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ORIGINAL ARTICLE

Pinocembrin Shows Antithrombotic Activity on the Thrombosis-Induced Experimental Rats

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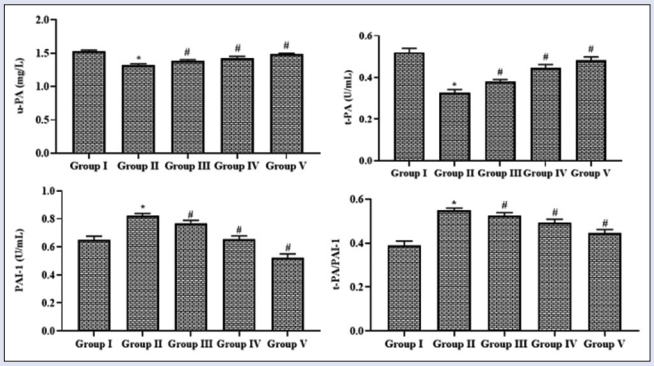
ABSTRACT

Background: Thrombosis is a major complication and is responsible for cardiovascular diseases (CVDs). The majority of CVDs like stroke, coronary syndrome, and vascular ailments are tightly connected to venous/arterial blood clots. The present research work was planned to address the curative benefits of pinocembrin against thrombosis in the experimental animal model. **Materials and methods:** The male Wistar rats were employed in this research work and the thrombosis was provoked to the rats via electrical shock by standard method. Animals were treated with 25 and 50 mg/kg of pinocembrin orally. The 20 mg/kg of aspirin was used as a positive control. The level of thrombin-provoked platelet aggregation was performed using the standard method. The plasma coagulation parameters were examined using an automated blood coagulation analyzer. The status of tissue factor pathway inhibitor (TFPI), thromboxane-B2 (TX-B2), 6-keto-PGF1α, and TXB2/6-keto-PGF1α (T/K) was investigated using assay kits. The levels of urokinase-type plasminogen activator (u-PA), tissue-type plasminogen activator (t-PA), PAI-1, and t-PA/PAI-1 were quantified using assay kits. **Results:** The pinocembrin treatment appreciably prolonged the coagulation parameter time periods like activated partial thromboplastin time, thrombin time, and TP. Pinocembrin also elevated the TFPI, 6-Keto-PGF1α, u-PA, and t-PA status and reduced the platelet aggregation, TX-B2, T/K, PAI-1, and t-PA/PAI-1 levels in the experimental rats. **Conclusion:** The findings of this work suggested that the pinocembrin exhibited potent antithrombotic activity and it could be a potential candidate to treat the thrombosis in the future.

Key words: Aspirin, coagulation, fibrinolysis, pinocembrin, thrombosis

SUMMARY

- The intrinsic and extrinsic cascades of the blood clot were effectively modulated by the pinocembrin, which is evidenced by the prolongation of TT, PT, and APTT assays.
- Pinocembrin also increased the TFPI, 6-Keto-PGF1α, u-PA, and t-PA and decreased the platelet aggregation, TX-B2, T/K, PAI-1, and t-PA/PAI-1 in the animals.



Abbreviations used: APTT: Activated partial thromboplastin time; CVDs: Cardiovascular diseases; PAI-1: Plasminogen activator inhibitor-1; PT: Prothrombin time; TT: Thrombin time; TX-B2: Thromboxane-B2.

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INTRODUCTION

Cardiovascular diseases (CVDs) are a group of ailments that arise in the cardiovascular system like venous thrombosis, pulmonary embolism, myocardial infarction, intravascular coagulation, and stroke, comprising many complications with increased mortality.[1] It was estimated that nearly 18 million mortalities were reported worldwide due to noncommunicable diseases. Thrombosis is the major complication that plays an imperative role in CVDs and severely endangers human health and life.[2,3] The increased thrombosis development is the prime most lethal cause of the CVDs that are typically provoked by imbalances in coagulation, irregular aggregation of platelets, and dysfunction of fibrinolysis mechanisms.[4]

Hemostasis is the common physiological process that controls blood loss during a traumatic injury. The hemostatic regulation of bleeding through thrombosis and the consequent disbanding via thrombolysis is the vital mechanisms in a front-line response to the trauma.[5] It comprises coagulation (formation of fibrin clots) and thrombolysis (dissolution of clots). In earlier years, several research reports demonstrated that thrombosis and thrombolysis expansively participate in the immune mechanisms.[6] The unbalance between thrombosis and thrombolysis can cause CVDs.[7] These vascular diseases are the foremost cause of mortalities around the world.[8] Platelets are the crucial players of the hemostatic reaction and also performs the antifibrinolytic actions via generating fibrinolytic inhibitors, for instance, plasminogen activator inhibitor-1 (PAI-1) that was comprised within the granules at more

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amounts. These inhibitors mediate the fibrinolysis inversely at various phases.[9]

The thrombotic disease is triggered by both thrombosis and thromboembolism. Vascular thrombosis is defined as three types microvascular, venous, and arterial thromboembolism.[10] It was already proved that blood clots could result in heart diseases if they occur in the coronary artery. The process of blood clotting primarily depends on the fibrin clot formation that results from the thrombin and fibrinogen interactions.[11] Thrombotic diseases are a serious risk to human life due to their increased mortality worldwide and their prevalence were increasing rapidly in recent times around the world due to an unhealthy lifestyle.[12,13]

The therapies for thrombotic diseases comprised of thrombolysis, surgery, interventional therapy, anticoagulant, and antiplatelet agents were employed.[14] The presently existing antithrombotic drugs like heparin, clopidogrel, aspirin, warfarin, and ticlopidine are the first-line medications and also it was well established with insufficient outcomes with adverse effects, like higher bleeding tendency and provoking gastrointestinal complications.[15,16] The insufficient success rate and higher adverse effects of these drugs have mystified the researchers. Hence, the exploration and discovery of novel antithrombotic agents in natural sources is still an imperative and challenging task. The highly complicated pathological mechanisms like thrombosis need potential remedies that can work on the manifold targets to proficiently control the fundamental mechanistic pathological events.[17]

Pinocembrin, a 5,7-dihydroxyflavanone, is the well-known flavonoid compound extensively found in honey, propolis, and many other plants like ginger and oregano.[18] Many previous reports highlighted that the pinocembrin exerts neuroprotective[19,20] and blood–brain barrier protective activities.[21] Pinocembrin also exerted many biological properties like antioxidant, antiapoptotic, and antiinflammatory activities.[22] Pinocembrin ameliorated the cognitive deficits,[23] improved memory and spatial learning,[24] and improved cardiac functions in the animal models.[25,26] On the contrary, the therapeutic actions of pinocembrin against thrombosis was not spoken yet. Accordingly, this research work was planned to address the therapeutic benefits of pinocembrin against thrombosis in the experimental animal model.

MATERIALS AND METHODS

Chemicals

Pinocembrin, benzocaine, aspirin, sodium citrate, and other reagents and chemicals were attained from Sigma-Chemicals, USA. The assay kits for the specific markers were purchased from the Thermofisher, USA, and Biocompare, USA, respectively.

Animals and treatment procedures

The 190–240 g weighing male Wistar rats were employed in this research work. The rats were acquired from the institutional animal house and then sustained in the infection-free cabins under organized laboratory situations with temperature (24 ± 2°C), air humidity (55 ± 5%), and 12 h dark/light sequence. Rats were fed with a regular rodent diet in throughout the experiments. All animals were habituated for a week in the laboratory with ambient conditions before the experiment initiation. The animals were administered 20 mg/kg of aspirin and 25 and 50 mg/kg of pinocembrin via oral route every day. The control animals were administered with pure water. After the 30 days period, the investigations were executed 5 h after the drug administrations.

Electrical-induced arterial thrombosis

The animals were administered with 20% of benzocaine via the intraperitoneal route. Then, in the Medline cervical incision route, the right carotid arteries of animals were exposed through blunt dissection and cleared the nearby tissues and vagus nerve. Then, the pinocembrin (25 and 50 mg/kg) and aspirin (20 mg/kg) were administered in the femoral vein intravenously. The control animals were administered with saline. Subsequent to the 5 min of administration, the carotid artery was put into the flow probe and touched with the electrode in a probe bottom (Yiyan Tech, China). The heart rate and blood flow range (beats/min) were detected through infrared detector. Afterward, 1 mA current was constantly conveyed for 10 min through an electrode connected with Animal Thrombosis Generator (Yiyan Tech, China). A carotid blood occlusive range (%) was examined in every 4 s via an infrared detector, and the mean times of occlusive thrombus development in the carotid artery were determined.

Assessment of thrombin-induced platelet aggregation

After the 5 min of thrombus formation, blood samples from both control and pinocembrin administered animals were gathered from the femoral artery in sodium citrate (3.8%). Then, the blood samples were subjected to examine the total platelet numbers with the aid of hematology analyzer (XS-800i, Sysmex, Japan). The platelet-rich plasma was prepared via centrifuging the sample at 3000 rpm for 10 min. Lastly, the 60 U/mL of thrombin stimulated platelet aggregation was detected as per the protocols described earlier by Zhao *et al.*[27] (2012), and the outcomes were displayed as a percentage (%) of platelet aggregation.

Assessment of thrombosis and thrombolysis parameters

The range of activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) of both control and pinocembrin supplemented animals were examined with the help of an automated blood coagulation analyzer (Beckman Coulter au5800, CA, USA). The levels of tissue factor pathway inhibitor (TFPI), thromboxane B2 (TXB2), 6-keto prostaglandin F1α (6-keto-PGF1α), and the ratio of TXB2/6-keto-PGF1α (T/K) in both control and pinocembrin administered animals were investigated using marker specific assay kits. (Thermofisher, USA). The urokinase-type plasminogen activator (u-PA), tissue-type plasminogen activator (t-PA), PAI-1, and t-PA/PAI-1 levels were quantified with the help of marker specific assay kits (Biocompare, USA).

Statistical analysis

The GraphPad Prism software was employed to assess the data statistically. The results were portrayed as mean ± SD of three measurements. One-way ANOVA and Tukey's *post hoc* assay were performed to detect the statistical variations between groups and $P < 0.05$ was fixed as significant. This research was approved by animal ethics committee of General Hospital of Northern Theater Command, ShenYang Province. (No.2021-106B,approved date:2021.09.23).

RESULTS

Effect of pinocembrin on the plasma coagulation parameters in the rats

Figure 1 illustrates the effects of pinocembrin on the plasma coagulation parameters like APTT, TT, and PT in the experimental rats. The

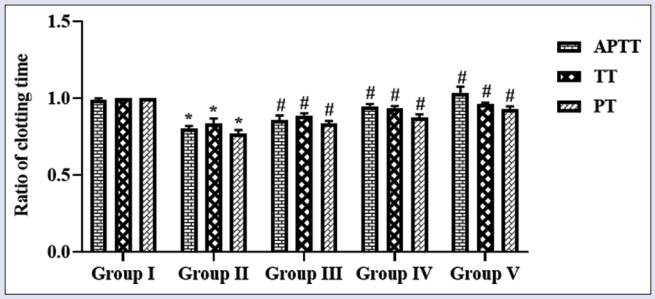

Figure 1: Effect of pinocembrin on the plasma coagulation parameters in the rats. Data were given as mean ± SD of three measurements. Data were statistically investigated by one-way ANOVA and Tukey's *post hoc* assay. Note: "*" signifies that data significantly vary at $P < 0.05$ from control and "#" signifies significantly vary at $P < 0.01$ from thrombosis rats

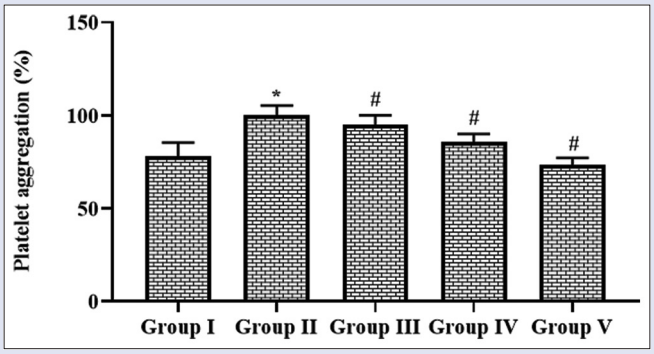

Figure 2: Effect of pinocembrin on the thrombin-provoked platelet aggregation in the rats. Data were given as mean ± SD of three measurements. Data were statistically investigated by one-way ANOVA and Tukey's *post hoc* assay. Note: "*" signifies that data significantly vary at $P < 0.05$ from control and "#" signifies significantly vary at $P < 0.01$ from thrombosis rats

pinocembrin treatment appreciably improved the time periods of APTT, TT, and TP in the experimental rats when compared with untreated normal rats. The supplementation of 25 and 50 mg/kg of pinocembrin potentially prolonged the APTT, TT, and TP periods more than the normal. The treatment with the 20 mg/kg of aspirin also effectively prolonged the time periods of APTT, TT, and TP in the rats. The outcomes of aspirin and pinocembrin supplemented experimental animals were found similar to each other, suggesting the anticoagulant activities of pinocembrin.

Effect of pinocembrin on the thrombin-provoked platelet aggregation in the rats

Figure 2 evidences the inhibitory action of pinocembrin against the thrombin-provoked platelet aggregation in the experimental rats. The decreased platelet aggregation was evidenced on the aspirin and pinocembrin administered in experimental animals. The administration of 25 and 50 mg/kg of pinocembrin appreciably decreased the thrombin-provoked platelet aggregation in the experimental animals. The 20 mg/kg of aspirin treatment also effectively decreased the thrombin-provoked platelet aggregation in the experimental animals. Both aspirin and pinocembrin treatment decreased the platelet aggregation, which is provoked by thrombin and these findings prove the therapeutic role of pinocembrin.

Effect of pinocembrin on the time of occlusion, TFPI, TX-B2, T/K, and 6-keto-PGF1α levels in the rats

Figure 3 exhibits the modulatory effects of pinocembrin on the time of occlusion, TFPI, TX-B2, T/K, and 6-keto-PGF1α status in the experimental animals. The time of occlusion was substantially prolonged by the 25 and 50 mg/kg of pinocembrin treatment in the experimental rats. The 20 mg/kg of aspirin also improved the time of occlusion in the rats. The status of TFPI and 6-keto-PGF1α in the animals was effectively improved by the 25 and 50 mg/kg of pinocembrin treatment when compared with the control animals. The status of TFPI and 6-keto-PGF1α was also enhanced by the 20 mg/kg of aspirin treatment. The 25 and 50 mg/kg of pinocembrin substantially decreased the TX-B2 and T/K status in the experimental animals, when related with untreated normal animals. The 20 mg/kg of aspirin treatment also reduced the levels of TX-B2 and T/K in the experimental animals. The outcomes of aspirin and pinocembrin were found similar with each other, suggesting the thrombolytic activity of pinocembrin.

Effect of pinocembrin on the levels of fibrinolytic parameters in the rats

Figure 4 demonstrates the modulatory effects of pinocembrin on fibrinolytic parameters such as u-PA, t-PA, PAI-1, and t-PA/PAI-1 in the experimental rats. Our findings demonstrated that the pinocembrin effectively modulated these parameters in the animals. The supplementation of 25 and 50 mg/kg of pinocembrin remarkably improved the u-PA and t-PA levels in the experimental animals. The status of u-PA and t-PA was also improved by the 20 mg/kg of aspirin treatment. The 25 and 50 mg/kg of pinocembrin treatment notably decreased the PAI-1 and t-PA/PAI-1 contents in the experimental animals. The aspirin treatment also diminished the PAI-1 and t-PA/PAI-1 contents in the experimental rats. A similar kind of outcomes was witnessed on pinocembrin and aspirin treatments, which proved the fibrinolytic activity of pinocembrin.

DISCUSSION

Thrombotic diseases are projected to contribute to about 30% of early mortalities worldwide.[28] Most of the CVD incidences like stroke, coronary syndrome, and vascular ailments are tightly connected to the venous/arterial blood clots, which direct to severe complications, long-term disabilities, and sudden deaths.[29] The preclinical examinations of the antithrombotic effects of new agents need the utilization of consistent and noteworthy investigational models of thrombosis. The electrolytic models of arterial thrombosis are the well-recognized animal modal and broadly employed to assess the effectiveness of new antithrombotic agents. The electrolytic damage could be presented to the primary surface of the arteries and generate an occlusive thrombus developed with erythrocyte, fibrin, and platelet that has the analogous morphological features that evidenced in the human vascular ailments. Both anticoagulant and antiplatelet medications were exhibited to interfere with the development of the thrombosis and fibrinolytic drugs affect their disbanding.[30] Here, we conducted the research work to address the antithrombotic action of pinocembrin in an electrical provoked animal thrombosis model.

The equilibrium of coagulation and anticoagulation systems plays essential functions in preserving the regular physiological functions. Under normal conditions, the combination of coagulation and anticoagulation systems could assure hemostasis during hemorrhage,

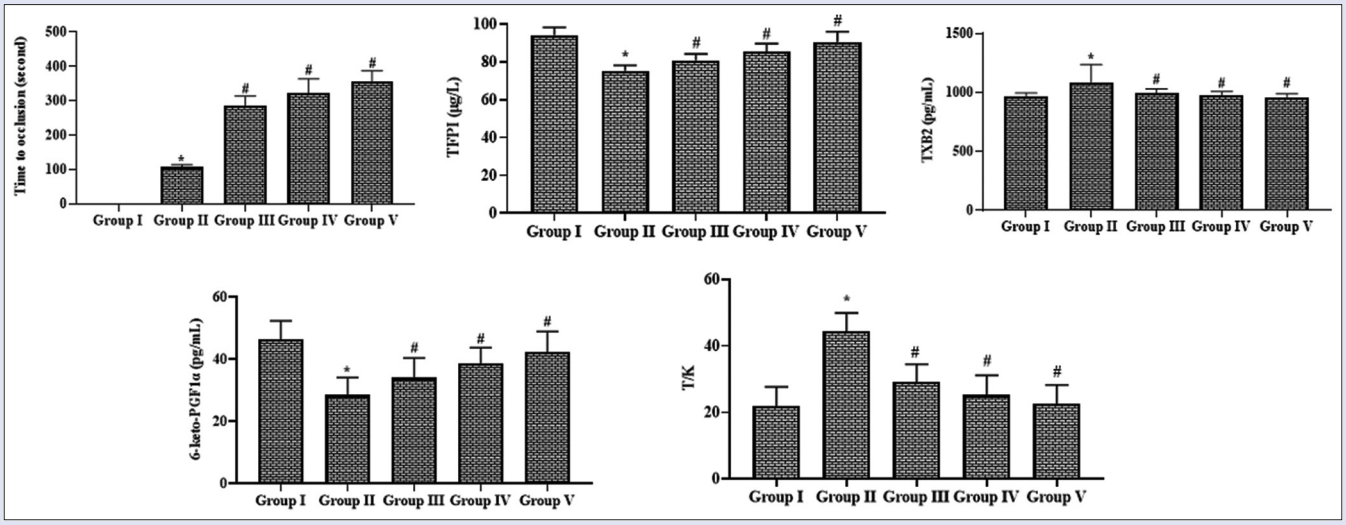

Figure 3: Effect of pinocembrin on the time of occlusion, TFPI, TX-B2, T/K, and 6-keto-PGF1α levels in the rats. Data were given as mean ± SD of three measurements. Data were statistically investigated by one-way ANOVA and Tukey's *post hoc* assay. Note: "*" signifies that data significantly vary at $P < 0.05$ from control and "#" signifies significantly vary at $P < 0.01$ from thrombosis rats

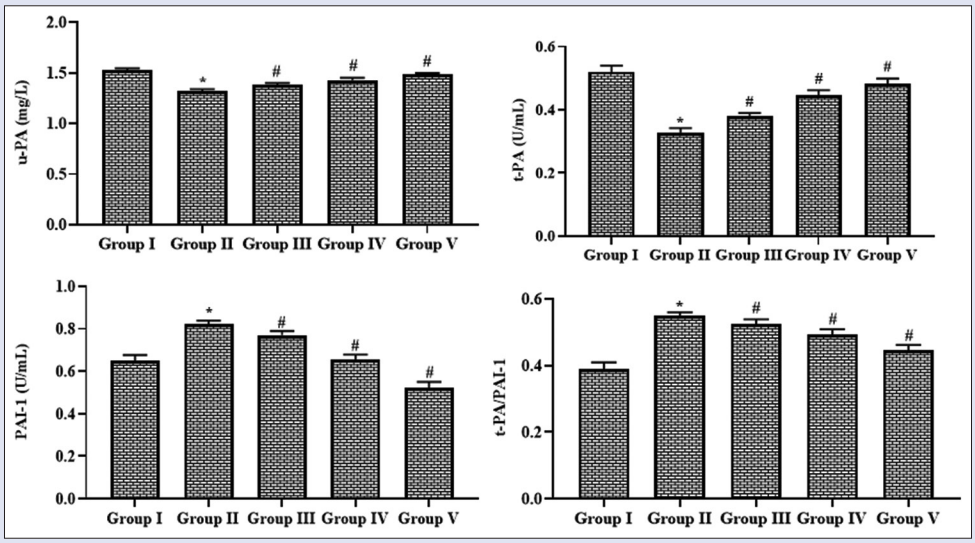

Figure 4: Effect of pinocembrin on the levels of fibrinolytic parameters in the rats. Data were given as mean ± SD of three measurements. Data were statistically investigated by one-way ANOVA and Tukey's *post hoc* assay. Note: "*" signifies that data significantly vary at $P < 0.05$ from control and "#" signifies significantly vary at $P < 0.01$ from thrombosis rats

controlling thrombus and blocking blood vessels to sustain physiological hemokinesis.[31] To treat thrombosis, many researchers tried to develop novel antiplatelet, anticoagulant, and thrombolytic drugs.[32] Along with the immense advancements in the treatment of thrombosis, many side effects and poor success rates are also still experienced. The natural sources are the hotspots of the reservoir of bioactive compounds to explore and develop a novel antithrombotic drug.[33] Pinocembrin is such a potential bioactive compound and demonstrated manifold pharmacological properties. Here, our findings witnessed that the pinocembrin demonstrated remarkable antithrombotic activity.

The TT, PT, and APTT are the extensively used parameters to examine the proficiency of the blood clotting pathway.[34] The APTT, PT, and TT are the crucial keys to demonstrate the blood-clotting level in the clinical sets.[35] As one of the vital markers, PT is highly sensitive and broadly utilized to assess and exhibit the plasma coagulation marker activities like coagulation factor-I, V, and X for extrinsic coagulation systems. APTT is also sensitive and extensively utilized to exhibit the coagulation marker levels like VIII, IX, and XI for the intrinsic clotting system.[36] Additionally, PT and APTT are utilized to assess the inclusive effectiveness of extrinsic and intrinsic clotting in the plasma.[37] Also, TT is exhibiting the third coagulation stage in plasma, and prolongation of TT demonstrates thrombin-regulated fibrin development inhibition.[38] Our findings evidenced that the pinocembrin treatment substantially prolonged APTT, TT, and TP periods in the experimental animals, which recommends the anticoagulant properties of pinocembrin.

TX-A2 and prostaglandins-I2 (PG-I2) are the incompatible metabolites of arachidonic acid and performs imperative functions in thrombosis. The over generation of TX-A2 enhance the thrombosis and platelet

aggregation, whereas PG-I2 has a contrary influence, and TX-B2 and 6-keto-PGF1α are the respective metabolites of them.[39] Hence, it was essential to preserve the equality of TX-B2/6-keto-PGF1α to regulate vascular resistance, platelet activity, and regional mobility.[40] It was already highlighted that the antithrombotic agents direct to the drastic diminution of TX-B2 and elevation of 6-keto-PGF1α levels.[41] In this investigation, we also witnessed that the pinocembrin supplementation could potentially diminish the TX-B2 status and elevate the 6-keto-PGF1α status in the rats, suggesting that the antithrombotic action of pinocembrin may be accredited to the regulation of active substances like TX-B2 and 6-keto-PGF1α in the vascular endothelium.

Under normal circumstances, the equality of coagulation, anticoagulation, and fibrinolysis were imperative in regulating normal blood circulation. The fibrinolytic system comprises the plasminogen activators and PAIs that consist of u-PA and t-PA.[42] As an activated form of plasminogen, the fibrinolysin controls the fibrinolysis process in the intrinsic fibrinolytic system via transforming insoluble fibrin into soluble products and thereby disbanding thrombus. t-PA and PAI-1 are the antagonistic modulatory markers, and t-PA is accountable for regulating the stimulation from plasminogen to fibrinolysin.[43] Additionally, PAI-1 is an essential fibrinolytic inhibitor and is accountable for 62% of the total PAI in plasma.[44] Moreover, the overexpression of PAI-1 could trigger impulsive fibrotic and thrombotic ailments. Subsequently, PAI-1 inhibition may be an essential process to promote blood flow and eliminate blood stasis.[45] The PAI-1 also participates in the hemostasis because it is the prime mediator of fibrinolysis. The antifibrinolytic property of PAI-1 is because of the t-PA inhibition and has encouraged an exploration for novel PAI-1 inhibitors in turn to treat the thrombotic ailments.

In the vascular system, t-PA is believed to be the essential motivator of fibrinolysis. It binds to the fibrin and transforms plasminogen into plasmin, which in turn initiates fibrin proteolysis.[46] Besides to the application of anticoagulant drugs to decrease the procoagulant activity, the thrombosis treatment also needs the utilization of thrombolytic therapies to regain the blood circulation in an occluded vessel. Similar to coagulation, fibrinolysis is also closely regulated by the array of receptors, cofactors, and inhibitors.[47] Plasmin is a chief fibrinolysin and is stimulated from the plasminogen via any of the proteases, that is, u-PA and t-PA. Although t-PA is generated and delivered by endothelial cells, u-PA is generated via macrophages, monocytes, and urinary epithelial cells. Both activators have exceptionally short half-lives in bloodstream, a 4–8 min because of the existence of high amounts of specific inhibitors like PAI-1. When compared with t-PA, u-PA has decreased affinity for plasminogen and under typical circumstances seems to act primarily in the extravascular sites. Both u-PA and t-PA are cleared by the liver subsequent to developing the complexes with LDL-receptor-like proteins.[48] Since plasmin enhances the activity of the activator via transforming single-chain t-PA and u-PA to their two-chain complements, plasminogen applies a positive response on its own stimulation.[49]

The deficiency of PAI-1 is categorized only by mild bleeding without impulsive bleeding incidents, while the increased plasma PAI-1 status has strong connections to the formation of thrombosis. The inhibition of PAI-1 could enhance thrombolysis. Various researchers have already tried to develop novel PAI-1 inhibitors.[50] Here, our findings witnessed that the pinocembrin treatment efficiently improved the u-PA and t-PA and abridged the PAI-1 status and prevented the binding of PAI-1 to the t-PA. Additionally, diminution of PAI-1 and increase of u-PA and t-PA demonstrated by pinocembrin may provide a new mechanism that expresses the promising therapeutic role of pinocembrin against thrombosis. The aggregation of platelets could trigger the development of a thrombus at the thrombus-vessel wall boundary.[51] The tissue factor is believed to be the vital contributor to blood thromogenicity in acute coronary ailments. The TFPI controls the extrinsic blood coagulation cascades via factor Xa-dependent hindering of TF-VIIa complexes and eventually regulates the development of thrombosis.[52,53] The findings of the current investigation witnessed that the pinocembrin effectively decreased the platelet aggregation and improved the TFPI in the experimental rats.

CONCLUSION

Our findings provide evidence that pinocembrin has the potential antithrombotic activity against electrical induced thrombosis in rats. The intrinsic and extrinsic cascades of the blood clot were effectively modulated by pinocembrin, which is evidenced by the prolongation of TT, PT, and APTT assays. Pinocembrin also increased the TFPI, 6-Keto-PGF1α, u-PA, and t-PA and decreased the platelet aggregation, TX-B2, T/K, PAI-1, and t-PA/PAI-1 in the animals. These findings recommend additional research in the future to promote pinocembrin as a potential candidate to treat thrombosis.

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Conflicts of interest

There are no conflicts of interest.

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