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Diosmetin Inhibits NETs Formation in Neutrophils Through Regulating Nrf2 Signaling

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Keywords: diosmetin | NETs | Nrf2 | thrombosis

ABSTRACT

Background: Neutrophil extracellular traps (NETs) are important pieces of equipment for neutrophils. Excess NETs play promoting roles in cancer-associated thrombosis (CAT). Therefore, directing NETs formation is a promising therapeutic strategy in thrombosis and related diseases. Diosmetin, an antioxidant flavonoid derived from dietary sources, might be involved in NETs formation and CAT.

Methods: Firstly, the tests of cell-free DNA and Immunofluorescence were applied to evaluate the NETs levels of neutrophils. Luminol-based chemiluminescence and the DCFH-DA probe were used to detect the levels of reactive oxygen species (ROS) in neutrophils. Then, network pharmacological analysis and molecular docking were used to predict potential target molecules of diosmetin. The RT-qPCR was performed to measure the levels of Nrf2 and HO-1. A series of functional assays of neutrophils were used to examine the effect of diosmetin on other neutrophil functions. Finally, an animal model of deep vein thrombosis was constructed to assess the effect of diosmetin on thrombosis.

Results: Diosmetin reduced NETs and ROS levels in neutrophils. Then, molecular mechanisms analysis suggested that Nrf2 might be the primary target of diosmetin. Diosmetin treatment increased the levels of Nrf2 and HO-1 in NETs-generating neutrophils. An inhibitor of Nrf2 diminished the negative effect of diosmetin on NETs generation. Lastly, the murine thrombosis model results indicated that diosmetin treatment reduced thrombosis via NETs formation.

Conclusion: Diosmetin exerts as anti-NETs effect through Nrf2 signaling in neutrophils, showing the therapeutic potential in thromboembolism and related pathological processes, such as CAT.

Lijuan Guo and Hongyang Luo contributed equally to the work.

1 | Introduction

Compared to healthy individuals, cancer patients show a much higher incidence of venous thromboembolism (VTE) and arterial events, collectively referred to as cancer-associated thrombosis (CAT) [1]. CAT is the second leading cause of death and has a positive relation with poor prognosis in patients with malignant tumors, with VTE being the main type [2]. Although blood hypercoagulability in cancer patients is driven by specific pathways, the mechanisms and risk factors in CAT are similar to those observed in non-malignant situations [3]. Up to now, growing evidence highlights the pivotal role of neutrophil extracellular traps (NETs) in promoting the formation of CAT and other thromboembolism [4]. NETs refer to the extracellular network released by neutrophils, consisting of DNA scaffolding, histone proteins, and many granule proteins (e.g., myeloperoxidase, elastase, matrix metalloproteinase 9, etc.) [5–7], as one main type of defense weapons in activated neutrophils during inflammation and related processes [8]. Several studies have reported elevated levels of NETs in CAT, as well as abnormally high abilities of NETs formation in peripheral neutrophils of cancer patients, such as those with non-small cell lung cancer [9, 10]. NETs are proven to bind von Willebrand factor (vWF) to trap platelets via histones, and the granules and cytosolic proteins in NETs can activate platelets exerting procoagulant functions, thereby promoting thrombus formation [11]. Therefore, targeting NETs is a hopeful therapeutic direction in CAT. DNase1 treatment, which degrades the DNA structure of NETs, has been found to protect the inferior vena cava against deep vein thrombosis [12]. However, direct or complete clearance of NETs might result in immunosuppression complications, limiting the clinical efficacy and widespread application. Novel and high-safety drugs remain to be developed.

Diosmetin is a flavonoid compound that exists in a variety of natural dietary plants [13]. Previous studies demonstrate the anti-apoptosis, anti-oxidative, anti-infective, and anti-shock effects of diosmetin in diverse experimental disease systems [14–16]. The reported target cells of diosmetin include tumor cells, various tissue cells, and macrophages [17–19]. However, the direct effect of diosmetin on neutrophils or NETs is still unknown. Other natural flavonoids, such as luteolin, have been reported to reduce the generation of NETs and protect vascular endothelial cells [20]. Diosmetin-containing Xuanfei Baidu Decoction (XFBD) has been reported to decrease NETs levels in acute lung injury [21]. Given the importance of ROS in the formation of NETs [22], we hypothesize that diosmetin may influence the generation and function of NETs.

In this study, we investigated the effect of diosmetin in the generation of NETs and its possible therapeutic effect in thrombosis. We first found that diosmetin attenuated NETs formation by inhibiting ROS production. Secondly, through network pharmacological analysis, molecular docking, and biochemical experiments, we focused on and identified Nrf2/HO-1 signaling as the target of diosmetin in the process of NETs formation. Additionally, we examined the impact of diosmetin on other ROS-related physiological functions of neutrophils. Our data showed that diosmetin did not affect the spontaneous apoptosis or chemotaxis of neutrophils. At last, in a murine thrombosis model, diosmetin-treated mice exhibited smaller thrombi and reduced NETs. In a word, our results suggested that diosmetin might mediate Nrf2 activity to reduce ROS and NETs production and show good therapeutic potential for anti-thrombosis.

2 | Results

2.1 | Diosmetin Weakens the Production of NETs

The study conducted in our Collaboratory demonstrates that XFBD restrains NETs formation in acute lung injury [22]. Our previous results about XFBD also support this finding (Figure S1). As an important flavonoid activity component of XFBD, diosmetin might also be involved in the process. We explored the effect of diosmetin in NETs generation by a widely recognized in vitro NETs-induced model (phorbol 12-myristate 13-acetate (PMA) stimulation) [23]. Firstly, we purified primary neutrophils from mouse bone marrow, with the purity of more than 90% (Figure 1A). Compared to control neutrophils, diosmetin-treated neutrophils displayed obviously reduced extracellular DNA reticular structure after PMA stimulation (Figure 1B). In addition, we identified the levels of cell-free DNA (cfDNA) in neutrophil culture media to assess the level of NETs. Both control and diosmetin-treated neutrophils displayed minimal cfDNA, whereas PMA triggered a notable elevation of cfDNA levels. While PMA combined with diosmetin significantly reduced the levels of cfDNA in neutrophil media (Figure 1C). These results suggested that diosmetin could weaken the ability of neutrophils to form and release NETs.

2.2 | Diosmetin Down-Regulates ROS Production During NETs Formation

Based on the principle that ROS production is a crucial event in the process of NETs formation [24], we suppose that diosmetin might decrease the levels of ROS to perform its inhibitory effect in NETs formation. Firstly, we tested the ROS levels in XFBD-treated neutrophils. DCFH-DA probe detection revealed that XFBD decreased the levels of ROS in PMA-stimulated neutrophils (Figure 2A). Then we evaluated the influence of diosmetin on ROS in activated neutrophils. Similarly, the levels of ROS in diosmetin-treated neutrophils were much lower than those in the control group after 4 h of PMA stimulation (Figure 2B). Considering respiratory burst as a crucial feature and resource of ROS in neutrophils [24], we also examined the effects of XFBD and diosmetin on this process. After stimulation, neutrophils rapidly produce a large number of ROS, which can be real-time detected by luminol/HRP-dependent chemiluminescence assay. As Figure 2C–F show, PMA triggered abundant ROS production within minutes, while XFBD or diosmetin pre-treatment dramatically inhibited this process. Compared to XFBD, diosmetin showed a greater reduction in ROS levels in both experimental systems. It supported diosmetin as one of the important active components in XFBD.

2.3 | Diosmetin Targets Nrf2-HO-1 Signaling to Inhibit NETs Formation

The above studies suggested that diosmetin might regulate the formation of NETs through ROS signaling in neutrophils.

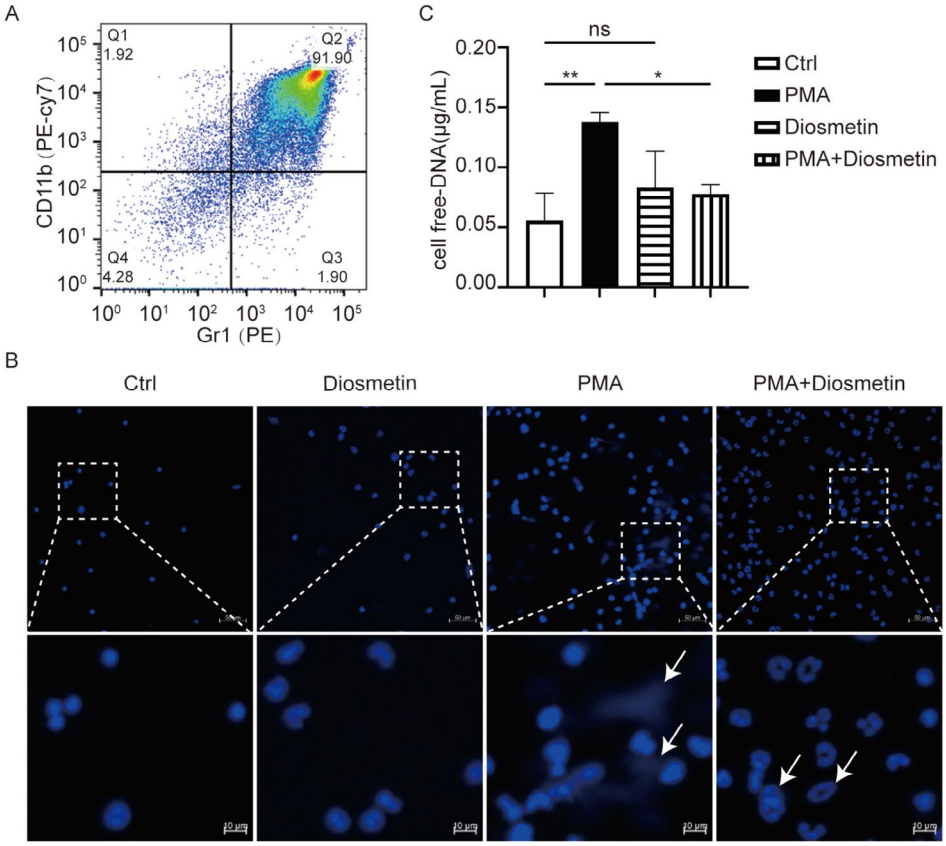

FIGURE 1 | Diosmetin weakens the production of NETs. (A) The purity of the extracted neutrophils was measured by flow cytometry. Neutrophils were labeled with CD11b (PE-Cy7) and Gr1 (PE) markers. (B) DAPI staining was used to detect the DNA of neutrophils after 4 h of PMA or PMA/Diosmetin stimulation. The white arrow indicates the DNA released out of cells. Scale bar = 10 μm. (C) The amount of cell-free DNA released into the culture supernatant of neutrophils was measured after 4 h stimulation with PMA or PMA + Diosmetin.

To identify potential targets of diosmetin in neutrophils, we resorted to network pharmacological analysis. From the corresponding data, we obtained the possible target genes of diosmetin, as well as the genes associated with NETs and ROS. A total of 59 common genes were found across the three gene groups (Figure 3A and Table S1). The protein–protein interaction network diagram indicated that Nuclear factor erythroid derived 2-like 2(Nrf2, gene name: *NFE2L2*) was one of the most prominently associated molecules (Figure 3B). Nrf2 is a key transcription factor that regulates anti-oxidative stress and can induce HO-1 transcription, which is involved in reducing ROS levels and anti-inflammatory processes [25]. Therefore, we speculated that diosmetin might mediate Nrf2 to regulate the generation of NETs. Next, we utilized an online molecular docking platform to simulate the interaction between Nrf2 and diosmetin, assessing the binding potential between the two molecules. The docking results showed that the vina score of the interaction between murine Nrf2 and diosmetin was −8.7, and diosmetin could form hydrogen bonds with Glu518 and Asp526 of Nrf2. For human Nrf2, the vina score was −7.6. Diosmetin could form hydrogen bonds with Asp21, Leu23, and Asp27 of human Nrf2 and π–π stacking with Trp24 of human Nrf2 (Figure 3C). In addition, the protein interaction network diagram revealed 13 other candidate molecules interacting with diosmetin. Therefore, we compared their vina scores using online molecular docking. Only prostaglandin-endoperoxide synthase (PTGS2) and matrix metalloproteinase 9 (MMP9) showed similar docking vina scores (−8.9 and −8.7) to Nrf2, while other candidates showed much lower or disparate scores in mouse and human proteins (Table S2). Although PTGS2 and MMP9 have been reported to be involved in NETs [26, 27], the two molecules appear to have minimal direct involvement in the regulation of NETs formation. Taken together, these findings suggest that diosmetin is likely to interact with Nrf2 to regulate ROS production and followed NETs formation.

Then we performed cellular experiments to test whether Nrf2 was involved in the action of diosmetin on NET formation. First, qPCR results displayed that diosmetin treatment increased the mRNA levels of Nrf2 and its downstream gene HMOX-1 (HO-1) (Figure 3D), which confirmed Nrf2 did participate in the process. Additionally, Immunofluorescence of anti-Nrf2 showed that Nrf2 levels in PMA and diosmetin co-treated neutrophils were much higher than those in PMA treated neutrophils (Figure 3E,F). Next, the pharmacological specific inhibitor of Nrf2 (ML385) was performed to investigate the effect of Nrf2. As shown in Figure S2, ML385 treatment largely impaired diosmetin-induced increases of HO-1 levels in activated neutrophils. Moreover, combined with diosmetin, ML385 treatment increased the ROS levels in PMA-activated neutrophils (Figure 3F). Meanwhile, ML385 partially reversed the inhibitory effect of diosmetin in NET formation (Figure 3H). These data indicated that diosmetin might activate Nrf2/HO-1 signaling to lower the ROS levels in neutrophils and then regulate the generation of NETs.

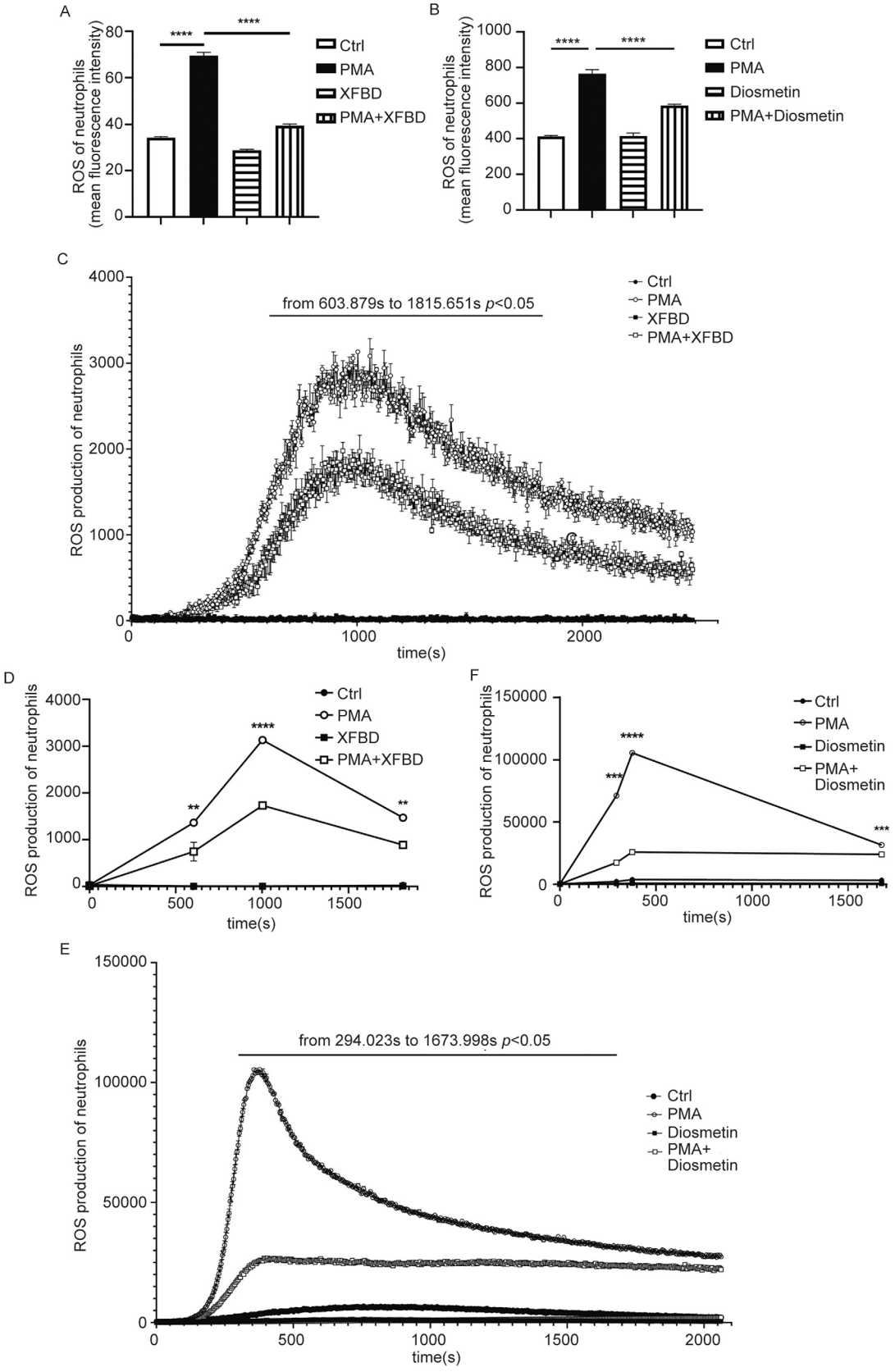

FIGURE 2 | Legend on next page.

2.4 | Diosmetin Does Not Affect Other Physiological ROS-Related Events of Neutrophils

Since ROS have been proved to be involved in some other physiological processes of neutrophils [28], we performed a series of functional experiments to check the possible effects of diosmetin. First, resting peripheral neutrophils have a short life span, and spontaneous apoptosis occurs in several hours. ROS displayed a promoting effect in this event [29]. Annexin V/PI staining was performed to examine the apoptosis rate of neutrophils. After culturing for 6, 12, or 24 h, diosmetin treatment didn't affect the rate of apoptotic neutrophils, as measured by flow cytometry (Figure 4A,B). During inflammation, neutrophils in bloodstream migrate to the site of infection, a process known as chemotaxis [30]. Our previous study identified ROS as physiological regulators of chemotactic migration [31]. Therefore, we investigated the effect of diosmetin on neutrophil chemotaxis, using transwell migration assay to examine up-to-down migration of neutrophils. As Figure 4C shown, in the lower chamber, there were similar amounts of diosmetin-treated neutrophils as control neutrophils. Next, an acute sterile peritonitis model was constructed to investigate neutrophil recruitment in vivo (Figure 4D). Four hours after thioglycolate (TG) solution injection, a large number of neutrophils were collected in peritoneal lavage. However, diosmetin treatment didn't weaken or enhance the recruitment of neutrophils (Figure 4E). These results indicated that diosmetin did not appear to influence spontaneous apoptosis or chemotaxis in neutrophils.

2.5 | Diosmetin-Mediated NETs Participate in the Regulation of Thrombosis in Mice

NETs have been identified to promote thrombosis and are involved in thromboembolism disease [32]. Therefore, we explored a murine deep vein thrombosis (DVT) model to study the potential function of diosmetin in thrombosis (Figure 5A). Thrombi from the inferior vena cava in the abdominal cavity of mice were collected 2 days after surgery. As Figures 5B,C and S3 show, thrombi were collected in almost every surgical mouse. However, shorter and lighter thrombi were obtained in diosmetin pre-treated mice. Even no macroscopical thrombi were found in two mice of the diosmetin-treated group. HE staining images of thrombi confirmed the results (Figure 5D). Next, we detected the levels of NETs in thrombi slices by staining for citrullinated histone H3 (citH3, a marker of NETs). Apparent positive signals of citH3 were detected in all the thrombi (Figure 5E), which supported the positive roles of NETs in thrombosis. To exclude the factor of neutrophil number in thrombi, the ratio of citH3 fluorescence to MPO (the marker protein of neutrophil) fluorescence was calculated. Our analysis showed that the citH3-to-MPO ratio in the diosmetin-treated group was much smaller than that in the control group (1.33 vs. 8.75). Meanwhile, the bone marrow neutrophils in both modeling groups were purified to test the NETs formation following PMA stimulation. DAPI staining results showed that neutrophils from the diosmetin group displayed much lower abilities of NETs formation compared to neutrophils from the control group (Figure 5F). Besides, we examined the conditions of the livers and kidneys in treated mice. The weights of the livers and kidneys were in the normal range (Figure S4A,B), and the ligated side kidney also showed a similar weight to that of the other one in the same mouse (Figure S4C). These results supported the good safety of diosmetin in clinical use. In summary, these data certified that diosmetin could reduce thrombosis through attenuating NETs generation.

3 | Discussion

This study explored the roles of diosmetin on neutrophils, with a particular emphasis on NETs formation, employing both in vitro and in vivo approaches. We found that diosmetin significantly reduced NETs by decreasing ROS. A mechanistic study revealed that diosmetin likely involves the Nrf2/HO-1 pathway, upregulating Nrf2 to enhance HO-1 transcription, thereby mitigating ROS and NETs levels. However, diosmetin showed no effect on other ROS-related physical functions of neutrophils, suggesting few complications in possible clinical use. In an animal thrombosis model, diosmetin reduced inferior vena cava thrombosis, exhibiting its possible therapeutic application in cancer-associated thrombosis.

The formation and regulation of NETs is a complex and heterogeneous process, depending on given pathogens or endogenous products [33]. In this study, we used PMA to trigger the generation of NETs. Besides, many stimuli could induce NETs, such as crystals or cytokines in the tumor microenvironment, bacteria, viruses, and immune complexes [34, 35]. Compared with other inducers, PMA-induced NETs are more stable and

FIGURE 2 | Diosmetin downregulates ROS production during NETs formation. (A) DCFH-DA probes were used to detect total ROS levels in neutrophils. After the DCFH-DA probe was pre-loaded into the neutrophils, the neutrophils were stimulated with PMA or PMA + XFBD for 4 h, and the green fluorescence signal intensity of the cells was detected. (B) With the same assay in A, the ROS levels in PMA or PMA/Diosmetin were measured. (C) Luminol-HRP was used to detect the instantaneous ROS levels of neutrophils produced by respiratory burst after PMA or PMA + XFBD stimulation (Before PMA, the cells had been pretreated by XFBD for 4 h). The horizontal line above the line chart indicated that the ROS levels produced by PMA + XFBD-treated neutrophils were significantly different from those in the PMA group during the detected period of 603.879 s-1815.651 s. (D) The line chart shows ROS levels of Figure C at four time points. These are the starting point of detection (0 s), the first time point with significant differences in ROS levels (603.879 s), the corresponding time point with peak ROS levels (1002.544 s), and the last time point with significant differences in ROS levels (1815.651 s). (E) Luminol-HRP was used to detect the instantaneous ROS levels of neutrophils produced by respiratory burst after 4 h of PMA or PMA + Diosmetin stimulation. The horizontal line above the line chart indicates that during the detected period of 294.023 s–1673.998 s, the ROS levels produced by PMA + Diosmetin-treated neutrophils were significantly different from those in the PMA group. (F) The line chart shows ROS levels of Figure D at four time points. These are the starting point of detection (0 s), the first time point with significant differences in ROS levels (294.023 s), the corresponding time point with peak ROS levels (376.638 s), and the last time point with significant differences in ROS levels (1673.988 s).

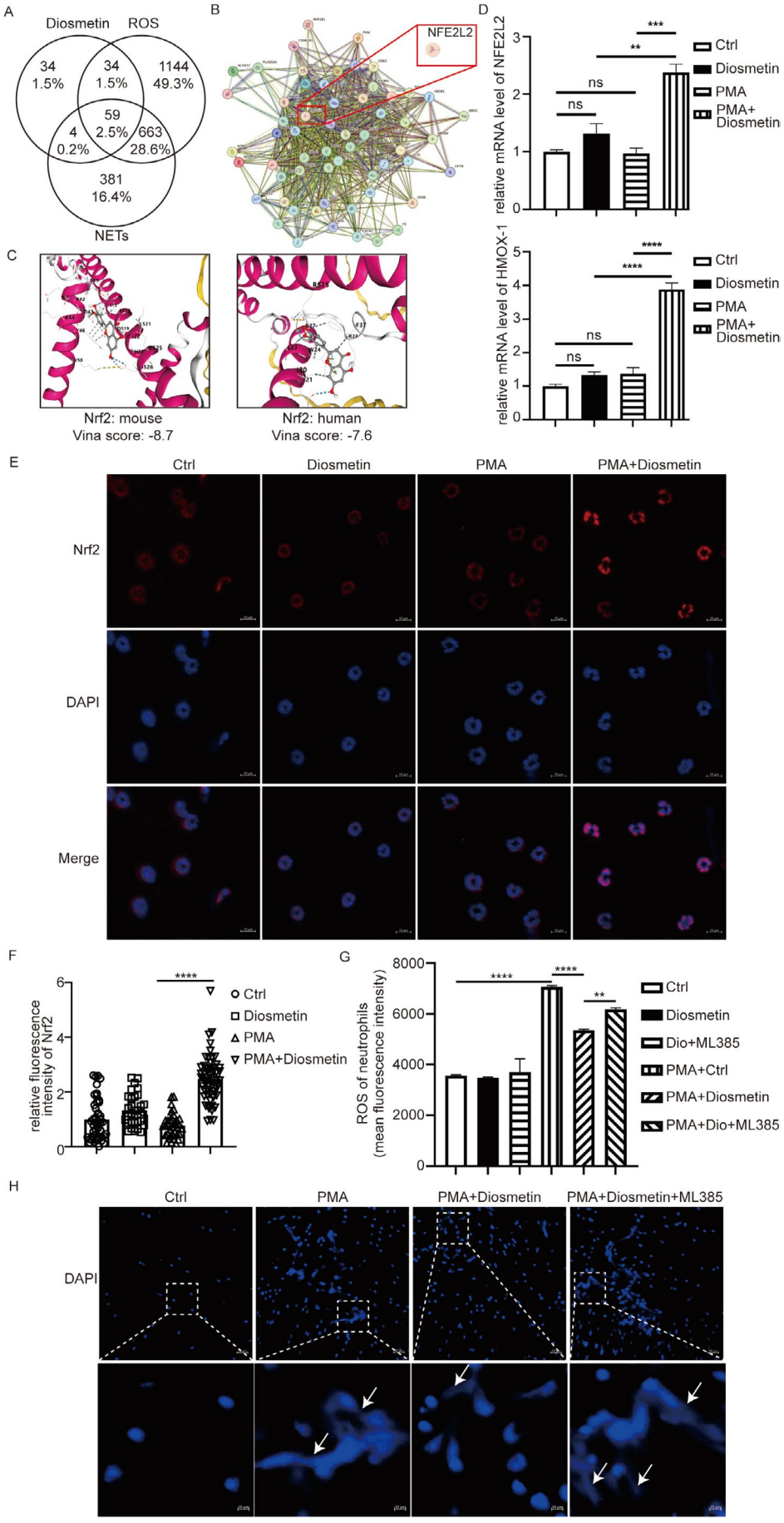

FIGURE 3 | Legend on next page.

prone to higher levels. In addition, some inducers need extra environmental conditions in NETs formation. These characteristics make PMA a commonly used stimulator in general cellular studies [36]. Different stimuli of NETs activate different receptors and subsequent signaling. For instance, PMA mediates MPO, NE, and some other signaling intermediates to trigger NETs production [37]. However, the intracellular ROS are verified as the common crucial factors in the process. Appropriate ROS promote neutrophils to produce NETs, while excessive ROS might break the balance of NETs production and then promote the pathological functions of NETs [22]. Therefore, screening ROS-related drug candidates has great potential in the treatment of abnormal NETs formation and related diseases. Diosmetin has shown anti-oxidative effects in several disease models. For example, diosmetin can regulate oxidative stress of endothelial cells and liver cells, mesenchymal transformation of epithelial cells, and various inflammatory processes by down-regulating ROS levels [38–40]. In this study, we first reported that diosmetin restricted NETs production and weakened ROS levels in neutrophils, which suggested that diosmetin might show therapeutic effects on NETs-related pathological conditions.

Given the role of ROS in mediating several physiological functions of neutrophils, we also examined the effects of diosmetin on these processes, including acute inflammatory recruitment. Our results displayed that diosmetin had few effects on these events of neutrophils. However, some literature suggests that diosmetin might affect neutrophil infiltration in acute lung injury [14, 21]. In these studies, neutrophil numbers in the lung were measured 2 days after inflammation. At that time, the number of tissue neutrophils might be influenced by some other factors such as macrophages, not only neutrophil recruitment. Since the recruitment of neutrophils predominantly occurs in the early period of acute inflammation, we collected cells 4 h after TG treatment to more accurately reflect the recruitment ability of neutrophils during acute inflammation. Besides, we also explored an ex vivo transwell experiment system to test the chemotaxis of neutrophils. Consistently, diosmetin treatment did not alter the number of neutrophils in the lower chamber. Taken together, our data verified that diosmetin was not involved in neutrophil movement in the early stage of acute inflammation. However, the therapeutic effects of diosmetin in various inflammatory diseases were determined by specific pathogens or disease types.

To study the action mechanism of natural products, network pharmacology and molecular docking are common and valuable tools. Based on our study aim, potential target genes of diosmetin, NETs-related genes, and ROS-related genes were analyzed in corresponding databases. In total, there were 14 candidates screened out. To narrow down the most probable targets, molecular docking was used to simulate the interactions between diosmetin and possible target proteins. Using both human and murine protein structures predicted by Alphafold, three molecules were filtered out, namely PTGS2, MMP9, and Nrf2. PTGS2 (prostaglandin-endoperoxide synthase), also known as cyclooxygenase (COX2), is a key enzyme in prostaglandin biosynthesis [41]. As reported, the inhibitor of PTGS2 decreases PGE2 levels to restore NETs formation after bone marrow transplantation [26]. Some studies suggest that NETs induce the activity of PTGS2 through toll-like receptors in metabolic dysfunction-associated steatohepatitis and gastric cancer [42, 43]. MMP9 (matrix metalloproteinase 9) is a specific component of tertiary particles in neutrophils, displaying the ability to degrade the extracellular matrix [9]. In NETs, MMP9 is a protease brought by NET DNA to defend against pathogens or cleave the extracellular matrix [27]. Meanwhile, considering the anti-oxidation of diosmetin in NETs production, PTGS2 and MMP9 seem not to be the main targets of diosmetin in regulating NETs formation. Nrf2, as the key anti-oxidative transcriptional regulator, could enhance the ability of cells to resist oxidative stress by promoting the expression of downstream molecules, such as HO-1 [44]. Therefore, Nrf2 is more likely to be the target of diosmetin in neutrophils. Our experimental results were consistent with the analysis, showing that diosmetin promotes the transcription of Nrf2 and its downstream molecule HO-1 in PMA-stimulated neutrophils. The inhibition of Nrf2 (MLA385 treatment) partially neutralized the effect of diosmetin on ROS and NETs. However, the ROS levels in the diosmetin, ML385, and PMA co-treatment group remained lower than those in the PMA treated group, which indicated that there might be some other molecules regulated by diosmetin in neutrophils. Some studies have reported that diosmetin might mediate the activity of NADPH oxidase to resist oxidative stress in mouse embryonic fibroblasts and neuronal co-cultures [45]. The roles of these mentioned molecules in NETs formation might be studied in the future.

A number of in vivo and in vitro studies reported the roles of diosmetin in diverse cancers development, including anti-proliferation, pro-apoptosis, anti-metastasis, and pro-radiotherapy sensitivity [46–49]. However, it is poorly understood about the possible roles of diosmetin on tumor-associated complications and tumor-associated immune cells, such as CAT and neutrophils. Thrombosis is a core promoting factor and an important death inducer in many diseases, including cancer [50]. Although the big advance of cancer treatment improves patient outcomes, the risk of CAT is still rising

FIGURE 3 | Diosmetin targets Nrf2-HO-1 signaling to inhibit NET formation. (A) Possible gene targets of diosmetin, genes related to NETs, and genes related to ROS were analyzed from multiple network databases and performed Wayne analysis. (B) Protein interaction analysis was performed on 59 possible common targets, as Wayne analysis showed. (C) Using CB-Dock2: An accurate protein-ligand blind docking tool to perform molecular docking of mouse or human Nrf2 proteins with diosmetin to predict their binding ability and amino acid sites of interaction. (D) RT-qPCR was used to detect the transcription levels of *NFE2L2* (encode protein: Nrf2) and *HMOX-1* (encode protein: HO-1) genes in neutrophils after PMA or PMA + Diosmetin stimulation. (E and F) Neutrophils were pre-treated with diosmetin for 2 h and then co-treated with PMA + Diosmetin for 15 min, and Nrf2 protein signals were detected by cellular immunofluorescence. Scale bar = 10 μm. (G) DCFH-DA probe was used to detect total ROS levels in neutrophils. Neutrophils were pretreated with Nrf2 inhibitor ML385 for 2 h and then co-stimulated with PMA or PMA + Diosmetin for 4 h. Finally, the DCFH-DA probe was loaded, and the intensity of the green fluorescence signal was detected. (H) Neutrophils were pretreated with ML385 for 2 h and later co-stimulated with PMA + Diosmetin for 4 h, and NETs levels were detected by DAPI. Scale bar = 50 μm. For enlarged pictures, the Scale bar = 10 μm.

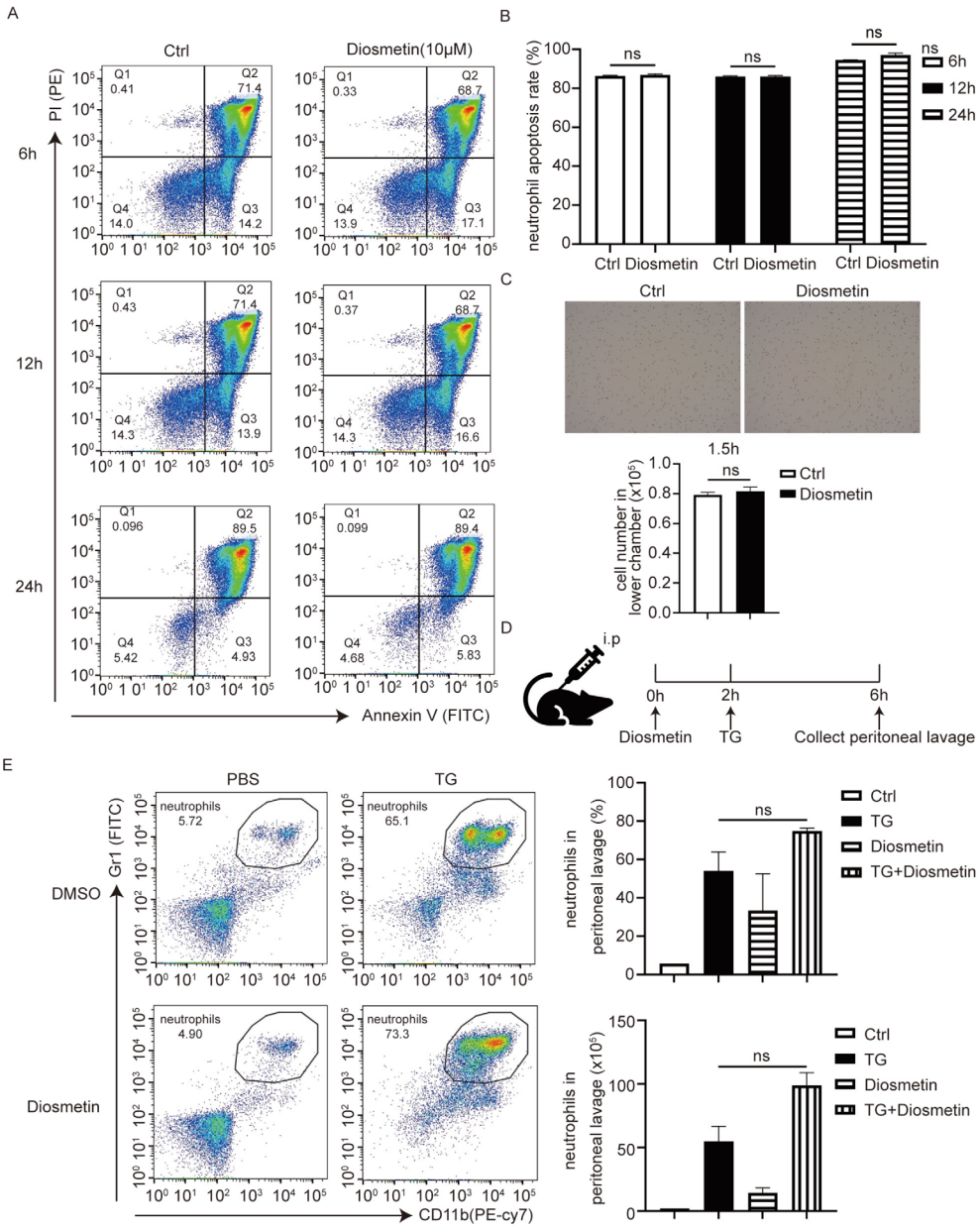

FIGURE 4 | Diosmetin does not affect other physiological ROS-related events of neutrophils. (A) The apoptosis rate of neutrophils was detected by flow cytometry. Neutrophils were cultured for a specified period of time (as shown in the figure) in the presence or absence of diosmetin, and the levels of Annexin V(FITC) and PI(PE) were detected. (B) The histogram showed the total apoptosis rate of neutrophils after diosmetin treatment for 6, 12, and 24 h (total apoptosis rate is the sum of Annexin V and PI). (C) The migration capacity of neutrophils was measured by the transwell system. Neutrophils were inoculated in transwell compartments (8 μm) and cultured for 1.5 h with or without diosmetin; cells in the pore of the plate below the compartments were counted. (D) Flowcharts of animal experiments showed the ability of neutrophils to be recruited in aseptic peritonitis in mice with or without diosmetin pretreatment (by intraperitoneal injection). (E) Mice with aseptic peritonitis were constructed and repeatedly washed with PBS to obtain peritoneal exfiltration solution. The percentage and quantity of neutrophils ($CD11b^+$(PE cy7) and $Gr1^+$ (FITC)) in peritoneal effluents were measured by flow cytometry.

in recent years [51]. Therefore, it is worth investigating the possible therapeutic strategy targeting CAT. The promoting roles of NETs in thrombogenesis have been well established. The extracellular DNA scaffolds released by neutrophils induce platelet activation and fibrin deposition, which accelerates the formation of thrombus [52]. Besides, some studies suggest that NETs can also interact with vascular endothelial cells to induce activation and injury of endothelial cells, thus exacerbating the formation of thrombi [10]. Our results showed that diosmetin significantly reduced the production of NETs and inhibited thrombus formation. These findings provided an experimental basis for the potential of diosmetin as an antithrombotic agent, suggesting its possible application value in the treatment of CAT.

The safety and toxicity of the drug is a key factor to limit its clinical application. Diosmetin is mainly found in several dietary

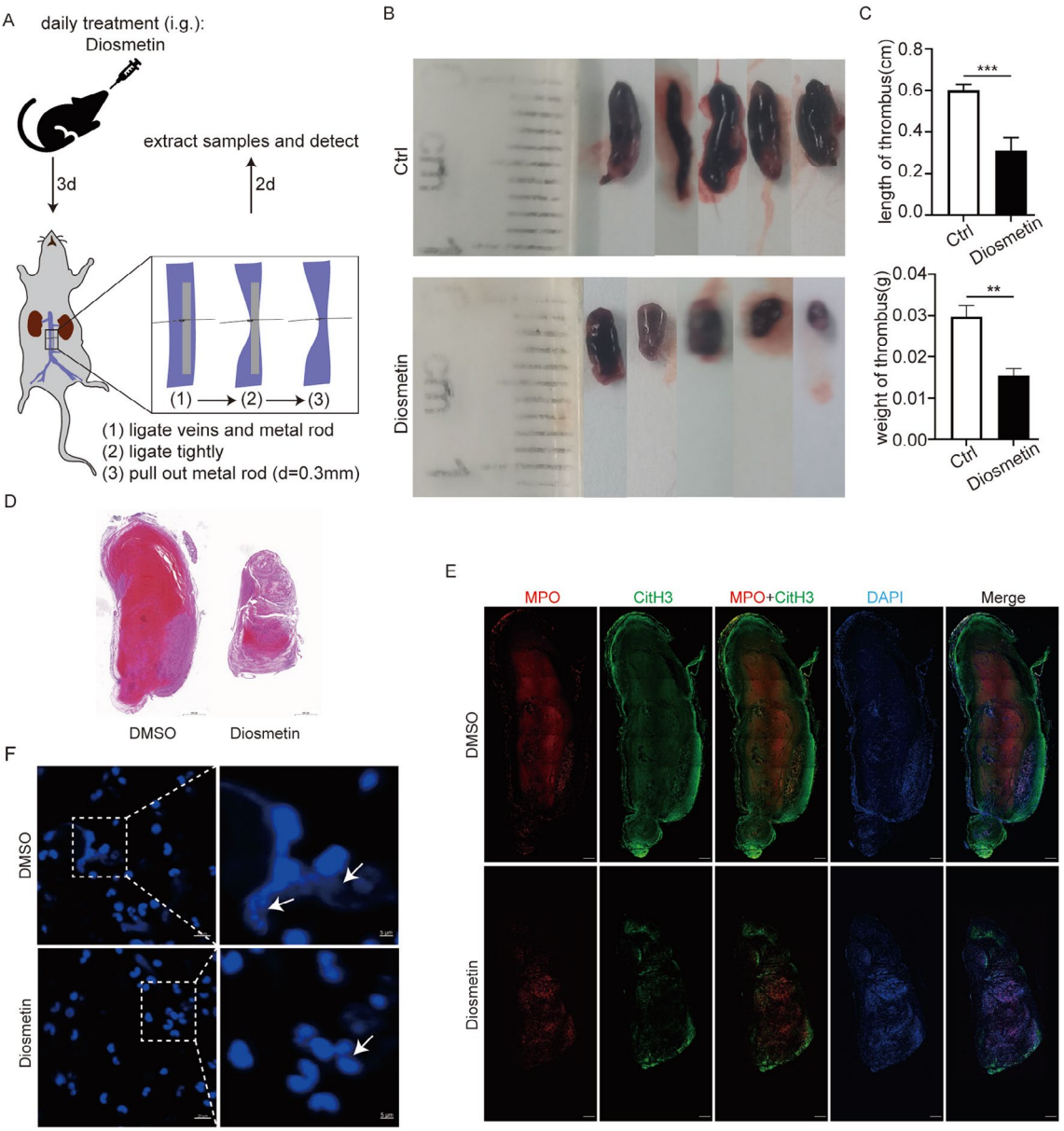

FIGURE 5 | Diosmetin-mediated NETs participate in the regulation of thrombosis in mice. (A) Deep vein thrombosis modeling flow chart. (B & C) Length and weight of the thrombus were observed and measured in an animal model of deep vein thrombosis. (D) HE staining was performed after the longitudinal incision of the thrombus. Scale bar = 500 μm. (E) Thrombus immunofluorescence staining was used to detect the level of NETs. CitH3 represents NETs levels, MPO represents the neutrophil number, and the ratio of citH3 to MPO represents the average NETs levels in each thrombus. Scale bar = 500 μm. (F) The bone marrow neutrophils were extracted from mice with deep vein thrombosis and stimulated by PMA for 4 h, followed by DAPI staining to detect the production of NETs. Scale bar = 20 μm. For the enlarged pictures, Scale bar = 5 μm.

plants, such as citrus fruits and beans [53]. Before performing experiments, we checked the dosages of diosmetin used in in vitro and in vivo studies. Literature indicates that the concentration of diosmetin could reach up to 250 μmol/L in in vitro systems and 2000 mg/Kg in mice, without any cytotoxicity [53]. We used much lower doses of diosmetin, specifically 10 μmol/L in cellular experiments and an oral administration dose of 50 mg/Kg in mice, even lower than some other pharmacological activity studies of diosmetin [54, 55]. These suggest that diosmetin possesses high safety in the potential application of anti-thrombosis. Of course, the safety of diosmetin in human beings still requires further investigation.

4 | Reagents and Methods

4.1 | Animals

6- to 8-week-old male C57BL/6J mice were used in this study, which were obtained from the Animal Experiment Center of Sichuan University. All animals were kept in the animal facilities of West China School of Basic Medicine and Forensic Medicine, Sichuan University, at an environmental temperature of 18°C–23°C, humidity of 40%–60%, with a 12-h light and 12-h dark cycle. All animal experiment procedures were approved by

the Ethics Committee of West China School of Basic Medicine and Forensic Medicine, Sichuan University.

4.2 | Isolation and Culture of Mouse Neutrophils

After the C57BL/6J mice were sacrificed, the femur and tibia were stripped. The bone marrow cells were harvested by flushing the marrow cavity with pre-chilled PBS using a 2 mL syringe. The cells were filtered through a 70 μm cell sieve and then used for Percoll (Cytiva, 17089109-1) gradient centrifugation (the Percoll concentration of the working solution in the centrifuge tube from bottom to top was 81%, 62%, 55%, and 50%, respectively) to isolate and purify neutrophils. After 4300 g and no-brake centrifugation for 30 min, the neutrophils were collected from the interface between the 81% and 62% Percoll layers. The Percoll liquid in the cell suspension was diluted with a large amount of PBS, and the neutrophils were obtained after centrifugation at 1500 rpm for 5 min. Neutrophils were cultured in 1640 medium containing a mixture of 10% FBS and 1% penicillin and incubated at 37°C with 5% CO_2 for 0.5 h prior to subsequent experiments.

4.3 | Detection of cfDNA

Neutrophils were treated with 100 nmol/L PMA (Sigma, P8139) and 10 μmol/L diosmetin (MCE, HY-N0125, CAS No. 520-34-3) for 4 h, and then centrifuged. The cell precipitate was washed three times with 1× PBS. According to the instructions of the cfDNA detection kit (GOIOO, PicaGreen, GY015), the detection reagent was added to the neutrophil suspension. After 20 min, the green fluorescence signal was measured by a multifunctional microplate reader. The excitation wavelength of the instrument was 480 nm, and the emission wavelength was 520 nm to evaluate the cfDNA level of neutrophils.

4.4 | Detection of Neutrophil Respiratory Burst

Neutrophils were co-treated with 10 μmol/L diosmetin or XFBD (a gift from State Key Laboratory of Component-based Chinese Medicine, Tianjin University of Traditional Chinese Medicine) for 4 h, and then centrifuged, and the cell precipitate was resuspended with Hank's buffer. The cells were incubated with 0.5 mmol/L isoluminol (MCE, HY-W016657) and 500 U/mL horseradish peroxidase (HRP) (Sangon Biotech, B110053) for 30 min away from light. A total of 200 μL cell suspension was taken into a 96-well plate followed by 100 nmol/L PMA in every pore. The chemiluminescence signal was detected immediately with a multifunctional microplate reader. The detection was repeated for 500 cycles.

4.5 | Detection of Total ROS in Neutrophils

Neutrophils were resuspended in serum-free, antibiotic-free 1640 medium, and the probe DCFH-DA (Adamas, 99119C) was added to the cell suspension in a certain proportion. The cells were incubated at 37°C, 5% CO_2 for 30 min, during which the cells were mixed every 5 min to ensure that the cells were fully mixed with the DCFH-DA probe. Subsequently, the cells were washed three times with the above 1640 medium and finally resuspended 100 nmol/L PMA and/or 10 μmol/L diosmetin were incubated with neutrophils for 4 h. After 4 h, the cells were washed and resuspended. The fluorescence intensity was measured by a multifunctional microplate reader. The excitation wavelength was 488 nm and the emission wavelength was 525 nm to evaluate the level of total ROS in neutrophils. To study the effect of Nrf2 on the ROS production of neutrophils, neutrophils were first pretreated with 5 μmol/L Nrf2 inhibitor (ML385, MCE, HY-100523) for 2 h and then treated with PMA/diosmetin/ML385 for 4 h. ROS levels in neutrophils were detected.

4.6 | Analysis of Network Pharmacology

The possible targets of diosmetin were obtained using TCMSP, Swiss TargetPrediction, and CTD databases. OMIM, Genecards, and DisGeNET databases were used to obtain NETs production and release-related protein genes (NETs) and ROS-related protein genes (ROS). Venny was used to obtain the intersection Venn diagram and 59 possible targets. Finally, a protein–protein interaction network (PPI) map of 59 possible target genes was obtained using the String database.

4.7 | Molecular Docking

We downloaded the entire predicted structures of mouse and human possible target proteins from the protein structure website (https://alphafold.ebi.ac.uk/) and downloaded the molecular structure of diosmetin (PubChem CID: 5281612) from the Pubchem website (https://pubchem.ncbi.nlm.nih.gov/). Then, molecular docking was performed on the docking platform: CB-Dock2: An accurate protein-ligand blind docking tool to predict the binding between protein and diosmetin [56, 57].

4.8 | RT-qPCR

According to the kit instructions, the total RNA of the cells was extracted with the UNlQ-10 Column Total RNA Purification Kit. Subsequently, YEASEN's cDNA Synthesis SuperMix (gDNA digester plus) (11141ES60) and Universal Blue qPCR SYBR Master Mix (11184) kits were used for reverse transcription and fluorescence quantitative PCR analysis of the extracted RNA. Threshold changes (CT) were used to analyze qPCR data, and β-actin was used as an internal reference to normalize the comparative CT values to analyze the transcriptional levels of target genes. The sequences of primers are shown in Table S3.

4.9 | Detection of Apoptosis

The extracted neutrophils were cultured with 10 μmol/L diosmetin in a cell incubator, during which neutrophils could undergo spontaneous apoptosis. PI (PE) and Annexin V (FITC) were stained according to the kit (YEASEN, 40302ES60)

instructions. The cells were washed twice with PBS to remove the excess dye. The cells were filtered with a 70 μm cell sieve, and the apoptosis was detected by flow cytometry.

4.10 | Transwell Cell Chemotaxis Assay

Neutrophils were resuspended in serum-free medium, and the cell density was adjusted to 3.5×10^5 cells/mL. A total of 150 μL cell suspension was inoculated in a 24-well transwell (8 μm) chamber. At the same time, 600 μL medium containing 10% FBS was added to the lower chamber. After treatment of neutrophils in the transwell chamber with diosmetin for 1.5 h, the culture medium of the lower chamber was collected, and the cell count was performed using a countstar cell counter to calculate the number of neutrophils in the lower chamber to evaluate the chemotaxis of neutrophils.

4.11 | Immunofluorescence

NETs detection: Extracted neutrophils were inoculated into 24-well plates containing polylysine-coated cell slides. Then, after 4 h of treatment with PMA (100 nmol/L) or PMA (100 nmol/L) + diosmetin (10 μmol/L) or XFBD, the cells were fixed with 4% paraformaldehyde for 15 min. Then the 5 μg/mL DAPI solution was used to stain the cells for 5 min.

Nrf2 detection: Neutrophils were also inoculated into a 24-well plate. The cells were pretreated with diosmetin (10 μmol/L) for 4 h in advance, and then treated with PMA + diosmetin for 15 min. After that, the neutrophils were fixed with paraformaldehyde for 15 min, and the excess paraformaldehyde was washed away with PBS. The cells were incubated with an Nrf2-specific antibody at 4°C overnight. Finally, the neutrophils were stained with DAPI (as above).

The cellular immunofluorescence was photographed and analyzed using a Zeiss LSM710 confocal laser microscope.

4.12 | Acute Recruitment of Neutrophils

C57BL/6J mice (aged 6–8 weeks) were randomly divided into four groups. Diosmetin (50 mg/kg) or DMSO was intraperitoneally injected into the mice. Two hours later, the mice were intraperitoneally injected with 3% thioglycolate or PBS. After 4 h, peritoneal lavage was collected. A total of 200 μL of peritoneal lavage fluid was taken from each mouse for cell counting using the countstar cell counting plate to calculate the total number of cells in each peritoneal lavage fluid. Then, the remaining peritoneal lavage fluid was centrifuged and resuspended, and the cell density was adjusted to 5×10^7–10×10^7 cells/mL. A 500 μL cell suspension was stained with CD11b (FITC) (BD Biosciences, 557396) and Gr1 (PE-Cy7) (BD Biosciences, 552985). The proportion of neutrophils was detected by flow cytometry. The number of neutrophils recruited into the peritoneal cavity of each mouse was calculated based on the proportion of neutrophils and the total number of cells in the peritoneal lavage fluid of each mouse.

4.13 | Murine Deep Vein Thrombosis Model

Male C57BL/6J mice aged 6–8 weeks were used. DMSO or diosmetin (50 mg/kg) was given to the mice by gavage daily starting 3 days before modeling, and an animal model was performed on the 4th day. After the mice were anesthetized with isoflurane, the abdominal cavity of the mice was opened to fully expose the inferior vena cava. The surgical suture was passed through the posterior end of the vein, and a metal rod with a diameter of 0.3 mm was placed next to the vein. The suture was ligated, and the metal rod was removed after ligation. Then the abdominal wall was sutured and disinfected. After 48 h, the mice were sacrificed to observe the formation of thrombi in the veins and to fetch out the thrombi. Thrombi were fixed in 4% paraformaldehyde after the length and weight were measured. Then the thrombus HE staining and MPO/citH3 staining were performed and analyzed.

4.14 | Data Analysis

Data were presented as mean or SEM. All comparisons were performed by Graphpad Prism 9. The data were analyzed using unpaired two-tailed *t*-test; One-way ANOVA (and nonparametric) and Two-way ANOVA (with Tukey test). A significance level of $p < 0.05$ was considered. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. All experiments were performed at least three times.

Author Contributions

The study was conceived by Jingyu Li, Xiaoyu Niu, Ning Huang, and Lijuan Guo. Experiments were conducted by Lijuan Guo, Hongyang Luo, Yuxin Lei, Leiyan Gu, Yaohui Wang, Siyuan Wang, Jingyu Li, Zihan Lian, Yuhao Li, Yanlan Xiang, Guanhua Du, Rui Shao, Xiaoxuan Tian, Han Zhang, Linjie Shen, Junli Chen, and Yi Wang. Data analysis was performed by Lijuan Guo, Hongyang Luo, Yuxin Lei, Leiyan Gu, and Jingyu Li. Figures were prepared by Lijuan Guo, Hongyang Luo, and Yuxin Lei. Funding was acquired by Jingyu Li and Xiaoyu Niu. The original manuscript was written by Lijuan Guo and Jingyu Li. Review and editing were performed by Jingyu Li, Lijuan Guo, Hongyang Luo, Yuxin Lei, Leiyan Gu, and Xiaoyu Niu.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.