IN VITRO AND IN VIVO ANTICOAGULANT ACTIVITY OF IMPERATA CYLINDRICA A NOVEL ANTICOAGULANT LEAD FROM NATURAL ORIGIN.

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ABSTRACT:
Anticoagulant monotherapy has been the basis of treatment for patients with atrial fibrillation, left ventricular thrombus, as well as for prevention or treatment of deep venous thrombosis and pulmonary embolism. Considering the adverse effects with existing anticoagulant therapy, alternative drugs from natural origin can help to get the new molecular lead as a hope towards the better efficacious and safe anticoagulant. The present investigation was planned to evaluate the in vitro and in vivo anticoagulant activity of Imperata cylindrica (IC). Methanolic extract of IC has demonstrated presence of tannins and polyphenols. It can be concluded from the present investigation that IC has exhibited significant anticoagulant activity in vivo and in vitro. Oral administration of IC after 1, 2 and 3rd hr have exhibited 4, 6 and 9 fold increase in prothrombin time when compared to base value and 6, 7 and 9 fold increase in prothrombin time at 10th, 30 and 60th min after i.v. administration when compared to base value. IC may be acting on the extrinsic cascade of clotting probably by binding with the antithrombin. IC could be a hope towards development of a novel anticoagulant with optimized efficacy and reduced side effects.

Keywords: anticoagulant, Imperata cylindrica, prothrombin time, ventricular thrombus
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INTRODUCTION
Anticoagulant monotherapy has been the basis of treatment for patients with atrial fibrillation, prosthetic heart valves, markedly reduced left ventricular function or left ventricular thrombus, as well as for prevention or treatment of deep venous thrombosis and pulmonary embolism (1).

Antiplatelet therapy is the cornerstone for both primary and secondary prevention therapies for ischemic events resulting from coronary atherosclerotic disease. Dual antiplatelet therapy (aspirin plus a thienopyridine, usually clopidogrel) has assumed a central role in the treatment of acute coronary syndromes and after coronary stent deployment. In addition to antiplatelet therapy, anticoagulant therapy might be indicated for stroke prevention in a variety of conditions that include atrial fibrillation, profound left ventricular dysfunction and after mechanical prosthetic heart valve replacement. For this reason, the use of triple antithrombotic therapy (a dual antiplatelet regimen plus anticoagulant) is expected to become more prominent, given an aging patient population (2).

The clinical use of glycosaminoglycans and particularly heparin is associated with various side effects such as bleeding tendency and induced thrombocytopenia. The extensive use of oral anticoagulant therapy has provided more in-depth knowledge of the associated adverse reactions and the potential risks (3).

However, non-haemorrhagic adverse reactions may also play a considerable role in both temporary and continuative therapy (4). Non haemorrhagic side effects include four principal types of adverse reactions (5, 6):

(a) Ecchymosis and purpura appearing in subjects with skin fragility (i.e. elderly or subjects under long-term
steroid therapy), or by an excessive anticoagulant effect.
(b) Maculo-papular, vesiculose, or urticating rashes which are extremely itchy.
(c) ‘Purple toes syndrome’, a rare syndrome.
(d) Skin necrosis, the most serious non-haemorrhagic side effects.

Insulin resistance (IR), which occurs in type 2 diabetes, appears to be a common precursor of both diabetes and macro vascular disease. Metabolic disturbances that commonly occur in patients with IR are atherogenic dyslipidemia, hypertension, glucose intolerance and a prothrombotic state. The prothrombotic state is characterized by increased fibrinogen levels, increased plasminogen activator inhibitor (PAI)-1 and different abnormalities in platelet function. Thrombosis thus gets promoted and thrombolysis is being retarded (7).

Considering the place of the anticoagulant therapy in the cardiovascular disorders and the adverse effects with existing anticoagulant therapy, alternative drugs from natural origin can help to get the new molecular lead as a hope towards the better efficacious and safe anticoagulant agent. Charak Sidhisthan in Charak Samhita mentions the reference of *Imperata cylindrica* (IC) commonly called as “Darbha” in 6th chapter and Shloka no. 83. The shloka states that, IC is being used while administering the blood enema which is administration of whole blood through rectum. The drug is mixed with the blood prior to administration explains the anticoagulant properties of the drug. The drug may have potential anticoagulant activity in vitro as well as in vivo. The present investigation was planned to evaluate the in vitro and in vivo anticoagulant activity of *Imperata cylindrica* (IC).

**MATERIAL**

**Plant Material**

The whole plant is collected from Raigurunagar, Pune. The crude material was shade dried and powdered in pulveriser.

**METHODS**

**Preparation of methanolic extract of imperata cylindrica (mic)**

The whole plant of *Imperata cylindrica* was shade-dried and powdered. The powdered material (100 g) was subjected to soxhlet extractor using methanol as a solvent. The extract was suspended in 1% CMC and used for pharmacological studies.

**Acute oral toxicity studies**

Adult albino mice of either sex were subjected to acute toxicity studies as per guideline (425) suggested by Organization for Economic Co-operation and Development (8). The mice were observed for 2 h for behavioral, neurological and autonomic profiles and for any lethality during next 48 h.

**Phytochemical screening**

Phytochemical screening of IC was performed for assessing the presence of tannins, flavonoids, saponins etc. as a qualitative testing (9).

**Total phenolic compound analysis (10, 11)**

The amount of total phenolics in MIC was determined with the Folin-Ciocalteu reagent. To 50 ml of each sample (three replicates), 2.5 ml 1/10 dilution of Folin-Ciocalteau’s reagent and 2 ml of Na2CO3 (7.5%, w/v) were added and incubated at 45°C for 15 min. The absorbance of all samples was measured at 765. Results were expressed as percentage of gallic acid.

**Total flavonoid content (12)**

The total flavonoid content was determined using the Dowd method. 5 mL of 2% aluminium trichloride (AlCl₃) in methanol was mixed with the same volume of the extract solution (0.4 mg/mL). Absorption readings at 415 nm after 10 minutes against a blank sample consisting of a 5 mL MIC solution with 5 mL methanol without AlCl₃. The total flavonoid content was determined using a standard curve with quercetin (0–100 mg/L) as the standard. Total flavonoid content is expressed as percentage of quercetin equivalents (QE).

**HPTLC fingerprinting study**

The test sample of IC was spotted in the form of band of width 6 mm with CAMAG µL syringe on precoated silica gel aluminium plate 60F₂₅₄ (10 cm × 10 cm with 0.2 mm thickness E. Merck, Germany) using CAMAG Linomat 5 applicator (Switzerland) fitted with a 100 µL syringe. The Linear ascending development was carried out in solvent system (20 mL) toluene: ethyl acetate: formic acid (5:4:0.5 v/v/v) in a glass twin through chamber (10×10 cm) previously saturated with mobile phase for 30 min. The HPTLC plate was allowed to run up to 80 mm from the point of application. HPTLC plate was dried in hot air oven at 60°C. Densitometric scanning was performed using CAMAG TLC scanner 3 in the absorbance mode at 280 nm and operated by winCATS software (V 1.4.3.6336).
The slit dimension was 5×0.45 mm with the scanning speed of 20 mm s⁻¹. Evaluation was done via peak area with linear regression.

**Pharmacological screening (13)**

**In vitro anticoagulant activity**

IC was tested for anticoagulant activity in human plasma. Blood was placed in 3.8% sodium citrate. Different concentrations of MIC were prepared in normal saline and Prothrombin time (PT) is being measured. 1–10 IU of heparin per 100 μL of human citrated plasma were employed as standard.

**In vivo anticoagulant activity**

**Oral route of administration**

Albino rats of either sex weighing 150–200g were used for the study. MIC was administered in doses of 100, 200 and 400 mg/ kg, p.o.

**Intravenous route of administration**

Albino rats of either sex weighing 150–200g were used for the study. Animals were anaesthetized and MIC in normal saline is introduced in femoral vein and the jugular vein is canulated to get the blood withdrawn. MIC was administered intravenously in the dose of 3, 10 and 30 mg/ kg. The blood was withdrawn at different intervals and PT was measured.

**RESULTS**

**Preparation of methanolic extract of Imperata cylindrica (IC)**

The yield of the yellowish green colored extract was found to be 3% w/w.

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**Table 1: Retention factor of peaks from fingerprinting of IC**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Peak No.</th>
<th>Retention factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.20</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.27</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0.37</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.50</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0.57</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>0.62</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>0.66</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>0.72</td>
</tr>
</tbody>
</table>

**Figure 1: HPTLC Figure printing of methanolic extract of IC**
In Vitro and In Vivo Anticoagulant Activity Of Imperata Cylindrica A Novel Anticoagulant Lead From Natural Origin.

Figure 2: HPTLC Figure printing of gallic acid

Figure 3: In vitro anticoagulant activity of IC
Values are expressed as Mean, analysed by ANOVA followed by Dunnett test, *p<0.05, **p<0.001
**Phytochemical screening:**

The preliminary phytochemical investigation of MIC demonstrated the presence of tannins and flavonoids.

Total phenolic content of MIC was found to be 30% GAE w/w.

Total flavonoidal content of MIC was found to be 0.7% QE w/w.

**HPTLC fingerprinting study**

The HPTLC fingerprints of MIC showed 8 well resolved peaks (Fig. 1) along with gallic acid (Fig. 2) having $R_f$ 0.37 (Table 1).

**Pharmacological screening**

**In vitro anticoagulant activity**

A concentration dependent increase in prothrombin time (PT) was observed after addition of MIC to citrated human plasma. Different concentrations of MIC 50, 100 and 150 μg of MIC were able to produce significant (p<0.05) prolongation in the prothrombin time as 30.21, 49.05 and 92 sec. respectively when compared to basic value as 13.87 sec (Fig. 3).

To determine the effect of IC on coagulation of blood after oral administration, the baseline blood was collected. Animals (n=5) were divided into different groups of 100, 200 and 400 mg/kg of MIC orally. Blood samples were collected from 1, 2 and 3rd hr of drug administration and PT was calculated. The results indicated that the onset of action of MIC was before 1st hr of drug treatment which produced 4 fold increase in PT compared to base reading. MIC had significantly (p< 0.05) prolonged the PT at 1, 2 and 3rd hr and exhibited 6 and 9 fold increase in PT when compared to base value. It can be concluded from the results that the duration of the action of MIC would be greater than 3 hr after oral administration (Table 2).

**Intra venous route of administration**

MIC has demonstrated significant anticoagulant activity when given intravenously in the dose of 3, 10 and 30 mg/kg, i.v. MIC exhibited 6 fold increase in PT when compared to base value at 10th min of drug administration. There was 7 and 9 fold increase in PT compared to base value at 30th and 60th min after administration of the drug which shows analogy with the 2nd and 3rd hr results after oral administration of MIC (Table 3).

**DISCUSSION:**

Chromatographic fingerprinting has been suggested to check the authenticity or provide quality control of herbal medicine. Chromatography has the advantages.

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**Table 2: In vivo anticoagulant activity of IC Oral route of administration**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>1st hr. Prothrombin Time (sec.)</th>
<th>2nd hr. Prothrombin Time (sec.)</th>
<th>3rd hr. Prothrombin Time (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1% CMC 1ml/kg, p.o.</td>
<td>13.29 ± 0.61</td>
<td>15.28 ± 0.52</td>
<td>13.87 ± 0.81</td>
</tr>
<tr>
<td>I</td>
<td>MIC 100 mg/kg, p.o.</td>
<td>41.33 ± 2.18**</td>
<td>60.34 ± 4.18**</td>
<td>96.10 ± 7.11**</td>
</tr>
<tr>
<td>II</td>
<td>MIC 200 mg/kg, p.o.</td>
<td>50.37 ± 3.91**</td>
<td>60.60 ± 4.11**</td>
<td>135.31 ± 9.01**</td>
</tr>
<tr>
<td>III</td>
<td>MIC 400 mg/kg, p.o.</td>
<td>60.23 ± 3.10**</td>
<td>95.16 ± 5.10**</td>
<td>140.12 ± 11.09**</td>
</tr>
</tbody>
</table>

Values are expressed as Mean, analysed by ANOVA followed by Dunnett test,

* p<0.05,
** p<0.001

**Table 3 Intra venous route of administration**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Prothrombin Time (Sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 min</td>
</tr>
<tr>
<td>Control</td>
<td>Saline 1ml/kg, i.v.</td>
<td>15.20 ± 1.01</td>
</tr>
<tr>
<td>I</td>
<td>MIC 3 mg/kg, i.v.</td>
<td>53.11 ± 1.98**</td>
</tr>
<tr>
<td>II</td>
<td>MIC 10 mg/kg, i.v.</td>
<td>82.19 ± 5.14**</td>
</tr>
<tr>
<td>III</td>
<td>MIC 30 mg/kg, i.v.</td>
<td>97.19 ± 2.66**</td>
</tr>
</tbody>
</table>

Values are expressed as Mean, analysed by ANOVA followed by Dunnett test,

* p<0.05,
** p<0.001
of separating a complicated system into relatively simple sub-system and then presenting the chemical pattern of herbal medicine in the form of a chromatogram. HPTLC fingerprinting is the best way for chemical standardization and it also helps to provides the comprehensive idea about the class of compound present in the plant. A simple and accurate fingerprinting method using CAMAG HPTLC Instrument has been developed for quality control of the IC. For the first time, HPTLC fingerprint was investigated and used for evaluation of IC collected from Rajgurunagar, Pune. Recent researches indicate that the polyphenols, being secondary metabolites, are present in rich amount in several plants. Many of them possess antioxidant, anti-inflammatary and several others therapeutic properties. Therefore this study also established HPTLC fingerprint for the methanol extract of IC.

In the present investigation of in vitro anticoagulant activity, prolongation in PT exhibited by MIC was found to be 65% when compared to prolongation produced by 4IU of heparin per 100 μL of human citrated plasma. Prolongation of PT after treatment of IC could explain its role in the extrinsic pathway of coagulation. Increased PT probably directs the mechanism of MIC towards inhibition of factors such as I, II, V, VII, X in the extrinsic cascade for coagulation.

It can be concluded from the in vivo anticoagulant activity that IC has significant anticoagulant activity when administered orally. The results indicated that the onset of action of MIC was before 1 hr of drug treatment which produced 4 fold increase in PT compared to base reading and the duration of the action of MIC would be greater that 3 hr after the oral administration.

When administered intravenously, the onset of action of MIC was found to be 10 min after the drug treatment and the duration of the action of MIC would be greater that 1 hr after the intravenous administration of MIC.

It can be concluded from the present investigation that IC has exhibited significant anticoagulant activity both in vivo after oral and intra venous administration and in vitro. IC may be acting on the extrinsic cascade of clotting probably binds with the antithrombin. The complex formed may be inhibiting the conversion of prothrombin to thrombin and finally inhibiting the conversion of the soluble fibrinogen to insoluble fibrin clot.

There is a great need of developing and optimizing the anticoagulant therapy using oral or long acting anticoagulants which will be for who must be treated lifelong and should be devoid of “off target” effects, such as liver dysfunction and other non haemorrhagic side effects. Challenges for the new anticoagulants remain their higher costs relative to heparins and vitamin K antagonists, and lack of appropriate antidotes. In such a situation IC could be the hope towards development of a novel anticoagulant with optimized efficacy and reduced side effects.

The potential of IC in the procoagulant stage in diabetes particularly type II and related micro and macro vascular complications needs to be screened.

In accordance with the fact that the drug having an anticoagulant as well as antiplatelet activity could reduce the side effects of the triple therapy. Further the antiplatelet activity of IC needs to be explored.

REFERENCES


