05 ml of drug extract was added in place of Tris buffer used in control protocol. All the procedures were done in triplicate. The amount of MDA formed was expressed as n mol/mg protein.

RESULTS AND DISCUSSION

3.1 Total flavonoid and total phenolic contents
The phytochemical analysis showed a total flavonoid 70.5±2.0 mg quercetin equivalent /g of extract and total phenol 41.5±2.29 mg gallic acid equivalent /g of extract

3.2 Clotting Time
Heparin treated standard group of rats gave a significant delay in blood clotting in clotting experiment with an average increase of clotting time by 0.46 minutes when compared to the untreated group of rats (Figure 1). But clotting time of MDG treated rats showed only a mild delay in clotting time of 0.08 minutes which is only 1.35% increase and statistically insignificant.

Table 1: Effect of MDG on clotting time

<table>
<thead>
<tr>
<th>Group</th>
<th>Clotting time (Minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6.0 ± 0.05</td>
</tr>
<tr>
<td>Standard</td>
<td>6.47 ± 0.1</td>
</tr>
<tr>
<td>MDG</td>
<td>6.09 ± 0.05</td>
</tr>
</tbody>
</table>

Fig. 1: Effect of MDG on Clotting Time

Values are expressed as mean ± SD for 5 animals: a = p<0.01, b = p<0.05 when compared to normal.

3.3 Prothrombin Time
The standard group of animals treated with heparin showed a delay in coagulation by 28% when compared to the normal group of animals (Figure 2). The treatment with MDG gave a delay in coagulation by 14.6% which is a significant elevation in comparison to the normal group.

Table 2: Effect of MDG on prothrombin time

<table>
<thead>
<tr>
<th>Group</th>
<th>Prothrombin time(seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>15 ± 0.5</td>
</tr>
<tr>
<td>Standard</td>
<td>19.2 ± 0.5</td>
</tr>
<tr>
<td>MDG</td>
<td>17.6 ± 0.5</td>
</tr>
</tbody>
</table>

Fig. 2: Effect of MDG on Prothrombin Time

Values are expressed as mean ± SD for 5 animals: a = p<0.01, b = p<0.05 when compared to normal.
3.4 Plasma recalcification time
Normal plasma recalcification time of PRP was 3.15 minutes. The standard tubes in which heparin were used as the standard substance showed no clotting of plasma even after a prolonged time period. The tubes in which different concentrations of MDG (100µg and 150µg) were added showed a delay of 0.15 minutes and 0.18 minutes respectively when compared to the normal (Figure 3).

Table 3: Effect of MDG on plasma recalcification time

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma recalcification time (Minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.15±0.05</td>
</tr>
<tr>
<td>Standard Heparin</td>
<td>No coagulation</td>
</tr>
<tr>
<td>MDG (100µg)</td>
<td>3.29 ±0.05</td>
</tr>
<tr>
<td>MDG (150µg)</td>
<td>3.33 ±0.05</td>
</tr>
</tbody>
</table>

Fig. 3: Effect of MDG on Plasma recalcification Time
Values are expressed as mean ± SD for 5 samples: a = p<0.01, b = p<0.05 when compared to normal

3.5 Platelet aggregation study
From the graph of platelet aggregation study, it can be seen that there was marked aggregation of PRP with usage of collagen, which was ameliorated in dose dependent manner up on treatment with MDG in two different concentrations of 100µg and 150µg (Figure 4). Collagen induced aggregation of PRP was brought down significantly as is indicated by the change in the optical densities.

Table 4: Effect of MDG on platelet aggregation study

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>Optical density</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Collagen</td>
<td>Collagen + MDG (100 µg)</td>
<td>Collagen + MDG (150 µg)</td>
</tr>
<tr>
<td>0</td>
<td>0.514</td>
<td>0.525</td>
<td>0.535</td>
</tr>
<tr>
<td>1</td>
<td>0.501</td>
<td>0.512</td>
<td>0.524</td>
</tr>
<tr>
<td>2</td>
<td>0.486</td>
<td>0.505</td>
<td>0.513</td>
</tr>
<tr>
<td>3</td>
<td>0.471</td>
<td>0.494</td>
<td>0.509</td>
</tr>
<tr>
<td>4</td>
<td>0.416</td>
<td>0.477</td>
<td>0.489</td>
</tr>
<tr>
<td>5</td>
<td>0.411</td>
<td>0.446</td>
<td>0.463</td>
</tr>
</tbody>
</table>

Fig. 4: Effect of MDG on Platelet aggregation study

3.6 Lipid peroxidation
The value of lipid peroxidation was increased by 78.19% in the control, which is highly significant in comparison to the normal in the experiment (Figure 5). On treatment with MDG, lipid peroxidation was brought down by 29.94% when compared to the control.

<table>
<thead>
<tr>
<th>Group</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal: Platelet alone</td>
<td>0.8155 ± 0.08</td>
</tr>
<tr>
<td>Control: Platelet + collagen</td>
<td>3.7393 ± 0.15</td>
</tr>
<tr>
<td>Test: Platelet + MDG + collagen</td>
<td>2.6207± 0.08</td>
</tr>
</tbody>
</table>

Table 5: Effect of MDG on lipid peroxidation

Values are expressed as mean ± SD for 5 samples: a = p<0.01, b = p<0.05 when compared to normal; c = p<0.01, d = p<0.05 compared to control.

The results of in vitro antiplatelet aggregation study revealed the property of MDG to reduce clumping of platelets in platelet rich plasma. Some of the proven antiplatelet aggregation agents like aspirin are having an effect of even irreversibly reducing platelet count in the blood. But unlike aspirin, MDG is not having such a property as assessed from the platelet count analysis done during the study. The property to act as antiplatelet aggregation agent may be due anyone of the mode of action of antiplatelet aggregation pathways as described in Figure 6. The mode of action can be classified into four types, namely COX-I inhibition, ADP antagonistic action, phosphodiesterase inhibition or GP IIb/IIIa antagonistic action[15].

One of the popularly used drugs namely Aspirin in low dose induces reversible inactivation of a key enzyme in platelet arachidonate metabolism through acetylation of a critical serine residue near its catalytic site. This key enzyme, cyclooxygenase (COX)-1, is responsible for the formation of prostaglandin (PG) H₂, the precursor of thromboxane (TX)A₂. Thromboxane (TX)A₂ is directly involved in platelet activation and aggregation functions.

Inhibition of adenosine diphosphate (ADP)-dependent platelet function by irreversible modification of the platelet P2Y₁₂ receptor through short-lived active metabolites, generated by liver cytochrome P-450 (CYP) isozymes is another mechanism under examination. These metabolites are found to form covalent bonds with critical cysteine residues within the receptor which inhibit ADP recognition of the receptor thus effectively preventing platelet aggregation.

Phosphodiesterase inhibitors can acts as vasodilators and antiplatelet agents. It inhibits adenosine uptake and cyclic GMP phosphodiesterase activity, which decreases platelet aggregation process.

Glycoprotein IIb/IIIa (also known as integrin αIIbβ₃) is an integrin complex found on platelets. It is a receptor for fibrinogen and von Willebrand factor and assists in platelet activation. The complex is formed through calcium-dependent association of GPIIb and GPIIIa, a required step in normal platelet aggregation and endothelial adherence. GPIIb/IIIa antagonists prevent fibrinogen attachment to activated GPIIb/IIIa receptors and, thus,
formation of fibrinogen bridges between platelets. Activation of GPIIb/IIIa constitutes the final common pathway of platelet aggregation. Studies are going on regarding GPIIb/IIIa blockers.

Phytochemical screening of extract of Desmodium gyrans revealed the presence of phenolic compounds and flavonoids. These secondary metabolites are known to have various biological activities of which can be credited to the medicinal properties of Desmodium gyrans. Although a study on the exact mode of action is still has to be done, various mechanisms as illustrated can be attributed as the possible mechanism by which MDG brings about inhibition of platelet aggregation as revealed in the present study.

Anticoagulant action of Desmodium gyrans was verified through clotting time and plasma recalcification experiments. Clotting of blood is delayed by a number of agents in blood which are having different mode of actions. Most of the agents depend on the inhibition of coagulation factors by one method or the other. Activation of antithrombin III is the mechanism of action of heparin and other anticoagulant drugs like Dalteparin, Lepirudin, Enoxaparin and Fondaparinux[16,17]. Antithrombin III (ATIII) is a 432 amino acid glycoprotein produced by the liver that inactivates several enzymes of the coagulation system. It contains three disulfide bonds and a total of four potential glycosylation sites. α-Antithrombin is the principal form of antithrombin found in blood plasma and has an oligosaccharide occupying each of its four glycosylation sites. A single glycosylation site remains consistently free in the minor form of antithrombin namely β-antithrombin. Its activity is amplified many times by heparin, which enhances the binding of antithrombin to factor II and factor X[18].

Another regularly used anticoagulant agent namely Warfarin inhibits vitamin K reductase, resulting in exhaustion of the reduced form of vitamin K (vitamin KH2). Since vitamin K is a cofactor for carboxylation of glutamate residues on the N-terminal regions of vitamin K-dependent proteins, this limits the gamma-carboxylation and subsequent activation of the vitamin K-dependent coagulant proteins [19,20]. The synthesis of vitamin K-dependent coagulation factors II, VII, IX, and X and anticoagulant proteins C and S are inhibited. Depression of three of the four vitamin K-dependent coagulation factors (factors II, VII, and X) results in decreased prothrombin levels and a reduced amount of generated thrombin. This reduces the thrombogenicity of clots [21]. Anticoagulant action of Desmodium gyrans can be anyone of these modes and opens up scope for further study on its mechanism of action.

CONCLUSION

Despite recent advances, there is still scope for safe, oral anticoagulants for both short term and long term therapeutic purposes. As observed, the normal coagulation function of the different blood coagulation factors can be influenced through diverse routes which have to be probed for demonstrating the role of Desmodium gyrans as an effective anticoagulant and antithrombotic agent.

REFERENCES


