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Crocin improves lower extremity deep venous thrombosis by regulating the PIM1/FOXO3a axis

Jun Chen, Yi Yang, Shuo Wang, Kaixin Zhang*

Department of Vascular Surgery, Suqian Hospital Affiliated to Xuzhou Medical University, (Nanjing Drum Tower Hospital Group Suqian Hospital), Suqian 223800, Jiangsu, China

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ABSTRACT

Lower extremity deep venous thrombosis (LEDVT) has a high incidence and mortality. Crocin has the potential to ameliorate thrombosis. The study aimed to clarify whether crocin affects LEDVT. Human umbilical vein endothelial cells (HUVECs) were exposed to thrombin and crocin (0, 5, 10, 20, 40, and 80 μM). Cell viability was assessed by MTT assay. Cellular behaviors were assessed using flow cytometry, TUNEL assay, and tube formation assay. The binding relationship between crocin and PIM1 was analyzed by molecular docking. The underlying mechanism of PIM1 was determined by reverse transcription-quantitative PCR, dual-luciferase reporter assay, and RIP. We found that crocin (5, 10, 20, and 40 μM) promoted thrombin-treated HUVEC viability in a dose-dependent manner. Crocin inhibited apoptosis and promoted the angiogenesis of HUVECs induced by thrombin. PIM1 was a target of crocin and was upregulated in patients with LEDVT and thrombin-treated cells. Foxo3a could interact with PIM1 and positively related to PIM1 expression. Moreover, the knockdown of PIM1 suppressed apoptosis and promoted angiogenesis in thrombin-HUVECs treated with crocin, while overexpression of Foxo3a reversed the effects. In conclusion, crocin inhibited apoptosis and promoted the angiogenesis of HUVECs induced by thrombin via the PIM1/Foxo3a axis, suggesting that crocin may be effective for LEDVT therapy.

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Introduction

Thrombus formation, the formation of blood clots, can occur in various parts of the body. However, one of the most common sites is within the deep venous system of the lower extremities (1). Lower extremity deep venous thrombosis (LEDVT) is a condition characterized by the abnormal clotting of blood within the deep veins of the legs. LEDVT is associated with significant morbidity and mortality, making it a considerable health concern (2).

LEDVT arises from a combination of factors, including endothelial injury, hypercoagulability (abnormal blood clotting), and blood stasis (poor blood flow) (3). The condition typically manifests as swelling, pain, and dilatation of the superficial veins in the lower limbs (4). In severe cases, LEDVT can lead to life-threatening complications such as pulmonary embolism, where a blood clot from the legs travels to the lungs, as well as post-thrombotic syndrome, which involves chronic leg pain and swelling (5). Early-stage LEDVT often presents with no symptoms, making it challenging to diagnose clinically. Current treatment options for LEDVT primarily involve anticoagulant therapy, thrombolytic agents, and, in some cases, surgical interventions. However, these approaches often require prolonged treatment durations and are prone to relapse (6). Therefore, there is a critical need to explore more effective therapeutic strategies for LEDVT.

Crocin is a water-soluble carotenoid compound that is commonly isolated from the fruit of Gardenia jasminoides and the stigma of Crocus sativus. It exhibits a wide range of pharmacological activities, including antioxidation, anti-inflammation, abirritation (soothing effect), antidepressant properties, and anti-apoptotic effects (7-9). Crocin has demonstrated potential for treating various human diseases, including colitis, malignancies, and neurological disorders (10-12). Previous research has suggested that carotenoid intake may reduce the risk of cardiovascular diseases (13). In addition, crocin has been shown to attenuate lipopolysaccharide (LPS)-induced thrombosis and prevent liver injury (14). However, the specific role of crocin in LEDVT remains unclear.

In this study, we aimed to investigate whether crocin could influence the development of LEDVT and elucidate the underlying molecular mechanisms involved. We conducted experiments to examine the effects of crocin on thrombin-induced human umbilical vein endothelial cells (HUVECs), which represent a relevant cellular model for studying LEDVT. Our results revealed that crocin effectively suppressed apoptosis (programmed cell death) and promoted angiogenesis (the formation of new blood vessels) in thrombin-treated HUVECs. To understand the molecular basis of these effects, we focused on the PIM1/Foxo3a axis as a potential mechanism through which crocin exerts its actions.

By investigating the interplay between crocin and the PIM1/Foxo3a axis, we aimed to provide novel insights into the therapeutic potential of crocin for LEDVT. Understanding the molecular mechanisms involved in crocin-mediated effects could contribute to the development of alternative treatment strategies for this challenging

* Corresponding author. Email: zkx15996757711@hotmail.com

condition. The findings from this study may pave the way for further research and clinical investigations, ultimately leading to the development of more effective therapeutic interventions for LEDVT patients.

In summary, LEDVT poses significant health risks and remains a major clinical challenge. Crocin, a water-soluble carotenoid with diverse pharmacological activities, has shown promise in various human diseases. However, its role in LEDVT has not been fully explored. Through our study, we aimed to shed light on the impact of crocin on LEDVT and elucidate the underlying molecular mechanisms involved. The findings presented here provide a basis for further investigation and highlight the potential of crocin as a novel therapeutic approach for LEDVT.

Materials and Methods

Participants

Written informed consent was signed by every subject. The study was approved by the Ethics Committee of Suqian Hospital Affiliated with Xuzhou Medical University. A total of 30 patients with LEDVT and 30 healthy controls participated in this study. The patients were diagnosed according to ultrasonography, phlebography, and D-dimer levels. None of the subjects had cardiovascular disease, organ dysfunction, autoimmune disease, and malignancy. Peripheral blood (2 ml) was collected from each participant on an empty stomach. Plasma was isolated from peripheral blood after adding anticoagulant heparin and centrifugation.

RT-qPCR

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, HiScript III 1st strand cDNA synthesis kit (Vazyme, Nanjing, China) was utilized to reverse transcribed total RNA (1 μg) into cDNA. qPCR was carried out using ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China) based on the manufacturer's instructions. GAPDH served as the internal control of PIM1 and Foxo3a, respectively. The fold change of mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from ATCC (Manassas, VA, USA). The cells were cultured at DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Life Technologies, Paisley, UK). All cells were maintained at 37°C with 5% CO_2.

To establish the LDEVT cell modeling, the cells were treated with 4U/ml thrombin (Roche, Basel, Switzerland) for 8 h. After that, the cells were exposed to different concentrations of crocin (0, 5, 10, 20, 40, and 80 μM; purity ≥98%, Yuanye Biotechnology, Shanghai, China; Figure 1A) for 24 h.

Cell viability

MTT assay was conducted. Briefly, the cells were cultured for 24 h after treating them with thrombin and crocin. Subsequently, the cells were incubated with 5 mg/ml MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) for 4 h. After incubating with 100 μl dimethyl sulfoxide for 15 min, absorbance was examined at 490 nm using a microplate reader (BioTek, Winooski, Vermont, USA).

Cell transfection

The cells were plated into 6-well plates and transfected with vector, PIM1 overexpression vector, Foxo3a overexpression vector, siRNA (si)-PIM1, and si-NC (GenePharma, Shanghai, China) using lipofectamine 2000. After 48 h, the cell transfection efficiency was detected using RT-qPCR.

Flow cytometry

Cell apoptosis was evaluated by flow cytometry using an Annexin V-FITC apoptosis detection kit (Beyotime, Shanghai, China). After transfection, Annexin V-FITC (5 μl), as well as PI reagent (10 μl), were added into resuspended cells and incubated for 20 min at 25°C. The apoptosis cells were detected on a CytoFLEX flow cytometer (Beckman Coulter, Miami, FL, USA).

TUNEL assay

After the cells washing with PBS, they were fixed with Immunol staining fix solution (Beyotime, Shanghai, China) for 0.5 h. Then the cells were incubated with precoated 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 2 min. All samples were incubated with 50 μl TUNEL solution at 37°C for 1 h in the dark. The stained cells were imaged using a fluorescence microscope (Microscopesmall, St. Louis, MI, USA).

Tube formation assay

Matrigel (50 μl) was incubated at 37°C for 1 h in 96-well plates. After the cells trypsinizing, they were added to the plates and incubated at 37°C for 6 h. The tube structures were imaged under a microscope (Olympus, Tokyo, Japan).

Prediction of the targets of Crocin

The Mol2 file of Crocin was downloaded from the TCMSP database. Then, the targets of crocin were predicted by the Pharmmapper database. The protein names were altered from the results of the Pharmmapper database using the UniProt database.

Molecular docking

The 3D crystal structure of PIM1 was obtained from the Protein Data Bank (PDB) database. The SDF file of Crocin was downloaded from the PubChem database. The affinities and interaction between crocin and PIM1 were evaluated by the AutodockVina 1.2.2 docking software.

Luciferase reporter assay

The interacted genes of PIM1 were predicted using the GeneMANIA database. To verify the targeting relationship between PIM1 and Foxo3a, the wild-type (WT) sites of Foxo3a 3'-UTR and mutantFoxo3a 3'-UTR were constructed with pmir-GLO plasmids (Promega, Madison, WI, USA). Then lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was utilized to co-transfected the WT plasmid or mutant plasmid along with si-NC and si-PIM1 into cells using. The luciferase activity was evaluated using a dual-luciferase reporter gene assay kit (Beyotime, Shanghai, China) at 48 h post-transfection.

RNA binding protein immunoprecipitation (RIP)

HUVECs were washed with PBS and lysed using an equal volume of RIP lysis buffer. After centrifugation, the supernatant was incubated with IgG and PIM1 antibodies at 4°C overnight and then continued to incubate with protein A/G beads at 4°C for 1 h. The beads were washed using PBS and total RNA was eluted for RT-qPCR to detect Foxo3a expression.

Statistical analysis

All data were assessed using the GraphPad Prism 7 statistical software (La Jolla, CA, USA) and presented as mean ± standard deviation (SD). Comparisons between the two groups were evaluated using an unpaired student's t-test, and comparisons among multiple groups were evaluated using one-way ANOVA. The correlation was evaluated by Pearson's correlation analysis. A P-value less than 0.05 was deemed to be statistically significant.

Results

Effects of crocin on cell viability

To explore the effects of crocin, we first screen the treatment concentrations of crocin. The results indicated that crocin did not affect cell viability at concentrations of 5, 10, 20, and 40 μM, and suppressed cell viability at concentrations with 80 μM (Figure 1B). We established a cell model in that HUVECs were treated with thrombin, and the results showed that thrombin-induced the suppression of cell viability (Figure 1C). Besides, cell viability was suppressed in the thrombin-induced cells, while crocin promoted cell viability in a dose-dependent manner (Figure 1D). Crocin at 80 μM was used for functional analysis.

Crocin inhibits thrombin-induced apoptosis and promotes thrombin-induced angiogenesis

To evaluate the effect of crocin, cell apoptosis and angiogenesis were detected. As shown in Figure 2A-C, thrombin treatment induced apoptosis, while crocin markedly suppressed thrombin-stimulated apoptosis. Angiogenesis was inhibited by thrombin, which was abrogated by crocin (Figure 2D).

PIM1 is a target of crocin

To analyze the molecular mechanism, we predicted the target genes of crocin using the Pharmmapper online database. We found that PIM1 is a potential target. Then, we performed the molecular docking analysis to assess the affinity of crocin for PIM1. The results illustrated that crocin could highly stable bind to PIM1 (Figure 3A). Then, we found that PIM1 expression was upregulated in thrombin-treated HUVECs, and crocin decreased PIM1 expression (Figure 3B). Besides, PIM1 expression was higher in patients with LEDVT than in normal subjects (Figure 3C).

Foxo3a is a target of PIM1

The results of bioinformatic analysis predicted that PIM1 could interact with Foxo3 (Figure 4A). Thus, we detected Foxo3a expression in a clinical sample. Foxo3a levels were increased in LEDVT, compared with the healthy individuals (Figure 4B). PIM1 and Foxo3a have a significant positive correlation in LEDVT (Figure 4C). After PIM1 vector transfection, PIM1 levels were increased, whereas PIM1 levels were reduced after si-PIM1 transfection (Figure 4D). Overexpression of PIM1 increased Foxo3a levels, whereas knockdown of PIM1 decreased Foxo3a levels (Figure 4E). The luciferase reporter assay showed that PIM1 increased and silenced PIM1 decreased luciferase activity in the WT-Foxo3a group, suggesting their targeting relationship (Figure 4F and G). The RIP assay confirmed the interaction between PIM1 and Foxo3a (Figure 4H). Thrombin treatment induced the upregulation of Foxo3a levels (Figure 4I).

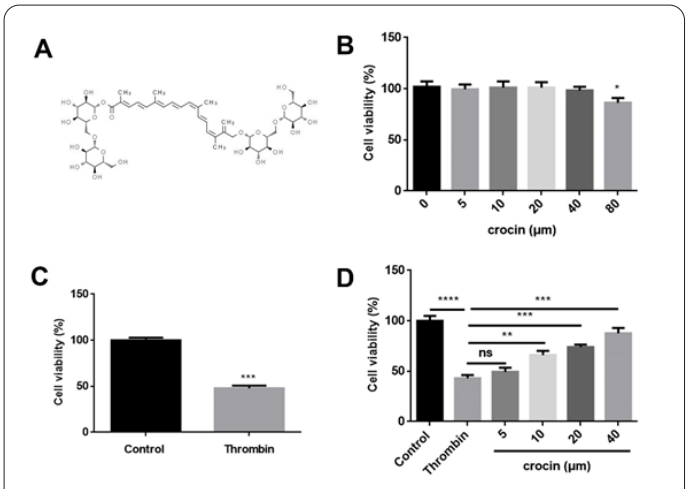

Figure 1. Effects of crocin on cell viability. (A) The chemical structure of crocin. (B) Cell viability was evaluated by MTT assay after 0, 5, 10, 20, 40, and 80 μM crocin treatment. (C) Cell viability was evaluated using MTT assay after thrombin-treated HUVECs. (D) HUVECs were treated with thrombin and 5, 10, 20, 40, and 80 μM crocin, and cell viability was assessed by MTT. ****P<0.0001. ***P<0.001. **P<0.01. *P<0.01. ns: no significance.

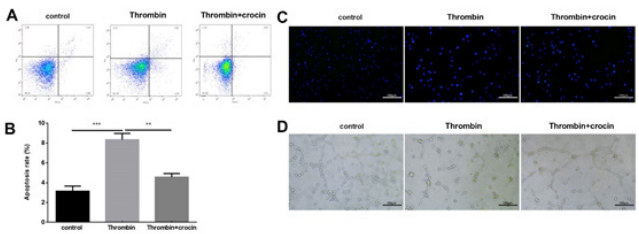

Figure 2. Crocin inhibits thrombin-induced apoptosis and promotes thrombin-induced angiogenesis. The apoptosis of thrombin and crocin-treated cells was evaluated using (A, B) flow cytometry and (C) TUNEL assay. (D) Angiogenesis was assessed after thrombin and crocin treatment by tube formation assay. ***P<0.001. *P<0.01.

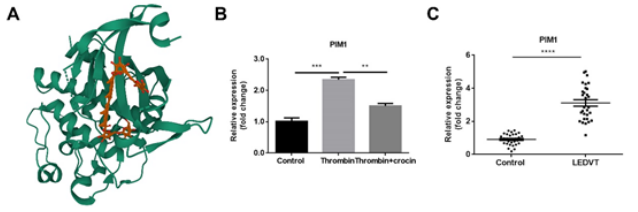

Figure 3. PIM1 is a target of crocin. (A) Molecular docking results of crocin and PIM1 protein. (B) PIM1 expression in HUVECs treated with thrombin and crocin. (C) The levels of PIM1 were examined using RT-qPCR in the plasma of patients with LEDVT (n-=30) and normal subjects (n=30). ****P<0.0001. ***P<0.001. **P<0.01.

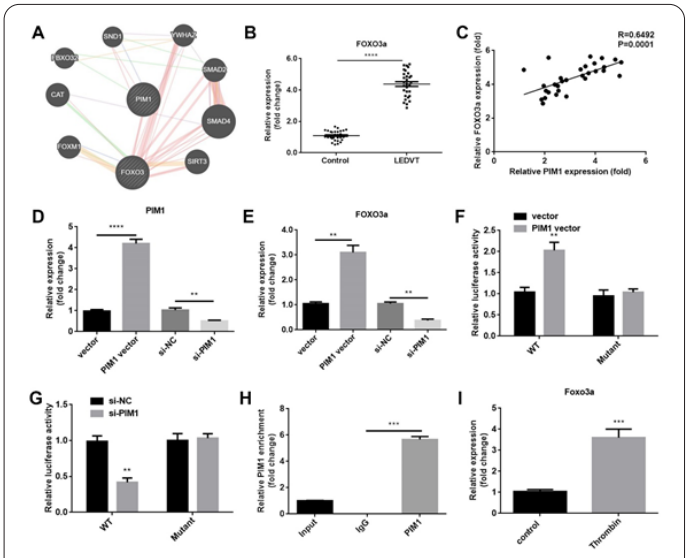

Figure 4. Foxo3a is a target of PIM1. (A) The PIM1-related genes were predicted using bioinformatic analysis. (B) Foxo3a expression was detected using RT-qPCR in the plasma of patients with LEDVT (n-=30) and normal subjects (n=30). (C) The relevance of PIM1 and Foxo3a was evaluated using Pearson's correlation analysis. (D) Transfection efficiency was measured using RT-qPCR after PIM1 overexpression or knockdown. (E) Effects of PIM1 on Foxo3a expression were detected by RT-qPCR. (F, G) The targeting relationship between PIM1 and Foxo3a was verified by dual-luciferase reporter analysis. (H) RIP determined the interaction between PIM1 and Foxo3a. (I) Foxo3a expression in HUVECs treated with thrombin and crocin. ****P<0.0001. ***P<0.001. **P<0.01.

Crocin inhibits apoptosis and promotes angiogenesis via the PIM1/Foxo3a axis

To confirm the underlying mechanism, apoptosis and angiogenesis capability were evaluated. Foxo3a expression was elevated after Foxo3a vector transfection (Figure 5A). The apoptosis was inhibited by PIM1 knockdown of thrombin-HUVECs treated with crocin, which was reversed by Foxo3a (Figure 5B-D). Silencing of PIM1 promoted angiogenesis, while enforced Foxo3a abrogated the promotion of thrombin-HUVECs treated with crocin (Figure 5E).

Discussion

Herein, we identified the influence of crocin on LEDVT and meantime explored the potential mechanism. We found crocin inhibited thrombin-induced apoptosis and promoted angiogenesis. Moreover, the PIM1/Foxo3a axis was the mechanism by which crocin served its function.

Various pharmacological activities of crocin are of great significance to human health. It has the potential to anti-tumor, anti-inflammation, reduce atherosclerosis and prevent neurological diseases (7, 15). Crocin is possible to prevent and treat thrombus because of its effects on promoting the growth and differentiation of endothelial cells (16). Tsantarliotou et al. have reported that crocin attenuates disseminated intravascular coagulation and septicemia as it has the effect of preventing thrombosis (14, 17). Thushara et al. have revealed that crocin effectively suppresses oxidative stress and apoptosis of damaged platelets, which is related to thrombosis and wound healing (18). However, the detailed effects of crocin on LEDVT remain unclear. Endothelial cells play crucial roles in vascular contraction and relaxation, platelet adhesion and aggregation, and intravascular immune response (19). Once endothelial cells are apoptotic, they will not only lose their antithrombotic function but also cause thrombosis formation (20, 21). In addition, endothelial cells are essential for angiogenesis (22). Thus, we focused on the effects of crocin on the apoptosis and angiogenesis of endothelial cells. Thrombin was utilized to treat HUVECs to establish the LEDVT cell model. We found that crocin suppressed thrombin-induced apoptosis, and meanwhile promoted thrombin-induced angiogenesis, suggesting that crocin may improve LEDVT.

To investigate the molecular mechanism. molecular docking was performed and the results indicated that crocin could function by binding to PIM1. As is well-known, PIM1 is an oncogene in numerous cancers, especially leukemia and prostate cancer (23, 24). Additionally, abnormally expressed PIM1 is associated with several diseases by regulating multiple cellular processes, such as growth, apoptosis, cell cycle, and metabolism (25). A recent study has reported that loss and inhibition of PIM1 attenuate thrombus formation (26). In this study, we found that PIM1 was highly expressed in patients with LEDVT and thrombin-treated cells, and crocin treatment decreased its expression. Knockdown of PIM1 further suppressed apoptosis and angiogenesis of HUVECs induced by thrombin and crocin. Taken together, crocin may attenuate LEDVT by targeting PIM1.

We predicted that PIM1 could interact with Foxo3. Foxo3a is a member of the FOXO family that mediates cell biological functions including apoptosis, proliferation, DNA damage, and tumorigenesis (27,28). Lou et al. have reported that Foxo3a is degraded by sirt1, which inhibits aging-induced DVT (29). Besides, Zhang et al. have reported that colchicine suppresses platelet activation and oxidative stress by upregulating Foxo3a, suppressing thrombus formation (30). These studies revealed a conflicting role of Foxo3a in thrombosis. Herein, we observed that Foxo3a was upregulated in patients with LEDVT. Besides, Foxo3a was a target of PIM1 and was positively regulated by PIM1. Overexpression of Foxo3a reversed the effects of PIM1 knockdown, suggesting that the PIM1/Foxo3a axis was the underlying mechanism of crocin-regulated

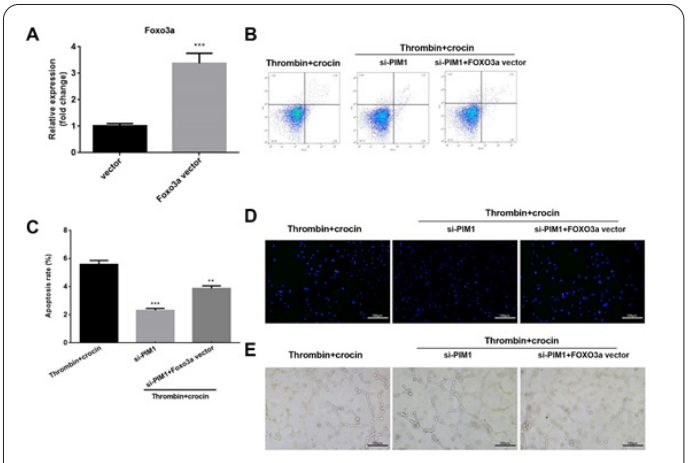

Figure 5. Crocin inhibits apoptosis and promotes angiogenesis via the PIM1/Foxo3a axis. (A) Transfection efficiency was measured using RT-qPCR after Foxo3a overexpression. Cell apoptosis was evaluated using (B, C) flow cytometry and (D) TUNEL assay. (E) Angiogenesis was assessed by tube formation assay. ***P<0.001. **P<0.01.

apoptosis and angiogenesis and the promoting thrombosis effects of Foxo3a.

The limitation of this study was that we only study the role of crocin using the *in vitro* cell model rather than *in vivo*. Whether crocin affected PIM1 to regulate the progression of LEDVT *in vivo* needs to be further investigated.

The current study provided valuable insights into the potential therapeutic effects of crocin in treating LEDVT. The research findings demonstrate that crocin exhibits inhibitory properties against apoptosis and promotes angiogenesis in thrombin-treated HUVECs. One significant discovery is the identification of PIM1 as a key molecular target for crocin. The study reveals that crocin binds to PIM1, a protein kinase, leading to the activation of the transcription factor Foxo3a. This activation of Foxo3a positively regulates its functions, thereby contributing to the anti-apoptotic and pro-angiogenic effects of crocin. Furthermore, the investigation uncovers the involvement of the miR-191-5p/SOX4 axis in Crocin's mechanism of action. Crocin is shown to modulate this axis, resulting in the inhibition of apoptosis and the promotion of angiogenesis. The intricate interplay between crocin, miR-191-5p, and SOX4 highlights the multi-faceted nature of crocin's therapeutic potential.

Conclusion

Overall, the findings from this study provide compelling evidence for the efficacy of crocin as a treatment option for LEDVT. By inhibiting apoptosis and facilitating angiogenesis through the regulation of the PIM1/FOXO3a axis and the miR-191-5p/SOX4 pathway, crocin demonstrates its ability to target key biological processes involved in LEDVT pathogenesis. These insights shed light on the potential of crocin as a promising therapeutic agent, opening up new avenues for the development of novel treatments for LEDVT. Further research and clinical investigations are warranted to validate and expand upon these findings, ultimately paving the way for the translation of crocin-based therapies into clinical practice for the benefit of patients suffering from LEDVT.

Ethical Compliance

Written informed consent was signed by every subject. The study was approved by the Ethics Committee of Suqian Hospital Affiliated with Xuzhou Medical University.

Conflict of Interests

The authors declared no conflict of interest.

Acknowledgments

This study did not receive any funding in any form.

Data availability statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

Author Contributions Statement

Jun Chen and Kaixin Zhang designed the study. Jun Chen, Yi Yang, and Shuo Wang performed the experiments and data analysis. Jun Chen is the major contributor to manuscript writing. All authors have read the manuscript.

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