

# Imperata cylindrica and Moringa oleifera: Antithrombotic Effect on Pulmonary Thromboembolism in Mice

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## ABSTRACT

**Background:** *Imperata cylindrica* and *Moringa oleifera* have been used widely as a traditional medicine to treat various diseases. These plants also have some compounds that are proven *in vitro* for their action to reduce platelet aggregation which has a major role in thrombosis pathogenesis. **Objective:** This study aimed to evaluate the effect of ethanolic extracts from *I. cylindrica* and *M. oleifera* as an antithrombotic agent in mice. **Methods:** The effects of *I. cylindrica* and *M. oleifera* on the pulmonary thrombosis *in vivo* and bleeding time parameters were examined. The two extracts were given orally for seven days prior to thrombosis induction. Survival rate was observed by the calculation of dead or paralysed mice and bleeding time was observed on amputated mice tails. **Results:** *I. cylindrica* and *M. oleifera* prevented paralyse and death caused by collagen-epinephrine induced pulmonary thrombosis in mice after 7 days pre-treatment in dose-dependent manner. The mouse tail bleeding time was significantly prolonged by administration of the extract, where *I. cylindrica* showed the most potent. **Conclusion:** These results suggest that the extract of *I. cylindrica* and *M. oleifera* have a potential activity as an antithrombotic agent *in vivo*.

**Key words:** Antithrombotic, *Imperata cylindrica*, *Moringa oleifera*, Survival rate, Pulmonary thromboembolism, Bleeding time.

## INTRODUCTION

Platelets play an important role in thrombus formation, including platelet adhesion, activation, secretion and aggregation.<sup>1</sup> Thrombus formation in the arterial or venous circulation can cause serious cardiovascular diseases for example atherothrombotic sicknesses, vascular blockage, myocardial and cerebral localized necrosis which can lead to death.<sup>2</sup> According to the World Health Organization (WHO), cardiovascular diseases is the global leading cause of death in the world.<sup>3</sup> Likewise one of the cardiovascular diseases, pulmonary embolism from the development of deep vein thrombosis (DVT) which is a cause of major mortality occurs when the thrombus breaks apart and causing occlusion pulmonary, making thrombosis to be a major health issue that needs attention.<sup>1</sup> The prevention of platelet aggregation will prevent the formation of thrombus and thrombosis occurrence. The antiplatelet efficacy for the prevention of thrombosis is indicated by its ability to block platelet activation and aggregation.<sup>4</sup> Aspirin as the most widely used antiplatelet has inhibition of platelet cyclooxygenase-1 (COX-1) activity, leading to reduced thrombosis occurrence and prolonged bleeding time. Resistance or variability in the response of aspirin treatment as the cause of treatment failure nowadays can be the reason to find alternative medicine to treat thrombosis especially to inhibit platelet aggregation.<sup>5</sup>

Currently, the treatment of herbal medicine is being a concern. But, antithrombotic agents from plants are still few. *Imperata cylindrica* and *Moringa oleifera* have been used widely to treat various diseases as traditional medicine. These plants also have some compounds which are proven *in vitro* studies to reduce platelet aggregation. *I.*

*cylindrica* extract was shown to inhibit adenosine diphosphate (ADP) – induced platelet aggregation, thrombin and collagen *in vitro*.<sup>6</sup> *M. oleifera* inhibited platelet aggregation throughout the five minutes and showed dose-dependent prevention.<sup>7</sup> In addition, treatment of *M. oleifera* extract in the previous study also showed a decrease platelet count in blood.<sup>8</sup> The mechanism of action of these plants may play different roles underlying its activity as an *in vitro* platelet aggregation inhibitor which has potential as a new antithrombotic agent. The development of antithrombotic agents from these plants requires further study. Therefore, this study aimed to evaluate antithrombotic activity *in vivo* of these plants to support *in vitro* studies that have shown the same activity.

## MATERIALS AND METHODS

### Chemicals

Collagen and epinephrine were purchased from Sigma-Aldrich (United States), saline was obtained from Euro-med (Indonesia). Carboxymethylcellulose was obtained from Brataco Chemical (Indonesia) and aspirin was purchased from Sigma-Aldrich (Tokyo, Japan).

### Plant Materials

The ethanolic extracts from roots of *I. cylindrica* and leaves of *M. oleifera* were obtained from Research Institute for Spices and Medicinal Plants, Bogor, Indonesia. The voucher specimens were deposited at botanical gardens plant conservation center (Number: B-1690/IPHx.3/KS/VI/2017).

### Experimental Animals

DDY (Deutschland, Denken, and Yoken) male mice were purchased from Veterinary IPB, Bogor,

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Indonesia. All the animals were acclimatized in a room under environmentally control at least a week before starting the experiments and also have free access to food and water. This research used experimental animals which were carried out according to ethics as set by Health Research Ethics Committees, Universitas Indonesia (Ethical Approval Number: 232/UN2.F1/ETIK/2017).

### Total Flavonoid and Phenolic Content Determination

Total flavonoid and phenolic determination in each extract were done by using the spectrophotometry method. Total flavonoid determination used the aluminium chloride colourimetry method by Soares et al (2015) with modification.<sup>9</sup> Working solutions of extracts were prepared by mixing 200 mg of the ethanol extracts with one millilitre of 0.5% w/v hexamethylenetetramine (HMT) solution. Twenty ml acetone was added followed by two ml of 25% HCl solution, then refluxed for 30 minutes and the filtrate was collected by filtering the solutions. Acetone and then ethyl acetate was added to the filtrate. Ten ml of working solution from the bottom layer of the resulting mixture to which was added one millilitre of  $AlCl_3$  (2%) in glacial acetic acid solution to obtain the final solution and then measured in Uv-Vis at  $\lambda = 510 \text{ nm}$ .<sup>10</sup>

Total phenolic content determination was done using Folin-Ciocalteu assay following the procedure of Aid et al (2015) with slight modification.<sup>11</sup> Two ml of dissolved extract in 95% ethanol as sample solution was mixed with 5 ml of aqua dest, then 0.5 ml of the Folin-Ciocalteu reagent was added to sample solution and mixed well, the solution was incubated for five minutes. After that, 1 ml of sodium carbonate (5% w/v) was added, and the solution was stored for one hour at room temperature. Then, the absorbance was measured at  $\lambda = 725 \text{ nm}$ .

### Phytochemical Screening

The presence of flavonoids and other metabolites such as alkaloid, saponin, terpenoid and glycoside were evaluated. The phytochemical screening indicated a notable presence of alkaloid, saponin, flavonoid, terpene and glycoside in both extracts, while tannin was only noticed in *M. oleifera* (Table 1).

### In vivo Antithrombotic Activity Assay

The experiment used two parameters, survival rate and bleeding time were done separately using different groups of five animals. All animals in both assays were treated with each extract for seven days by oral gavage daily. Survival rate and bleeding time were determined in the treated mice according to the protocols described by Saputri et al (2017) with slight modification. A pulmonary thromboembolism model was carried out to evaluate the survival rate of mice, while a tail bleeding assay was used to observe the prolongation of bleeding time.<sup>2</sup>

### Pulmonary Thromboembolism Model

The treatment of mice were divided into groups as follow in Table 2. Twenty four hours after the last treatment, each actively treated mice was injected with a mixture of collagen (700  $\mu\text{g}/20 \text{ g BW}$ ) and epinephrine (42  $\mu\text{g}/20 \text{ g BW}$ ) solution in the tail vein to induce pulmonary thrombosis.<sup>13</sup> While vehicle controls were given an injection of saline solution. After 15 minutes, the paralyzed mice was observed and the number of dead mice was recorded. While the protection percentage was calculated as described by Diminno and Silver method (1983).<sup>12</sup>

$[1 - (\text{number of dead or paralyzed} / \text{total number of mice})] \times 100$

### Tail Bleeding Assay

The treatment of mice were divided into groups as follow in Table 3. Five hours on the last day of treatment, animals were anaesthetized with ether by inhalation route and then placed in a horizontal position.

**Table 1: Phytochemical compounds identified in different extracts.**

Metabolite	Reagent	<i>I. cylindrica</i> extract	<i>M. oleifera</i> extract
Alkaloids	Bourchardat	+	+
	Dragendorff	+	+
	Mayer	+	+
Tannins	$FeCl_3$ 3%	-	+
	NaCl-Gelatin	+	+
Saponins	HCl 2N	+	+
Flavonoids	Mg+HCl	+	+
Terpenoids	Lieberman	+	+
	Bouchard	+	+
Glycosides	Molisch	+	+

**Table 2: The treatments of pulmonary thromboembolism model.**

Groups	Number of Mice (n)	Treatments 1 <sup>st</sup> -7 <sup>th</sup> days	24 hours after the last day	
<i>Imperata cylindrica</i>	Vehicle	1.5 % CMC	0.9 % saline injection	
	Aspirin	5	0.208 mg/20 g BW	Collagen-epinephrine injection
	D1		2.8 mg/20 g BW	
	D2		5.6 mg/20 g BW	
	D3		11.2 mg/20 g BW	
<i>Moringa oleifera</i>	Vehicle	0.5 % CMC	0.9 % saline injection	
	Aspirin	5	0.208 mg/20 g BW	Collagen-epinephrine injection
	D1		2.8 mg/20 g BW	
	D2		5.6 mg/20 g BW	
	D3		11.2 mg/20 g BW	

Note: D1: Dose 1; D2: Dose 2; D3: Dose 3; CMC: Carboxymethylcellulose; BW: Body Weight.

**Table 3: The treatments of tail bleeding assay.**

Groups	Number of Mice (n)	Treatments 1 <sup>st</sup> -7 <sup>th</sup> days	5 hours on the last day	
<i>Imperata cylindrica</i>	Vehicle	1.5 % CMC	Tail Bleeding Assay	
	Aspirin	5		0.208 mg/20 g BW
	D1			2.8 mg/20 g BW
	D2			5.6 mg/20 g BW
	D3			11.2 mg/20 g BW
<i>Moringa oleifera</i>	Vehicle	0.5 % CMC	Tail Bleeding Assay	
	Aspirin	5		0.208 mg/20 g BW
	D1			2.8 mg/20 g BW
	D2			5.6 mg/20 g BW
	D3			11.2 mg/20 g BW

Note: D1: Dose 1; D2: Dose 2; D3: Dose 3; CMC: Carboxymethylcellulose; BW: Body Weight.

One cm segment of the tail was amputated and the ends of severed tails were immediately immersed in a falcon tube filled with isotonic saline (0.9%). Bleeding time was monitored for 20 minutes. Although the bleeding stops before 20 minutes, monitoring was still being done to detect any re-bleeding.<sup>14</sup>

### Data Analysis

The bleeding time data was collected and analyzed statistically with IBM SPSS (Statistical Program for Social Science) ver. 22. To analyze

the distribution of normality and homogeneity, data was tested by the Shapiro-Wilk and Levene. Then, Kruskal-Wallis or one way ANOVA was used to analyze the significant difference in overall data and Mann-Whitney or LSD was used to analyze the significant difference between the treatment groups. The value of  $p < 0.05$  was considered to be statistically significant.

## RESULTS

### Total Flavonoid and Phenolic Content Determination

The total flavonoid and phenolic content of *I. cylindrica* and *M. oleifera* extracts can be seen in Table 4.

### Survival Rate Calculation

The survival rate was calculated and the results were obtained as in Table 5. Positive control of aspirin increased survival rate up to 100%. Extracts of *I. cylindrica* and *M. oleifera* treatments increased the survival rate up to 20%-60%.

### Bleeding Time Observation

The results of bleeding time observation can be seen in Table 6. Overall, the groups treated with the extract can prolonged bleeding time and were significantly different with the vehicle controls and did not significantly different with aspirin controls ( $P < 0.05$ ). Except for dose 3 of *I. cylindrica* extract, prolongation of bleeding time showed a significant difference compared with aspirin control. Figure 1 showed that all doses in both extracts significantly increased the bleeding time more than 100% compared with vehicle group.

## DISCUSSION

### Analysis of Total Flavonoid-Phenolic and Its Antithrombotic Effect

The total flavonoid and phenolic content of *I. cylindrica* and *M. oleifera* extracts can be seen in Table 4. The results showed the extracts contained flavonoid and phenolic compounds. Total flavonoid

**Table 4: Total flavonoid and phenolic determination.**

Extracts	Parameter	Content (% w/w)
<i>Imperata cylindrica</i>	Total flavonoid	0.002
	Total phenol	1.610
<i>Moringa oleifera</i>	Total flavonoid	0.020
	Total phenol	5.410

**Table 5: Survival rate analysis.**

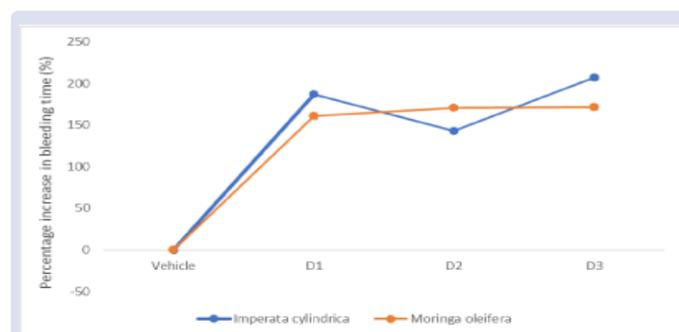
Groups	(%) survival rate	
<i>Imperata cylindrica</i>	Vehicle	0
	Aspirin	100
	D1	40
	D2	40
	D3	60
<i>Moringa oleifera</i>	Vehicle	0
	Aspirin	100
	D1	20
	D2	40
	D3	60

Note: Vehicle (0.5% CMC volume: 0.3 ml/20 g BW), Aspirin (0.208 mg/20g BW), *Imperata cylindrica* D1 (2.8 mg/20 g BW), *Imperata cylindrica* D2 (5.6 mg/20 g BW), *Imperata cylindrica* D3 (11.2 mg/20 g BW), *Moringa oleifera* D1 (2.8 mg/20 g BW), *Moringa oleifera* D2 (5.6 mg/20 g BW), *Moringa oleifera* D3 (11.2 mg/20 g BW). D1: Dose 1; D2: Dose 2; D3: Dose 3; CMC: Carboxymethylcellulose; BW: Body Weight.

**Table 6: Bleeding time observation results.**

Groups	Bleeding time	
	Average (min) ± SD	
<i>Imperata cylindrica</i>	Vehicle	6.37 ± 2.14
	Aspirin	17.06 ± 1.63*
	D1	18.28 ± 2.48*
	D2	15.45 ± 1.77*
	D3	19.54 ± 0.65* #
<i>Moringa oleifera</i>	Vehicle	6.31 ± 1.43
	Aspirin	16.16 ± 3.97*
	D1	16.44 ± 3.09*
	D2	17.08 ± 2.26*
	D3	17.11 ± 3.07*

Note: Vehicle (0.5% CMC volume: 0.3 ml/20 g BW), Aspirin (0.208 mg/20g BW), *Imperata cylindrica* D1 (2.8 mg/20 g BW), *Imperata cylindrica* D2 (5.6 mg/20 g BW), *Imperata cylindrica* D3 (11.2 mg/20 g BW), *Moringa oleifera* D1 (2.8 mg/20 g BW), *Moringa oleifera* D2 (5.6 mg/20 g BW), *Moringa oleifera* D3 (11.2 mg/20 g BW). \*Significantly difference  $P < 0.05$  as compared to the vehicle group. #Significantly difference  $P < 0.05$  as compared to the positive control (Aspirin) group. SD: standard deviation; D1: Dose 1; D2: Dose 2; D3: Dose 3; CMC: Carboxymethylcellulose; BW: Body Weight.



**Figure 1:** The percentage increase in bleeding time of *Imperata cylindrica* and *Moringa oleifera*. D1: Dose 1; D2: Dose 2; D3: Dose 3.

content was measured by  $AlCl_3$  colourimetric method. The principle involved in this method is that  $AlCl_3$  with the C-4 keto groups or either C-3 or C-5 hydroxyl group of flavonoids structure can form acid-stable complexes. It also with the ortho dihydroxyl groups in the A- or B-ring of flavonoids will form acid-labile complexes.<sup>15</sup> In total phenolic content used Folin-Ciocalteu method, a blue chromophore complex was formed by reaction between phosphomolybdic and phosphotungstic acid in the Folin-Ciocalteu reagent with phenolic groups in extracts.<sup>16</sup> The presence of these compounds was assumed to have the contribution to its activity as an antithrombotic agent *in vivo*. In a recent study, Jantan et al (2011) has examined that platelet activity could partly be due to the total phenolic content, so it can be a potential source for cardioprotective agents.<sup>17</sup> As well known, the phenolic compounds may reduce platelet aggregation by inhibiting Reactive Oxygen Species (ROS). In platelets, ROS are produced during platelet activation in the receptor-mediated signalling pathways. ROS induces changes in  $Ca^{2+}$  intracellular and may play as the second messenger on platelet activation induced by collagen or thrombin.<sup>18</sup> The inhibitory effect of collagen-induced platelet aggregation by some flavonoids has been investigated *in vitro* about its ability to bind competitively with the thromboxane A2 (TXA2) receptor.<sup>19</sup>

*M. oleifera* has the highest total flavonoid and phenolic content, followed by *I. cylindrica*. However, the antithrombotic effectiveness shown by the treatment of *M. oleifera* extracts was not better than others indicating there was no association between the content of these compounds and their effectiveness as an antithrombotic agent. The assumption was because of other compounds contained in the *I. cylindrica* have

a stronger potential in preventing platelet activation and aggregation thus prolonging the bleeding time and decreasing mortality due to thrombosis induced by collagen and epinephrine.

### Analysis of Survival Rate

The survival rate was calculated and the results were obtained as in Table 5. Positive control of aspirin increased survival rate up to 100%. Extracts of *I. cylindrica* and *M. oleifera* treatments increased the survival rate up to 20%-60%.

Induction methods of thrombosis using collagen (700 µg / 20 g) and epinephrine (42 µg / 20 g) were able to induce 100% mortality in mice. Collagen is a thrombogenic component also present in subendothelial vessels that induce platelet activation through the α2β1 (GPIa / IIa) receptors and glycoprotein receptor VI.<sup>20</sup> Epinephrine activates the platelets through their bonding with adrenergic α-2 receptors.<sup>21</sup>

Calculation of survival rate showed improvement compared with vehicle control. Dose 11.2 mg/20 g of each extract were the optimum doses in this study which was able to increase the survival rate to 60%. The effectiveness of each extract as an antithrombotic agent is assumed to be related to its ability to inhibiting the platelet aggregation of collagen-induced or other agonist-induced as already mentioned in the *in vitro* study. Inhibition of platelet aggregation may prevent further formation of thrombus and thrombosis occurrence.

### Bleeding Time Observation

The results of bleeding time observation can be seen in Table 6. Overall, the groups treated with the extract can prolonged bleeding time and were significantly different with the vehicle controls and did not significantly different with aspirin controls (P < 0.05). Except for dose 3 of *I. cylindrica* extract, prolongation of bleeding time showed a significant difference compared with aspirin control.

*I. cylindrica* prolonged the bleeding time and was statistically different from vehicle controls. The prolongation of bleeding time at dose 5.6 mg/20 g is shorter than dose 2.8 mg/20 g. The average bleeding time of *I. cylindrica* extract treatment in dose 11.2 mg/20 g showed longer bleeding time prolongation than aspirin control. Based on *in vitro* test, *I. cylindrica* showed inhibition of platelet aggregation induced by ADP, thrombin and collagen. The compounds contained in this plant had been studied and considered to be responsible for this activity.<sup>22</sup>

*M. oleifera* extract has the same effectiveness potency as antithrombotic along with aspirin in their activity to prolong the bleeding time. *M. oleifera* extract showed inhibit platelet aggregation *in vitro* caused by agonists such as adenosine diphosphate, collagen, and epinephrine. The inhibitory mechanism was related to its activity in inhibiting the cyclooxygenase pathway in arachidonic acid metabolism.<sup>23</sup> However, the specific compounds which were responsible for their acts were not known certainly. *M. oleifera* contains several major bioactive compounds such as kaempferol 3-O-glucoside (astragaloside), quercetin 3-O-glucoside (isoquercetin) and cryptochlorogenic acid indicating this plant has a high content of polyphenols.<sup>24</sup> Some natural plant contents with antiplatelet activity including the phenolic groups may reduce platelet aggregation by inhibiting ROS.<sup>25</sup>

Observation of bleeding time was done to investigate the hemostatic function. Bleeding time is a measure of the primary hemostasis where platelets plugs are formed. When platelet plug formation is inhibited, the process of wound closure, as well as its time of bleeding will become longer.<sup>26,27</sup>

### CONCLUSION

In conclusion, the present study revealed that extracts of *Imperata cylindrica* and *Moringa oleifera* had potential activity in preventing thrombosis. The *in vivo* study showed that treatment of each extract could increase the survival rate in male mice induced by collagen-

epinephrine as a thrombotic agent and prolong the bleeding time with the optimum dose was 11.2 mg /20 g BW.

### ABBREVIATIONS

WHO: the World Health Organization; DVT: deep vein thrombosis; COX-1: cyclooxygenase-1; ADP: adenosine diphosphate; DDY: Deutschland, Denkan, and Yoken; HMT: Hexamethylenetetramine; TXA2: Thromboxane A2; CMC: Carboxymethylcellulose; BW: Body Weight; ROS: Reactive Oxygen Species.

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### CONFLICTS OF INTEREST

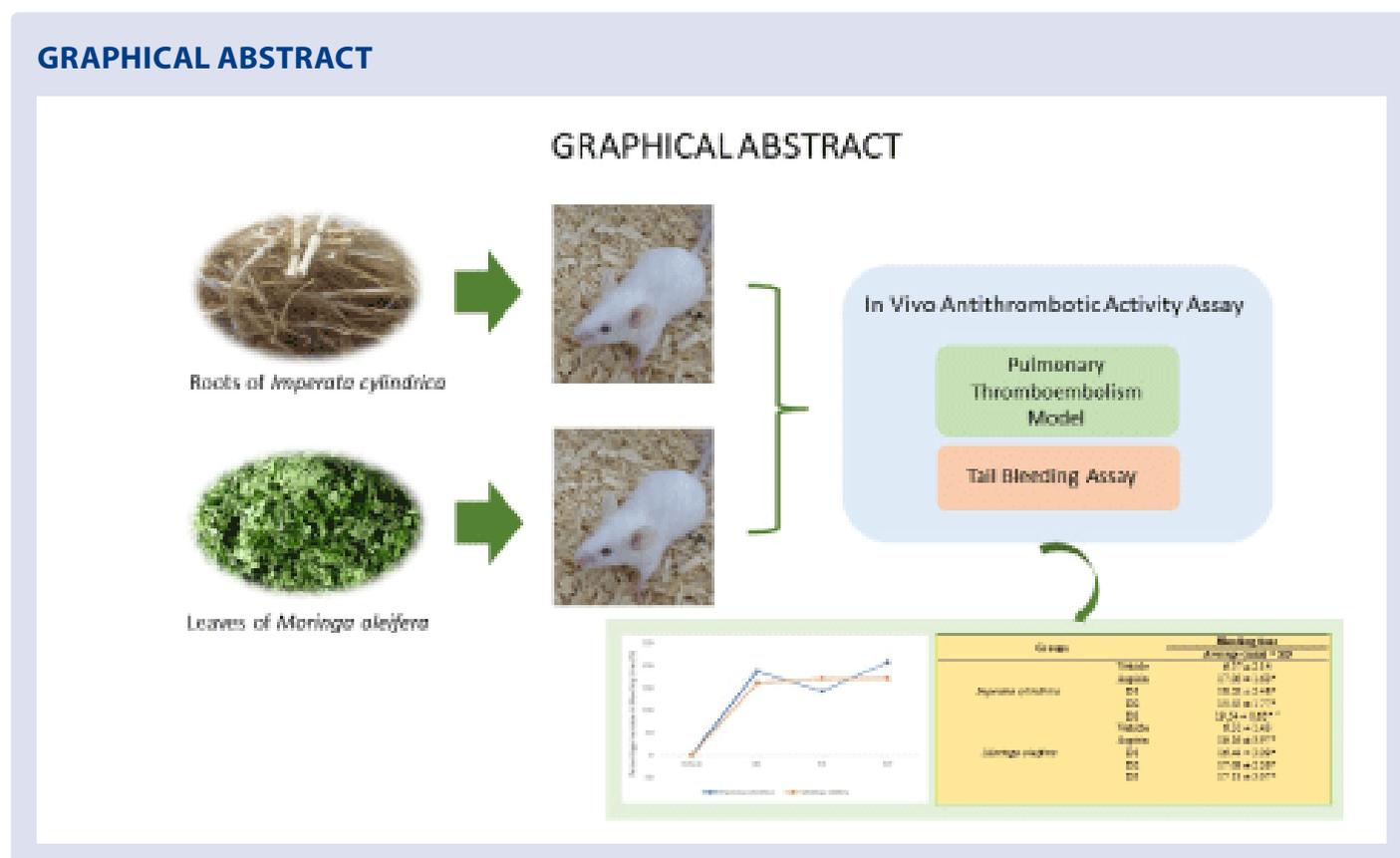
The authors declare there is no conflicts of interest.

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## GRAPHICAL ABSTRACT



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