CHAPTER 6
ANTITHROMBOTIC EFFECT OF PROTEOGLYCANS AND SOME PLANT EXTRACTS
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Introduction

Intra vascular thrombosis is known to be the final catastrophic event in atherosclerosis (96). It occur immediately after the rupture of atheromatous plaque. Experimental and clinical evidences have established the role of platelets, as a central participant in the final thrombotic event (139). Further, the sequential activation of series of serine proteinases which culminate in the generation of thrombin and subsequent thrombin catalysed conversion of fibrinogen into insoluble fibrin (220) also play a major role in thrombosis.

Thus anticoagulant and antiplatelet therapy in atherosclerosis is gaining much importance as it limit arterial thrombosis. Aspirin which is a cyclo-oxygenase inhibitor has been tried with good results (221). As fibrin is an important component of both venous and arterial thrombi anticoagulant drugs which reduce the formation fibrin should be effective in prevention and treatment. Evidences are however increasing that anticoagulant drugs are indeed effective in preventing thromboembolism.

Many plant products have been reported to have antithrombotic potential. Ajorn (172), Resveratrol (171) etc are known to inhibit platelet aggregation mediated by the inhibition of cyclo-oxygenase pathway in platelets. It is generally aggred that most antioxidant and anti-inflammatory agents are also posses antithrombotic effect.

In the present study we investigate the anticoagulant and antiplatelet effect of proteoglycans as well as A. galanga, K. galanga and R. damascena extracts. A.
*galanga* and *K. galanga* are known anti-inflammatory agents (191, 192). Our previous study has revealed their anti-oxidant efficacy in vitro. *R. damascena* has also showed antioxidant and anti-inflammatory activity in our studies. Thus the study of these plant extracts on their anticoagulant and anti platelet properties will be beneficial in the atherosclerotic disease pathology.

**Materials and methods**

*(Chapter 2.1.11, 2.1.12, 2.13)*

**Anticoagulant activity**

Anticoagulant effect was studied by the activated prothrombin time (APTT) method and plasma recalcification method under slow centrifugation. For this 1 mg / kg body wt. of uronic acid equivalent of each proteoglycan fractions were individually given intra venously to rabbit through ear vein. 70% ethanol extract of *A. galanga* and *K. galanga* were tried in two doses i.e. 5 and 10 mg / kg body wt. *Rosa damascena* acetone fraction was given at 10 and 15 mg / kg body wt. through the same route. Heparin which is known anticoagulant is used as reference standard.

4.5 ml blood was drawn to test tubes containing 0.5 ml M/10 sodium oxallate solution. Plasma was collected by centrifugation at 1500 rpm for 5 minutes in a remi centrifuge.

**Plasma recalcification method**

Plasma recalcification time was calculated by the addition of M/100 CaCl₂ to the plasma warmed previously at 37°C as described in the materials and method section (2.1.11a).
Activated prothrombin time method

APTT was calculated followed by the addition of commercial thromboplastin reagent to the prewarmed plasma at 37°C (chapter II 2.1.1b).

Platelet aggregation studies in vitro

Aggregation of platelet rich plasma with ADP as agonist was studied in a Lumi aggregometer as per the method described in chapter II (2.1.12). Each proteoglycan fraction as well as plant extracts were tested and compared to that of heparin, which was the reference standard.

Study on platelet arachidonate path way in vitro

The Malondialdehyde produced in the cyclooxygenase pathway in platelets by the induction of Diethylmaleimide was studied in vitro (chapter II 2.1.13). Efficacy of plant extracts as well as proteoglycan fraction were evaluated in comparison with aspirin which is a known inhibitor of cyclo-oxygenase pathway in platelets.

Results

The anticoagulant activity of proteoglycan fraction was given in table 6-1. The normal time of Rabbit blood for recalcification was found to be 47 ± 4.4 minute and the activated prothrombin time was 12 ± 3.1 minute. Administration of heparin delayed the time to 139 ± 3.2 minute and 76 ± 1.4 minute respectively. Among the 3 proteoglycans studied PGA treated blood plasma coagulated with in 105 ± 4.6 sec. after the addition of CaCl₂ and in 49 ± 1.5 sec. after the addition of thromboplastin. The recalcification time of PGB treated blood plasma was 82 ± 3.7 sec. and that of PGC treated blood plasma was 57 ± 3.4 sec. the APTT for PGB was 25 ± 2.3 sec.
### Table 6-1. Anticoagulant activity of proleoglycans

<table>
<thead>
<tr>
<th>Treated group</th>
<th>Activated prothrombin time in seconds</th>
<th>Plasma recalcification time in seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rabbit blood</td>
<td>12 ± 3.1</td>
<td>47 ± 4.4</td>
</tr>
<tr>
<td>Heparin treated blood</td>
<td>76 ± 1.4</td>
<td>139 ± 3.2</td>
</tr>
<tr>
<td>PG fraction 'A' treated blood</td>
<td>49 ± 1.5</td>
<td>105 ± 4.6</td>
</tr>
<tr>
<td>PG fraction 'B' treated blood</td>
<td>25 ± 2.3</td>
<td>82 ± 3.7</td>
</tr>
<tr>
<td>PG fraction 'C' treated blood</td>
<td>17 ± 2.6</td>
<td>57 ± 3.4</td>
</tr>
</tbody>
</table>

Values are average ± SD of triplicate experiments (triplicate tubes)
Table 6-II. Anticoagulant activity of plant extracts

<table>
<thead>
<tr>
<th>Treated group</th>
<th>Activated prothrombin time in seconds</th>
<th>Plasma recalcification time in seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal blood</td>
<td>15 ± 2.2</td>
<td>43 ± 2.14</td>
</tr>
<tr>
<td>Heparin treated blood</td>
<td>79 ± 2.18</td>
<td>142 ± 4.32</td>
</tr>
<tr>
<td>A. galanga 70% ethanol extract 5 mg / kg body wt treated blood</td>
<td>14 ± 1.3</td>
<td>46 ± 2.4</td>
</tr>
<tr>
<td>A. galanga 70% ethanol extract 10 mg / kg body wt treated blood</td>
<td>16 ± 2.14</td>
<td>42 ± 2.3</td>
</tr>
<tr>
<td>K. galanga 70% ethanol extract 5 mg / kg body wt treated blood</td>
<td>18 ± 2.2</td>
<td>43 ± 1.31</td>
</tr>
<tr>
<td>K. galanga 70% ethanol extract 10 mg / kg body wt treated blood</td>
<td>15 ± 1.7</td>
<td>43 ± 1.5</td>
</tr>
<tr>
<td>R. damascena acetone fraction 10 mg / kg treated blood</td>
<td>52 ± 4.17</td>
<td>92 ± 3.12</td>
</tr>
<tr>
<td>R. damascena acetone fraction 25 mg / kg treated blood</td>
<td>51 ± 2.12</td>
<td>97 ± 4.32</td>
</tr>
</tbody>
</table>

Values are average ± SD of triplicate experiments (triplicate tubes)
and that of PG ‘C’ was 17 ± 2.6 sec.

Data given in the table 6 II shows the anticoagulant effect A. galanga, K. galanga and R. damascena. The plasma recalcification time in normal group was 43 ± 2.14 sec. and APTT was 15 ± 2.2 sec. The time was delayed in Heparin treated group to 142 ± 4.32 and 79 ± 2.18 respectively. Administration of A. galanga and K. galanga could not delay the coagulation time. Administration of Rosa damascena AF was found to have significant effect on coagulation as it delayed plasma recalcification time to 92 ± 3.12 and 97 ± 4.32 sec. by 10 mg / kg wt and 50 mg / kg body wt respectively. In APTT study the time delay was 52 ± 4.17 and 51 ± 2.12 sec. by 10 and 50 mg / kg wt of AF respectively. The anticoagulant activity was not dose dependent.

Anti platelet aggregatory activity

The extent aggregation (% transmission) of control (curve 1 of Fig. 6-II) was 40% while proteoglycan ‘A’ with 25 µg, the % transmission (curve 2&3) was higher than that of control. With higher concentration the extent of aggregation reduced depending up on the dose (curve 4, 5 and 6). On the other hand with the addition of heparin (Fig. 6-I) there was no acceleration of aggregation compared to control and the inhibition was not dose dependent. From 50 µg to 125 µg concentration of (curve 3,4,5 and 6) heparin added, the % transmission was constant. Addition of PG ‘C’ had no significant effect on platelet aggregation (curve 2 and 3 of Fig 6-III) whereas PG ‘B’ significantly inhibited the extent of aggregation at higher concentration only (curve 4, 5 and 6 of Fig 6-III).

Extent of aggregation was found unchanged by the addition of A. galanga
Fig 6-I: Effect of heparin on ADP induced platelet aggregation

Curve 1 – Control
Curve 2 – 25 µg
Curve 3 – 50 µg
Curve 4 – 75 µg
Curve 5 – 100 µg
Curve 6 – 125 µg
Fig 6-II: Effect of PG 'A' on ADP induced platelet aggregation

Curve 1 – Control  Curve 4 – 75 μg
Curve 2 – 25 μg     Curve 5 – 100 μg
Curve 3 – 50 μg     Curve 6 – 150 μg
Fig 6-III: Effect of Proteoglycan 'B' and 'C' fractions on ADP induced platelet aggregation

Curve 1 - Control
Curve 2 - 100 µg PG 'C'
Curve 3 - 150 µg PG 'C'
Curve 4 - 100 µg PG 'B'
Curve 5 - 125 µg PG 'B'
Curve 6 - 150 µg PG 'B'
Fig 6-IV: Effect of *A. galanga* and *K. galanga* extracts on ADP induced platelet aggregation

Curve 1 – Control
Curve 2 – 100 µg *A. galanga*
Curve 3 – 100 µg *K. galanga*
Fig 6-V: Effect of AF of *Rosa damascena* on ADP induced platelet aggregation

Curve 1 – Control
Curve 2 – 10 μg
Curve 3 – 20 μg
Curve 4 – 30 μg
Curve 5 – 40 μg
Curve 6 – 50 μg
even at 100 mg concentration (Fig 6 IV, curve 2) compared to control curve (curve 1). On the other hand addition of *K. galanga* accelerated the extent of aggregation.

Addition *R. damascena* AF was found to inhibit the ADP induced platelet aggregation (Fig 6 V). The extent of aggregation of control curve was 60% (curve 1). While addition of 10 µg to 50 µg of AF inhibited the extent of aggregation in a dose dependent manner (curve 2-5).

Effect on arachidonic acid metabolism

Study was conducted to find out whether the anti platelet effect of AF was due to the inhibition of arachidonate metabolism. In the *in vitro* assay system M.D.A formation in platelets was found to be inhibited by the addition AF. The concentration needed for 50% inhibition was 130 µg / ml. Compared to aspirin, a known inhibitor, AF was found less effective (Fig. 6 VI).

Discussion

Anticoagulant therapy is the only mode of effective treatment for intravascular thrombosis. Heparin has been a choice for decades, but the bleeding and other complications encountered in heparin therapy prevented its long term use. Aspirin, another anticoagulant and anti platelet aggregating agent, is a well known clinically accepted anti thrombotic drug which is used widely in thrombotic disorders. It has also complications associated with its side effect. Thus anticoagulant and anti platelet aggregating agents are well warranted to decrease the severity the thromboembolic disorders.

In the present study proteoglycan fraction ‘A’ has shown to possess potent anti coagulant property *in vivo*. It delays plasma recalcification time to 105 ± 4.6 sec.
Figure 6.VI: Effect of *R. damascena* AF on MDA formation in Platelet Arachidonate pathway
and activated prothrombin time to $49 \pm 1.5$ sec. when compared to normal blood. The efficacy is significant but less, compared to heparin, which delay plasma recalcification time of to $139 \pm 3.2$ sec. and APTT to $76 \pm 1.4$ sec. when considering the clinical complications of heparin PG 'A' seems advantage over heparin. Since PG 'A' is a relative compound of heparin the mode of their action may be the same.

More over in vitro studies on ADP induced platelet aggregation has revealed anti platelet aggregatory effect of this proteoglycan fraction. Though its lower concentrations shows some accelerating effect, higher concentration effectively inhibit platelet aggregation in a dose dependent manner. Heparin on the other hand inhibit platelet aggregation irrespective of dose. Higher concentrations of heparin decrease the percent transmission more or less in a similar fashion. Thus it can be assumed that PG 'A' is much more effective than heparin as an anti platelet aggregatory agent.

Among the plant extracts studied, AF of *Rosa damascena* shows significant anticoagulant activity in vivo. Compared to heparin it has less effect. In vitro AF inhibit ADP induced platelet aggregation in a dose dependent manner at 50 μg concentration AF produce 80% inhibition. The effect is far better than that of heparin. Further AF inhibit MDA formation in platelets. The IC 50 value for AF to inhibit MDA formation is 130 μg / ml. Compared to aspirin (IC 50; 25 μg / ml) AF is less active. MDA is believed to be formed in the cyclo-oxygenase pathway in platelets. Hence AF can be thought to inhibit cyclo-oxygenase pathway in platelets. Since AF, has shown to posses antioxidant and anti-inflammatory effect in our earlier studies its antithrombotic potential seems to be similar to the aspirin. Which is a known antioxidant, and antiinflammatory antiplatelet aggregatory agent.
Thus from the overall studies anticoagulant and anti platelet aggregating potential of PG 'A' and AF of *Rosa damascena* have been revealed. This may be beneficial in atherosclerotic cerebrovascular disease process and other thromboembolic disorders.